Norwegian University of Life Sciences

Acknowledgements

The work presented in this thesis was conducted at the Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, with Prof. Vincent Eijsink and Dr. Geir Mathiesen as supervisors.

First, I would like to thank my supervisors Vincent Eijsink and Geir Mathiesen. Vincent, for your great optimism and productive input regarding my thesis. Geir, your enthusiasm and exceptional guidance never cease to amaze. You always have time for your students, making us feel as if your only task is to help and support us.

I would also like to thank my fellow master students Lise Øverland and Stefan Marinai for interesting and productive discussions during the course of our lab work, and all the other members of the PEP group for all help and motivation. Together, you provided a great working environment.

Finally, I would like to direct special thanks to Dr. Inger Øynebråten for help with everything regarding the mouse trials and the immunological aspects of this thesis, and to Elin Ørmen and Hilde Raanaas Kolstad at the Imaging centre, UMB, for all help regarding microscopy.

Ås, May 2013

Guro Kruge Urdal

Abstract

The aim of this study was to develop the lactic acid bacterium (LAB) *Lactobacillus plantarum* WCFS1 as delivery vector for a human mucosal vaccine against human immunodeficiency virus (HIV). LAB are known for their wide use in the food industry, especially in food and beverage preservation, and are natural inhabitants of the human gastrointestinal (GI) tract. *L. plantarum* WCFS1 has probiotic and immunomodulating effects and can persist in the human tract for up to seven days. *L. plantarum* and other LAB are regarded as safe for oral consumption and are thus subjects of extensive research regarding their potential as *in situ* delivery vectors for heterologous proteins. In several previous studies on the ability of recombinant bacteria to activate an immune response through the mucosal immune system, specific immune responses have been detected.

In the present study, the HIVp24 fusion protein consisting of a HIV antigen and a cytokine, was expressed and anchored to the surface of *L. plantarum* WCFS1 using different anchors: a lipoanchor, an LPxTG-type anchor and a LysM-type anchor. Expression and surface display of the fusion protein was achieved, but with varying results depending on the anchor type. The variation in results emphasises the need to optimise the combination of signal peptide, anchor type, target protein and host strain for each heterologous protein that one wishes to express and display. A replicon change, presumably leading to higher plasmid copy numbers, was performed to increase production of HIVp24. Production was increased, but surface display of HIVp24 decreased. Surface display was analysed using flow cytometry and confocal microscopy. The surface structure of the various recombinant strains was closer investigated through scanning electron microscopy, which showed that strains producing HIVp24 fused to an anchor sequence, and in particular the strain expressing HIVp24 with the LPxTG-type anchor, had a disrupted, blistery surface. Finally, the recombinant strains were tested in a mouse model. ELISPOT analysis indicated development of tolerance, but final conclusion cannot be made before additional experiments have been done.

Our data show that *L. plantarum* WCFS1 is able to express and anchor the HIVp24 fusion protein to the surface using homologous signal peptides and anchors, which is an important achievement in its own right. So far, immune responses in mice have not been detected for any of the recombinant strains, but further analyses are in progress. There is a great need and potential to optimise the secretion efficiency, delivery dosage and route of immunisation in further development of this promising system for preparing an oral vaccine for HIV.

Sammendrag

Målet med denne studien var å bruke melkesyerbakterien *Lactobacillus plantarum* WCFS1 som en leveringsvektor for en vaksine mot HIV infeksjoner. Melkesyrebakterier er kjent for deres brede anvendelse i næringsmiddelindustrien, samt deres naturlige tilstedeværelse i det menneskelige mage-tarmsystemet. *L. plantarum* WCFS1 har probiotiske og immunmodulerende effekter og kan bli værende i den menneskelige tarmkanalen i opptil syv dager. *L. plantarum* og andre melkesyrebakterier regnes som trygge for oralt forbruk og er dermed gjenstand for omfattende forskning angående deres potensial som *in situ* leveringsvektorer av heterologe proteiner. Det har blitt gjennomført flere studier som undersøker rekombinante bakteriers evne til å aktivere en immunrespons gjennom mucosa, og en har klart å fremkalle spesifikke immunresponser gjennom forsøk i dyremodeller.

I dette studiet ble et fusjonsprotein bestående av et HIV-antigen og et cytokin, kalt HIVp24, uttrykt og ankret til overflaten av *L. plantarum* WCFS1. Tre forskjellige ankere ble benyttet: et lipoanker, et LPxTG-type anker og et LysM-type anker. Ekspresjon og deteksjon av fusjonsproteinet på overflaten ble oppnådd, men med varierende resultater avhengig av ankerproteinet. Variasjonen i resultatene understreker behovet for å optimalisere kombinasjonen av signalpeptid, ankertype, heterologt protein og vertsstamme for hvert heterologt protein en ønsker å uttrykke. Det ble utført et replikonbytte, hvilket var antatt å øke antall plasmider i cellen, som igjen kan øke ekspresjonen av HIVp24. Uttrykket ble økt, men mengden av HIVp24 på overflaten ble redusert. Mengden av HIVp24 på overflaten ble analysert ved hjelp av væskestrømscytometri og konfokalmikroskopi. Stammenes overflatestruktur ble nærmere undersøkt gjennom skanningelektronmikroskopi, som viste at stammer som uttrykker HIVp24 koblet til en ankersekvens, spesielt stammen som uttrykte HIVp24 med LPxTG-anker, hadde en byllete overflate. Til sist ble de rekombinante stammene testet gjennom museforsøk. Resultatene av ELISPOT-analysen indikerte utvikling av toleranse, men ingen endelige konklusjon kan settes før flere analyser er gjort.

Våre data vier at *L. plantarum* WCFS1 er i stand til å uttrykke og forankre HIVp24 fusjonsproteinet til overflaten ved hjelp av homologe signalpeptider og ankere, hvilket er en viktig prestasjon i seg selv. Så langt har en ikke klart å detektere immunresponser i mus for noen av stammene, men videre analyser er under utvikling. Det er et stort behov og potensial for å optimalisere sekresjonseffektiviteten, dosering og immuniseringsrute for videre utvikling av dette lovende systemet for å utvikle en oral vaksine mot HIV.

Abbreviations

AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
BSA	Bovine serum almunin
CCL17	Chemokine (C-C motif) ligand 17
CFU	Cell forming units
DC	Dendritic cell
dNTP	Deoxyribonucleotide triphosphate
ddNTP	Di-deoxyribonucleotide triphosphate
ELISPOT	Enzyme-linked immunosorbent spot
GI tract	Gastrointestinal tract
GALT	Gut-associated lymphoid tissue
Gag p24	Group-specific antigen p24
GRAS	Generally recognised as safe
HIV	Human immunodeficiency virus
HIVp24	Fusion protein of CCL17 and Gag p24
HLA	Human leukocyte antigen
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
LAB	Lactic acid bacteria
M cell	Microfold cell
MALT	Mucosa-associated lymphoid tissue
MCS	Multiple cloning site
MHC	Major histocompatibility complex
PCR	Polymerase chain reaction
PFA	Paraform aldehyde
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
sIgA	Secreted immunoglobulin A
SP	Signal peptide
SPase	Signal peptidase

Contents

1 INTRODUCTION

Lactic acid bacteria (LAB) are known for their wide use in the food industry, especially in food and beverage preservation. They have been used to ferment dairy products, meats and vegetables since ancient time, and are still important in such preservation techniques today. LAB are natural inhabitants of the human gastrointestinal (GI) tract, where some are believed to act as probiotics. Many strains of LAB have a high tolerance for acidic environments, and such strains thus have the ability to effectively survive the passage through the stomach. Because of the characteristics mentioned above, LAB have been identified as potential live delivery vectors for therapeutic proteins such as antigens, enzymes and antibodies. Several expression systems have been developed for *in situ* production of secreted or cell surface-anchored heterologous proteins. The ultimate goal of the present study was to explore the possibility of exploiting the LAB *Lactobacillus plantarum* WCFS1 as a delivery vehicle for an HIV antigen. To achieve this, attempts were made to anchor a HIV antigen to the surface of the bacteria using homologous anchoring mechanisms.

1.1 Lactic acid bacteria

Lactic acid bacteria are Gram positive cocci, coccobacilli or rods with a low G+C content. They are non-sporeforming and able to grow both anaerobically and aerobically. Functionally, the bacteria are characterised by their ability to produce lactic acid through fermentation of hexose sugars (Makarova et al. 2006). Production of lactic acid acidifies their environment which explains why LAB are important for food preservation. LAB genera of great importance for food preservation are *Lactococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc*, and *Lactobacillus* (Makarova et al. 2006). The low pH caused by lactic acid production protects food and beverages against food spoiling organisms and food pathogens. Many LAB have also been shown to produce bacteriocins, small peptides that inhibit growth of competing Gram positive bacteria occurring in the same ecological niche (Eijsink et al. 2002), which also can contribute to inhibition of food pathogens. Important food pathogens inhibited by LAB include *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Salmonella typhimurium* and *Shigella flexneri* (Meira et al. 2012; Yu et al. 2013). Most LAB have obtained a GRAS (Generally Recognised As Safe) status, because they are non-pathogenic, non-invasive and found in food products. Several of

the species found in food habitats are also natural inhabitants of the mucosal surfaces of humans, such as the colon, small intestine and vagina (Makarova et al. 2006).

The genus *Lactobacillus* holds bacteria of great functional and phylogenetic diversity. Most lactobacilli are aero-tolerant and anaerobic, and all are strictly fermentative (Hammes & Vogel 1995). Bacteria of the genus are of great importance in the food and beverage industry, both regarding preservation and contributions to flavour and texture. Because they are considered potential health promoting microorganisms in the human GI tract, lactobacilli are marketed as part of several probiotic products (Kleerebezem et al. 2010). Probiotics are defined by the World Health Organisation as "*live microorganisms which when administered in adequate amounts confer a health benefit on the host*" (FAO/WHO 2002). Strains of *Lactobacillus* are regarded as probiotics because they have several extracellular characteristics that enables them to interact with and influence host GI tract (Kleerebezem et al. 2010; Lebeer et al. 2008; Turner et al. 2004). Lactobacilli are also able to reside and persist naturally on the mucosal surfaces of the host. Genomes of several *Lactobacillus* strains have been sequenced during the last decade, including the genomes of many of those thought to have probiotic effects.

Due to the qualities mentioned above, including the GRAS status, a great amount of research is currently being conducted to investigate the potential of strains of the genus *Lactobacillus* as *in situ* delivery vectors of heterologous proteins in humans. Several strains have been engineered to function as delivery vectors of heterologous proteins for therapeutic purposes, such as enzymes, antigens, pathogen receptors and antibodies (Turner et al. 2004).

1.1.1 *Lactobacillus plantarum* WCFS1

Lactobacillus plantarum WCFS1 is a facultative heterofermentative lactic acid bacterium found in several different environmental niches, such as dairy and meat products, plant and vegetable fermentations, and the GI tract and oral cavity of humans (Ahrne et al. 1998; Axelsson & Ahrné 2000; Vaughan et al. 2002). The ability to utilise such diverse niches is reflected in the *L. plantarum* genome, which contains a large number of regulatory and transport functions, suggesting large adaptability (Kleerebezem et al. 2003). *L. plantarum* WCFS1 is a single colony isolate of *L. plantarum* NCIMB8826, isolated from human saliva (Kleerebezem et al. 2003). It was the first *Lactobacillus* to have its genome sequenced, and analysis of the circular 3.3 Mb chromosome has revealed 3.042 predicted protein-encoding

genes (Siezen et al. 2012). Over 200 of the proteins were predicted to contain an N-terminal signal sequence, making them part of the bacterium's extracellular proteome. The majority of these proteins were predicted to be anchored to the cell surface. Of the anchored proteins, most were attached to the cell surface through single N- or C-terminal transmembrane anchors, and the rest (in descending order) were anchored through lipoprotein anchors, LPxTG-type anchors or other cell-wall binding domains such as LysM domains (Kleerebezem et al. 2003). Details about these anchors are found in section 1.4.

The key to a bacterium's probiotic effects lies in its ability to interact with and persist in the GI tract of the host, and this ability is thought to depend on the bacterium's extracellular proteome. Previous studies on *L. plantarum* WCFS1 have shown that the bacteria survive the passage through the GI tract and are able to colonise the digestive tract to high levels, and persist for up to 7 days (Marco et al. 2009; van Bokhorst-van de Veen et al. 2012; Vesa et al. 2000). Several strains of *L. plantarum* have probiotic effects on humans and animals, such as lowering cholesterol levels in the liver and blood serum (Huang et al. 2013; Yu et al. 2013), suppressing the clinical characteristics of high-fructose-induced metabolic syndrome (Park et al. 2013), prevention of atopic disease (Kalliomaki et al. 2001) and immunomodulatory effects resulting in protection against influenza virus infection (Kechaou et al. 2013). Probiotic effects observed for *L. plantarum* WCFS1 include adjuvant effects (van Baarlen et al. 2009) and triggering of immune responses in host cells (Troost et al. 2008). It has also been suggested that adherence to intestinal mucosa in itself is the basis of a probiotic effect of *L. plantarum* because this leads to competitive exclusion of pathogens (Boekhorst et al. 2006; Marco et al. 2006). For example, binding of pathogenic bacteria to mannose residues in the mucosal cell layer is known to play a key role in their virulence. *L. plantarum* can adhere to mannose residues in the mucosa and in that way protect against pathogenic microbes through competitive exclusion (Pretzer et al. 2005).

Intense research has been conducted over the last years to create efficient gene expression systems enabling the use of *L. plantarum* and other bacteria as food grade "factories" for production of heterologous proteins. The immunomodulatory effects of *L. plantarum* WCFS1, and the fact that it is a natural inhabitant of the human GI tract, makes this bacterium a very promising vector for vaccine delivery.

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

One expression system for use in *Lactobacillus* is the pSIP system, which was developed by Sørvig et al. (2003) to create a controllable protein expression system in LAB. The system has minimal basal expression activity, and when induced, it has the potential to yield the highest protein expression levels ever observed in LAB, using a one-plasmid system and without integrating genes into the chromosome of the host (Mathiesen et al. 2004; Nguyen et al. 2012; Sørvig et al. 2005a).

The expression system is based on genes involved in production of the class II bacteriocin sakacin P in *Lactobacillus sakei* (Brurberg et al. 1997). The system is incorporated in one plasmid and consists of two operons, one encoding the components for peptide-pheromone triggered two-component signal transduction, and one for expression of the target gene (originally sakacin P). The two-component signal transduction operon consists of a promoter (P_{sppIP}) and the genes encoding a transmembrane histidine kinase (HK) and a response regulator (RR). The other operon consists of a different promoter (P_{sppA}) and the target gene. The target gene is in the site originally held by sakacin P and the cognate immunity gene. The plasmid also contains a erythromycin resistance gene (*ermB*), a the replication determinant for *E. coli* (pUC(GEM)$_{rep}$), and the narrow host replicon 256$_{rep}$ which works in a few *Lactobacillus* species, like *L. sakei* and *L. plantarum* (Sørvig et al. 2003). Figure 1.1 shows a graphic presentation of the prototype plasmid. Based on this prototype, several expression plasmids have been constructed, containing different promoters and replicons and enabling use of the plasmid system in other lactobacilli.

In the natural genetic determinant for sakacin P prodcution, activation of the P_{sppA} promoter and, thus, expression of the sakacin P structural gene is initiated when the system senses the peptide SppIP. SppIP has no antimicrobial properties; it only functions as a pheromone, and induces expression of the bacteriocin through a cell-density dependent mechanism (Eijsink et al. 2002). The gene encoding this pheromone is part of the same operon as the HK and RR. The promoter controlling the operon (P_{sppIP}) has some leakage (Risøen et al. 2000), resulting in very low, basal production of HK, RR and SppIP during growth. When the pheromone concentration in the medium reaches a "threshold value", the pheromone interacts with the membrane-located receptor domain of the HK, leading to autophosphorylation of the HK's cytoplasmic domain. The cytoplasmic domain of the HK then activates the RR through phosphorylation, enabling the phosphorylated RR to bind to specific sequences in both the P_{sppIP} and the P_{sppA} promoter, leading to induction of transcription. To ensure full control of

gene expression in the pSIP system, the gene encoding SppIP was deleted (Fig. 1.1). The P_{sppA} promoter is under very tight control; thus there is no transcription of the target gene unless pheromone is added to the culture (Sørvig et al. 2005a). This set-up results in a tightly controlled system. The fact that expression of HK and RR is induced simultaneously with expression of the protein of interest gives an autoinduction loop that leads to explosive transcription once the system is induced (Brurberg et al. 1997; Mathiesen et al. 2004; Sørvig et al. 2003; Sørvig et al. 2005a).

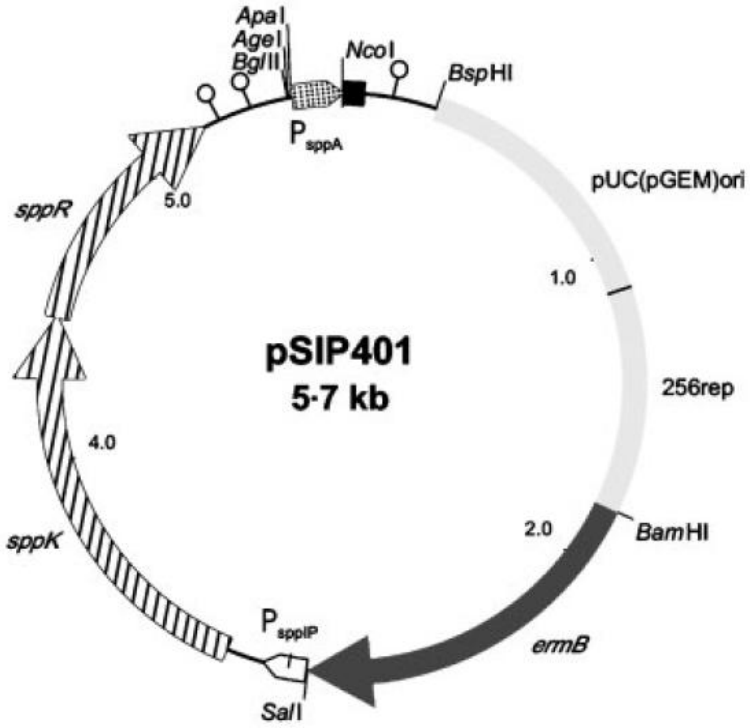


Figure 1.1: Graphic presentation of the pSIP401 plasmid. Light-grey regions, replication determinants; dark-grey regions, erythromycin resistance marker; vertically hatched regions, genes encoding HK, histidine protein kinase (*sppK*) and RR, response regulator (*sppR*); dotted region, inducible P_{sppA} promoter; white region, inducible P_{sppIP} promoter; lollypop structures, transcriptional terminators; black boxes, multiple cloning site (MCS) (SphI, XbaI, XhoI, EcoRI, KpnI, SmaI, NarI, HindIII). All restriction sites shown are unique. The figure and most of the legend text are taken from Sørvig et al. (2005a). A target gene can be translationally fused to the P_{sppA} promoter using *NcoI* and a restriction endonuclease from the MCS.

All plasmids used and constructed in the present study are derived from pSIP401, which is depicted in Figure 1.1. To develop this vector for secretion of proteins, Mathiesen et al. (2008) constructed a new plasmid called pUsp45, where a "secretion cassette" was incorporated downstream of the P_{sppA} promoter. This cassette was designed for allowing easy

exchange of its components and is depicted in Figure 1.2. For fusion of the signal peptide (SP) to the promoter, the *NcoI* restriction site downstream of the P_{sppA} promoter has been changed to *NdeI*, and the *SalI* restriction site originally present in the promoter was removed to enable construction of a unique *SalI* site in the linker region between the SP and secreted protein (Mathiesen et al. 2008).

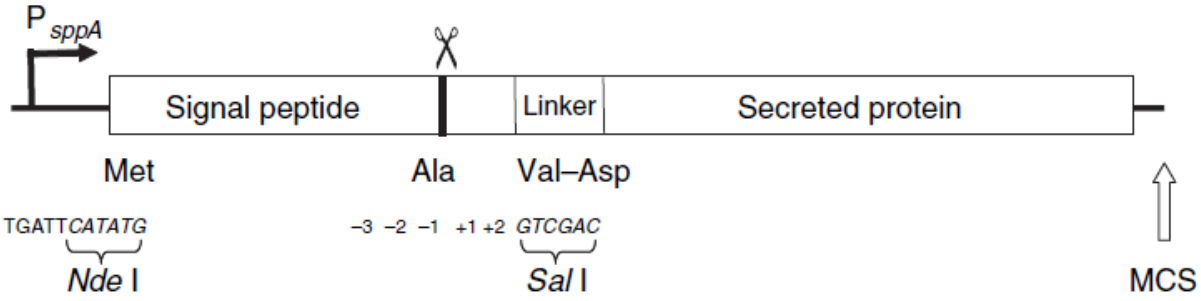


Figure 1.2: Schematic overview of the secretion cassette used in pSIP vectors. The P_{sppA} promoter is translationally fused to a signal peptide using an *NdeI* site. The signal peptide is cloned including the first two amino acids of its cognate mature protein. In between the SP and the protein of interest there is a linker area that encodes Val and Asp and creates a *SalI* restriction site. The scissor marks the cleavage site of the signal peptidase. MCS is the multiple cloning site, including *EcoRI* and *Acc65I*. The figure is taken from Mathiesen et al. (2008).

Sørvig et al. (2005a) constructed several pSIP expression vectors, with varying combinations of promoters and replicons. Through these experiments, it was discovered that the performance of the expression system relied greatly on the combination of replicon, the target gene, the promoter and the host strain. Further studies showed that by replacing the narrow host range 256_{rep} replicon (Sørvig et al. 2005b) with the broad range and high plasmid copy number $SH71_{rep}$ replicon from *Lactococcus lactis* (de Vos 1987), plasmid copy numbers in *L. plantarum* increased two- to eight-fold and target gene expression was greatly increased (Maudal 2012; Sørvig et al. 2005a). This change of replicon thus has a great potential to increase the production of proteins in *L. plantarum* (Maudal 2012).

The pSIP secretion system has been further developed to include plasmids encoding genes to be anchored to the cell surface. Fredriksen et al. (2010; 2012) inserted sequences of anchor proteins with the target genes to allow anchoring of the target gene to the cell wall or the plasma membrane. This allows surface display of heterologous proteins using homologous anchor proteins.

1.3 Secretion of proteins in Gram-positive bacteria

Gram positive bacteria are considered promising host organisms for secretion of heterologous proteins due to their lack of an outer membrane. The lack of an outer membrane enables these bacteria to release proteins directly into the extracellular environment after transport across the cytoplasmic membrane. Seven main protein secretion pathways have so far been characterised in Gram positive bacteria (Kleerebezem et al. 2010):

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

The Sec pathway (I) is the major secretion pathway of Gram positive bacteria. Proteins targeted for this pathway contain an N-terminal signal peptide (SP) with highly conserved features (Kleerebezem et al. 2010). The pathway is described in more detail below. Whereas Sec translocates unfolded proteins, the Tat pathway (II) translocates already folded proteins. The proteins targeted for Tat pathway contain a highly conserved twin-arginine motif (Palmer & Berks 2012). The FEA (III) pathway translocates flagellar components, and the FPE (IV) pathway translocates proteins involved in exogenous uptake of DNA across the bacterial cytoplasmic membrane (Chen & Dubnau 2004). The Holin pathway (V) transports holins; small integral membrane proteins which lack a signal peptide. The holins are involved in secretion of muralytic enzymes and play important role in bacterial autolysis (Wang et al. 2000). Peptide efflux ABC transporters (VI) are a subfamily of proteins involved in export of antimicrobial peptides and proteinaceous substrates, such as bacteriocins, peptide pheromones (such as SppIP), lantibiotics and competence peptides (Diep et al. 1996; Håvarstein et al. 1995). The last of the pathways, the Wss secretion pathway (VII), secrets WXG100 (ESAT-6) family proteins (Fagerlund et al. 2010).

Kleerebezem et al. (2010) screened the sequenced genomes of 13 lactobacilli for genes of all the pathways mentioned above, and found that these species only seem to use the Sec (I), FPE (IV), holin (V) and peptide-efflux ABC (VI) pathways (Kleerebezem et al. 2010). Genes

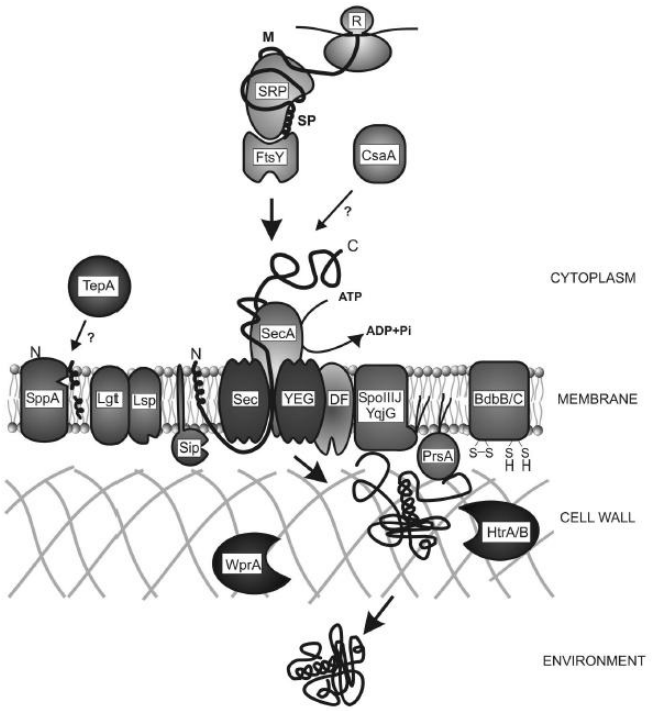
encoding the main factors involved in the FEA, Wss and Tat secretion systems were not found.

1.3.1 The major secretion pathway: Sec

The Sec pathway is the major pathway for protein secretion in Gram positive bacteria and *L. plantarum*. It is also the major pathway for proteins that end up in the plasma membrane. The pathway is based on the protein transport complex called Sec translocase, which mediates protein transport across the plasma membrane (Driessen & Nouwen 2008). All preproteins targeted for the Sec pathway contain an N-terminal signal peptide (SP). The SP typically consist of three regions: the N region containing positively charged amino acids, the H region containing a stretch of 15-25 hydrophobic amino acids, and the C region which contains a signal peptidase (SPase) cleavage site (Driessen & Nouwen 2008). A model depicting the Sec-dependent secretion system is shown in Figure 1.3. The polypeptide is synthesised by ribosomes in the cytosol and the N-terminal SP is recognised by a signal recognition particle (SRP). A second protein called FtsY then binds to the SP-SRP complex and helps stabilising the unfolded polypeptide together with SRP until it reaches the Sec translocase located in the cytoplasmic membrane (Tjalsma et al. 2004). The Sec translocase consists of a membrane embedded protein-conducting channel called SecYEG (composed of SecY, SecE, SecG) and an ATPase motor protein called SecA. Translocation of the preprotein across the cytoplasmic membrane is mediated by ATP hydrolysis by SecA and the proton motive force (PMF) across the membrane. Sec A binds to the membrane bound SecYEG translocase before the preprotein is transferred from the SRP to the SecYEG-bound SecA. ATP binds to SecA and is then hydrolysed, resulting in SecA release from the preprotein and insertion of the preprotein into the translocation pore of the translocase. Complete translocation of the preprotein through the translocation pore is mediated through PMF and cycles of ATP hydrolysis by SecA.

The signal peptide is removed by membrane bound signal peptidases (SPases) during or right after protein translocation across the cytoplasmic membrane. This is possible because the hydrophobic region of the SP allows the preprotein to bind temporarily to the cytoplasmic membrane post translocation. The SP is cleaved by either a Type-I or a Type-II SPase. Type-I SPases recognise an AxA↓A cleavage site (van Roosmalen et al. 2004) and Type-II SPases recognise an L-x-x-C lipobox cleavage site (Sutcliffe & Harrington 2002). In the latter case, the Cys residue of the lipobox is bound to the lipid bilayer through a thioether linkage, which

results in the lipoprotein being covalently bound to the plasma membrane. Proteins cleaved by Type-I SPases may end up in the culture medium, but may also be retained in the plasma membrane or the cell wall, as explained below. Notably, proteins may contain SPs devoid of a SPase cleavage site; in this case, the protein is retained in the plasma membrane leading to non-covalent anchoring of the protein.



Figur 1.3: The main components involved in Sec-dependent translocation in Gram positive bacteria. Preproteins targeted for translocation via the Sec pathway are transported across the plasma membrane through Sec translocase and released into the extracellular environment. More details are given in the text. Sec translocase consists of a membrane embedded protein-conducting channel (SecYEG) and an ATPase motor protein (SecA). Nb: Genes encoding the SecDF-proteins appear not to be present in *Lactobacillus* genomes, including *L. plantarum* WCFS1 (Kleerebezem et al. 2010). The figure is taken from Tjalsma et al. (2004).

Protein secretion efficiency is highly dependent on the combination of signal peptide (SP) and target protein, especially for secretion of heterologous proteins (Brockmeier et al. 2006; Caspers et al. 2010; Dieye et al. 2001; Mathiesen et al. 2008; Perez-Martinez et al. 1992; Slos et al. 1998). The secretion efficiency is also highly host specific (Perez-Martinez et al. 1992). Thus, when constructing a bacterial delivery vector of therapeutic proteins, where high secretion of the heterologous protein might be required, the factors mentioned above must be carefully considered and optimised.

1.4 Anchoring of proteins in Gram-positive bacteria

After a protein has been translocated, it can be released into the surroundings or become bound to a part of the cell surface. Gram positive bacteria have several mechanisms enabling attachment of proteins to the cell surface, leaving the proteins anchored covalently or non-covalently to the cell envelope. Natural protein anchoring mechanisms in principle allow researchers to display heterologous proteins on the surface of the bacteria (Desvaux et al. 2006; Turner et al. 2004). The anchoring mechanisms of surface bound proteins are usually divided into four categories based on their interaction with the cell envelope (Desvaux et al. 2006):

I. Transmembrane proteins
II. Lipoproteins
III. LPxTG-containing proteins
IV. Cell wall binding proteins

Figure 1.4 shows a schematic overview over the various anchoring mechanisms.

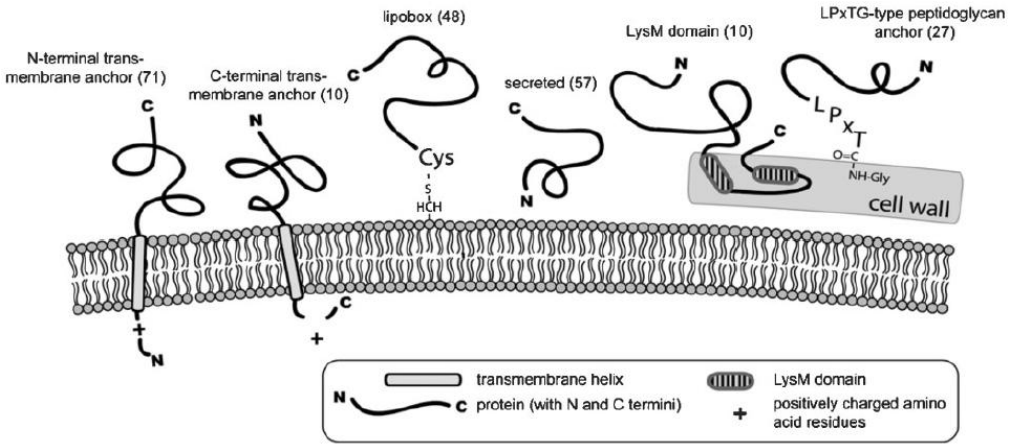


Figure 1.4: Schematic overview of surface anchored proteins found in *L. plantarum*. Several types of cell surface anchored proteins exist. Membrane anchored proteins can possess an N- of C-terminal transmembrane helix (left two proteins), or possess a lipobox motif enabling covalent anchoring to the cell membrane (third protein from the left). Cell wall binding proteins are either covalently bound to the peptidoglycan via an LPxTG motif (far right protein) or non-covalently attached to the cell wall via LysM domains (second protein from the right). More details can be found in the text. The figure is taken from (Boekhorst et al. 2006).

1.4.1 Transmembrane proteins

Transmembrane proteins are bound to the plasma membrane through a C- or N terminal hydrophobic transmembrane domain, often a helix (Desvaux et al. 2006). The proteins can be single-pass transmembrane proteins, which only cross the membrane once, or multi-pass membrane proteins which cross the membrane several times (i.e. with more than one transmembrane helix). N-terminally anchored transmembrane proteins contain an N-terminal signal peptide (SP). If the SP is cleaved by signal peptidase I (SPaseI), the protein is secreted. If the SP is left uncleaved, due to lack of an efficient SPase cleavage site in the C-terminal part of the SP, the SP will function as a membrane anchor domain and the protein will be attached to the plasma membrane (Tjalsma et al. 2004). Some proteins might also contain a C-terminal anchor domain. Such transmembrane proteins may be retained in the plasma membrane even though the N-terminal SP is cleaved off (Tjalsma et al. 2004). The natural functions of transmembrane proteins are mostly related to extracellular processes, such as cell-envelope metabolism, signal transduction, competence, transport and protein turnover (Kleerebezem et al. 2010).

1.4.2 Lipoproteins

Another method that bacteria use to attach proteins to the cell surface is to covalently bind them to the cell membrane. These lipoproteins are covalently anchored to the plasma membrane through a series of reactions. Covalent anchoring of the lipoproteins is made possible because they possess a signal peptide which targets the proteins to transport via the Sec secretion pathway. The signal peptide contains all the domains characteristic for Sec pathway SPs, namely the N, H and C domains. The C-region of the SP contains a lipobox motif [L-(A/S)-(A/G)-C] which directs the protein to a biogenesis machinery after transport (Hutchings et al. 2009). The biogenesis machinery contains a diacylglyceryl transferase (Lgt) which adds a diacylglyceryl group to the conserved Cys residue in the lipobox. Addition of the diacylglyceryl group generates a thioether linkage between the Cys residue and the lipid bilayer, which results in the lipoprotein being covalently anchored to the plasma membrane (Hutchings et al. 2009). Post linkage, the SP is cleaved N-terminally of the Cys-residue by Type II SPase. Many lipoproteins are ABC-transporters, but many are also involved in antibiotic resistance, cell-envelope homeostasis, adhesion, sensory processes and protein secretion, folding and translocation (Kleerebezem et al. 2010). A recent study has shown that it is possible to use lipoprotein anchor motifs to display heterologous proteins the bacterial cell surface of *L. plantarum* (Fredriksen et al. 2012).

1.4.3 LPxTG-containing proteins

LPxTG-containing proteins are proteins which contain an LPxTG motif for covalent anchoring to the cell wall. Such proteins are covalently anchored to the cell wall through activity of the transpeptidase sortase (SrtA) (Marraffini et al. 2006). The LPxTG motif is located in the C-terminal part of the protein, followed by a stretch of hydrophobic residues and a positively charged amino acid, which probably function as a membrane anchor domain. The membrane anchor domain keeps the protein from being released from the bacteria and thus allows recognition of the LPxTG motif by SrtA. SrtA recognises the LPxTG domains, cleaves the protein between the Thr and Gly residues in the motif and covalently binds the threonine carboxyl group to a glycine residue in the peptidoglycan (Marraffini et al. 2006). Most lactobacilli possess sortases and proteins with LPxTG domains (Boekhorst et al. 2005). Several studies have been conducted in which heterologous proteins have been anchored to the bacterial cell surface using protein anchors containing LPxTG-like motifs (Fredriksen et al. 2010; Kajikawa et al. 2011; Turner et al. 2003).

1.4.4 Cell wall associated proteins

Many proteins on the surface of bacteria are non-covalently bound to the cell wall through various attachment domains or through protein-protein interactions with other cell wall bound proteins. Such proteins may contain special binding domains such as lysin motif (LysM) domains or choline-binding domains. Proteins bound through a LysM domain are mostly extracellular enzymes involved in bacterial cell wall metabolism (Buist et al. 2008). Proteins containing choline-binding domains bind to choline in cell wall teichoic- and lipoteichoic acids (Kleerebezem et al. 2010). Other types of cell wall associated domains have also been recognised, such as Type II cell-wall binding domains, S-layer homology domains and so-called GW-modules (Desvaux et al. 2006).

1.5 The human mucosal immune system

The mucosal surfaces cover the surfaces of the ducts of exocrine glands, the urogenital tracts, the aerodigestive tract, the eye conjunctiva and the inner, which marks the main entrance point of pathogens into the human body. The mucosal surfaces mark the first line of defence, and the mucosa layer consists of epithelial cells covered with mucus, which together create a physical barrier against microorganisms from the exterior, yielding both mechanical and chemical protection against potential pathogens. To ensure adequate immune responses, mucosal surfaces are guarded by highly specialised immune cells from both the innate and

adaptive immune system. Delivery of mucosal vaccines can thus be oral, rectal, vaginal or nasal (Holmgren & Czerkinsky 2005).

The mucosa is heavily populated with immune cells, which are concentrated in and circulating between compartments of lymphoid tissue, making up the mucosa-associated lymphoid tissue (MALT). The MALT constitutes the largest mammalian lymphoid system and is compartmentalised into anatomically defined lymphoid microcompartments. These microcompartments include Peyer's patches, mesenteric lymph nodes, the appendix and solitary follicles in the intestine, as well as tonsils and adenoids at the entrance of the aerodigestive tract (Holmgren & Czerkinsky 2005).

Due to the compartmentalisation of the MALT, specific mucosal inductive sites (where the antigens are sampled) are linked with particular effector sites (where the immune response is executed). Hence, the route and mode of immunisation has large effects on where the final immunity is located. Immunisation through the oral route has been shown to result in a substantial antibody response in the small intestine, ascending colon and mammary and salivary glands (Kozlowski et al. 1997; Quiding et al. 1991). A drawback of oral immunisation is an increased risk for development of oral tolerance, since the immune system is adjusted to tolerate food-antigens that transit through the GI tract (Ryan et al. 2001). Another way of administering mucosal vaccines is through nasal immunisation, which has been proven to result in high antibody production in the respiratory tract and the genital mucosa, in addition to local immune responses (Johansson et al. 2001). Nasal immunisation seems to be the most effective immunisation route to date in regard of antigen multitude and antibody response (Wells & Mercenier 2008). Vaginal and rectal immunisation yield a local antibody response, and constitute, together with nasal immunisation, the most promising routes for vaccination against pathogens entering through the reproductive and oral tracts, such as sexually transmitted pathogens (Holmgren & Czerkinsky 2005).

The lymphoid tissue in the gut is called the gut-associated lymphoid tissue (GALT). It consists mainly of Peyer's patches and mesenteric lymph nodes and is separated into two parts; effector sites and induction sites (Lea 2005) (Fig. 1.5). The effector sites are located outside the organised lymphoid tissues, and consists of intraepithelial lymphocytes spread around the in the mucosa, such as lymphocytes, antigen presenting cells (APC) and IgA-producing plasma cells in the lamina propria (the thin connective tissue beneath the epithelial

layer). It includes both active cells and memory cells. The induction sites constitute the organised lymphoid tissue, such as Peyer's patches and mesenteric lymph nodes.

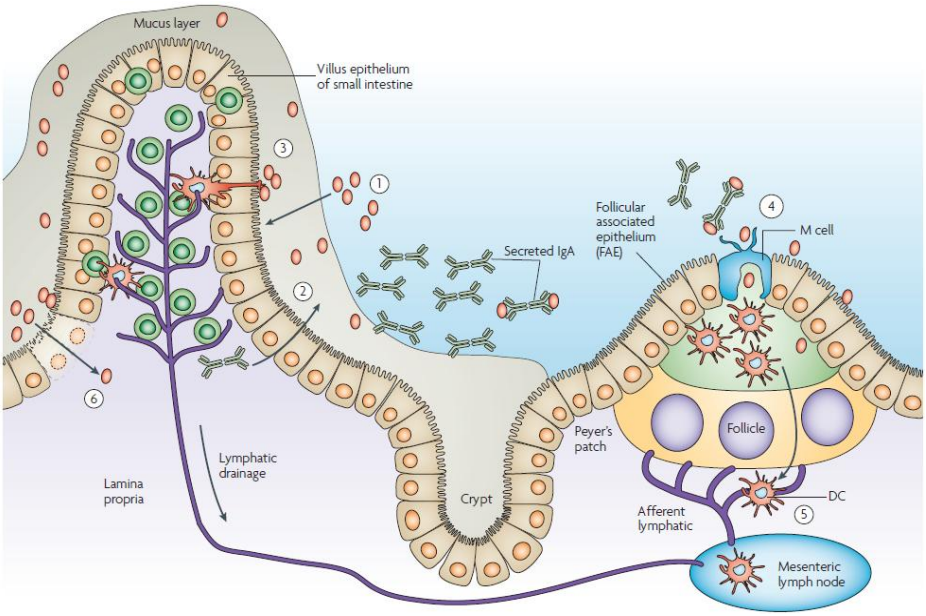


Figure 1.5: Interaction of bacteria with the human intestinal mucosal immune system, the GALT. Bacteria can be detected through the effector sites of the GALT (step 1-3 and 6) or through the induction sites of the GALT (step 4-5). Antigens from the bacteria can come into contact with the epithelial layer (step 1) and further be sampled by dendritic cells (step 3) or bound to IgA secreted from plasma cells (step 2). Bacteria can also be sampled by M cells in the Peyer's patches through trancytosis, where the antigen can induce an immune response when released to APCs in the dome of the Peyer's patch. Dentritic cells (DCs) can phagocytose the bacteria and move from the dome to the mesenteric lymph node to prime T-cell responses to the presented antigen (step 5). Bacteria can gain access to the lumina propria if the epithelial layer is damaged (step 6), and be sampled by DCs present in that area. The figure is taken from Wells and Mercenier (2008).

A Peyer's patch is a collection of immune cells located beneath the epithelial cell layer in the small intestine (Fig. 1.5). The cells of the Peyer's patches are sites of antigen sampling and constitute the main contact point between the lumen and the lymphoid tissue. The cells responsible for the antigen sampling are called microfold cells (M cells) and are located in the epithelial cell layer above the lymph tissue (Fig. 1.5, step 4). They are phagocytes, shipping components from the lumen and into the lymphoid tissue through transcytosis (Lea 2005), enabling the immune system to control the contents of the lumen at all times. The M cells have a characteristic shape: facing the lymphoid tissue, there is a large intraepithelial pocket, where antigen-presenting cells (APC) such as macrophages, dendritic cells (DCs) and lymphocytes meet the phagocytised material. All the transcytosed material is immediately detected by APC, mostly DC, which move to follicles of B cells and their surrounding and T cells located immediately beneath the M cells, to interact with the naïve lymphocytes.

APCs presenting antigens to naïve lymphocytes mark the beginning of the primary immune response. APCs phagocytose and degrade the antigen in the cytosol before displaying epitope peptides on their surface through human leukocyte antigen (HLA) molecules. The epitopes are then presented to naïve B cells and $CD4^+$- and $CD8^+$ T-cells. Epitopes of exogenous antigens (antigens sampled from the cell exterior) are presented through HLA class II molecules and detected by $CD4^+$ T cells, while epitopes of endogenous antigens (such as viruses or other intracellular pathogens) are presented through HLA class I molecules and detected by $CD8^+$ T cells. Class I HLA molecules are present in all cell types, but HLA class II are only present in APCs and lymphocytes (Ryan et al. 2001). B and T cells activated by APCs in the Peyer's patches move to the mesenteric lymph nodes, which are the largest lymph nodes in the body, located in the connective tissue connecting the intestines to the abdominal wall (Lea 2005). From the mesenteric lymph nodes, the B and T cells migrate through the lymph circulation system and differentiate into effector and memory cells at specific mucosal sites. The final localisation of the activated cells depends on the immunisation site, as discussed earlier. Memory B cells generate high affinity antigen receptors against the antigen to ensure a rapid immune response upon the next encounter. Activated $CD8^+$ cytotoxic T cells kill infected host cells, while activated $CD4^+$ T cells mediate antibody production, regulate immune responses and activate phagocytic cells of the innate immune system. $CD4^+$ T cells can further proliferate into T helper cells. Two main types of T helper cells exist, T helper 1 (T_H1) and T helper 2 (T_H2), which are regulated by cytokines secreted by cells of the innate immune system, but also reciprocally regulated by cytokines secreted by one another (Lea 2005).

The type of immune response generated by the mucosal immune system depends on the nature of the antigen, the APCs involved and the local microenvironment (Holmgren & Czerkinsky 2005). APCs detecting "danger signals" from pathogens initiate a T_H1 response, which mediates cellular immunity and stimulates bactericidal activity against intracellular agents infecting the host (such as viruses). A T_H2 cell response mediates a humoral immune response which stimulates B cell antibody production (such as secreted IgA (sIgA)) and mediate protection against helminthic parasites, allergens and soluble antigens (Lea 2005; Ryan et al. 2001). T_H2 cells are also typically generated towards non-pathogenic agents that should be tolerated, resulting in active suppression of systemic immunity and oral tolerance (Holmgren & Czerkinsky 2005).

1.6 Bacteria as live vectors for mucosal delivery of microbial antigens

Vaccination is one of the most effective strategies of combating infectious disease (Mielcarek et al. 2001). Subcutaneous, systemic immunisation is the most used method today, but an increasing amount of vaccines for administration through the mucosal route, especially oral and nasal, are becoming available. Oral delivery of vaccines holds great advantages relative to systemic delivery. Oral vaccines show less side effects, are able to modulate both mucosal and systemic immune responses, and immunisation through the mucosal route is much more easily performed (Bermudez-Humaran 2009; Mielcarek et al. 2001). Oral immunisation does not require sterile needles and syringes, and does thus not require trained medical personnel (Bermudez-Humaran & Langella 2010). Avoiding needles and syringes also lowers the risk of disease transmission between patients during immunisations. Furthermore, oral administration strongly reduces the costs of vaccination programs, and leads to increased consent from the patients (Giudice & Campbell 2006). These are all important factors for obtaining successful mass vaccination programs. A drawback of mucosal vaccines, especially orally administered vaccines, is that they require more and stronger doses of antigen to be effective, which increases the risk of tolerance (Bermudez-Humaran 2009).

The first attempts of creating vaccines using bacterial delivery vectors for mucosal delivery were made in the 1980s (Formal et al. 1981), using attenuated pathogenic microorganisms such as *Shigella, Listeria, Mycobacterium* and *Salmonella*. These bacteria are well adapted to interact with the mucosal surfaces and modulate an immune response, but they are not entirely safe for use in humans, as they can regain their pathogenic potential when present in the gut environment (Bermudez-Humaran et al. 2011). This is particularly relevant for children, elderly and immunosuppressed patients.

As an alternative to attenuated pathogens, non-pathogenic, recombinant food grade bacteria are now being developed as delivery vectors for therapeutic proteins (Tarahomjoo 2012). Lactococci and lactobacilli are excellent candidates as delivery vectors of mucosal vaccines, as they have a GRAS status (due to their extraordinary safety profile). As pointed out above, strains of these genera have safely been consumed as food preservatives since ancient times and are natural inhabitants of both food products and the human GI tract. Several strains of these genera also have adjuvant- and mucosal adhesive properties and low intrinsic immunogenicity (Pouwels et al. 1998). Interestingly, the use of recombinant bacteria also gives the possibility of designing multivalent vaccines, by displaying antigens from several

different infectious agents in the same bacterium. Such a strategy could eventually reduce the number of necessary vaccinations (Moore et al. 2001).

Most of the LAB-based recombinant vaccines constructed so far use *Lc. lactis* as the delivery vector, but there is an increasing focus on using *Lactobacillus spp.* instead. Lactobacilli have certain advantages over lactococci when it comes to vaccine delivery. Lactobacilli, especially *L. plantarum,* can persist longer in the GI tract than lactococci. Lactobacilli have a higher tolerance for acid and bile, enabling longer exposure of the antigen to the host and thus potentially increasing the immune response (Seegers 2002). Notably, too long and persistent exposure is not necessarily beneficial, as prolonged continuous contact with the antigen yields an increased risk of developing immunologic tolerance rather than obtaining an immune response (Lea 2005).

No recombinant LAB vaccines are yet commercially available, but several trials using mouse models show positive results. Bermudez-Humaran et al. (2005) and Ribelles et al. (2013) have successfully protected mice against human papillomavirus type 16-induced tumors using lactococci expressing the E7 antigen. Kajikawa et al. (2012) have successfully induced an immune response towards HIV in mice, using a *Lactobacillus acidophilus* strain coexpressing HIV-1 Gag and FliC from *Salmonella enterica* serovar Typhimurium. Marelli et al. (2011) documented prevention of infectious diarrhoea in mice using a *Lc. lactis* expressing rotavirus antigen. Vintini and Medina (2011) accomplished an enhanced antigen-specific immune response with a pneumococcal antigen expressed in *Lactobacillus casei*. In addition to vaccines towards infectious pathogens, vaccines are also being developed to fight different types of allergies (Ohkouchi et al. 2012).

It is now generally accepted that mucosal vaccines can elicit both mucosal and systemic immune responses, and that such novel vaccines could be advantageous over many existing vaccines. The main obstacle concerning mucosal vaccines are the difficulties in attaining high T-cell responses to gain long-term “immunological memory” for long-term protection, not just production of sIgA (Wells & Mercenier 2008). Through further research one hopes to overcome these obstacles and to create efficient mucosal vaccines for use in humans. One promising approach is to co-express cytokines with the antigen, to push the immune response into the desired direction (Bermudez-Humaran et al. 2005; Cortes-Perez et al. 2007; Steidler et al. 1998).

1.7 Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS)

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), has been the cause of death for close to 30 million people since the beginning of the epidemic in the early 1980's, and today, 34 million people are infected by HIV worldwide (WHO). There is no cure for the disease; however there are antiretroviral medications available which can prevent and delay development of AIDS for HIV infected individuals. Such antiretroviral treatment is resource-demanding and expensive; treatment is available in industrialised countries but scarcely available in Africa, where about 60 % of today's HIV infected people live. Furthermore, currently available antiretroviral medications may give harsh side effects and induce resistance and even intolerance in the patients. There is therefore a need for alternative treatment strategies, such as a prophylactic or therapeutic vaccine.

Most vaccines developed until now use an approach that mimics natural protective immunity by using inactivated or modified forms of the infectious agent. This approach cannot be used for HIV due to safety issues, and other approaches mimicking natural protective immunity have proven unsuccessful, much due to the virus' escape mechanisms for avoiding detection by natural protective immunity mechanisms. The HIV virus displays extreme genetic variability and the high mutation rate during replication complicates recognition by the adaptive immune system (Walker & Burton 2008). Upon infection, HIV also down regulates mechanisms important in HIV epitope display on the surface of infected cells, complicating recognition by the immune system even further (Kirchhoff 2010).

In 1984, the CD4 protein present on the surface of T-helper cells was proven to be the cell entry receptor for the virus (Dalgleish et al. 1984; Klatzmann et al. 1984), partly explaining why HIV infection results in loss of $CD4^+$ T-cells. $CD4^+$ T-cells are crucial in maintaining the function of the human immune system; depletion of $CD4^+$ T-cells thus disables efficient protection against infectious diseases and eventually results in AIDS (Grossman et al. 2006). Deng et al. (1996), immediately followed by several other research groups, later discovered that HIV also requires co-receptor CCR5 for cell entry. T-cells harbouring both receptors are thus the main target cells of HIV. $CD4^+$ T-cells that express co-receptor CCR5 constitute the major T-cell population of the gastrointestinal (GI) and genital mucosa, which is also the major entry point for an HIV infection. Upon infection, the virus infects the cells in the

mucosa very rapidly, before spreading the infection systemically through the lymphoid system. Despite this "perfect" infection strategy, the stage of initial mucosal infection is also the virus' most vulnerable stage (Haase 2010). Vaccines targeting mechanisms occurring during this stage are therefore believed to be the potentially most efficient, because they would prevent acquisition and systemic infection by the virus and save $CD4^+$ T-cells (Haase 2010). This can be done by developing "*a vaccine that can establish and maintain high-frequency, tissue-based, functionally differentiated $CD8^+$ T-cells at potential sites of infection*" (Picker et al. 2012). $CD8^+$ T-cells are the cytotoxic T cells mainly responsible for killing virus infected cells. Studies have shown that immunisation through the mucosal route, such as immunisation through the GI tract using LAB, can elicit mucosal immunity and development of mucosal cytotoxic ($CD8^+$) T-cells towards the antigen (Holmgren & Czerkinsky 2005; Kajikawa et al. 2012; Klavinskis et al. 1996; Staats et al. 2001). Delivering an HIV antigen in a bacterial vector can thus be a successful strategy to prevent HIV infection.

1.8 The HIV-antigen used in this study

The HIV antigen used in the present study is a truncated protein from the so-called "group specific antigen" (gag) p24 from HIV-1 (see below), fused to a murine version of the C-C motif chemokine ligand 17 (CCL17). CCL17 can bind to cells presenting CCR4, such as $CD4^+$ T cells, $CD8^+$ T cells, natural killer cells, macrophages and subsets of dendritic cells (Inngjerdingen et al. 2000; Ness et al. 2006; Sallusto & Lanzavecchia 1999). CCL17 has also been shown to comprise an important role in development of T-cells in the thymus (Alferink et al. 2003). The idea behind fusing CCL17 to truncated Gag p24 is that the chemokine, as part of the fusion protein attached to the bacterial cell envelope, will promote T-cell responses towards the delivered antigen.

In HIV, Gag p24 is the protein making up the capsid enclosing the RNA strands of the virus (shown as dark blue "beads" labelled "capsid" in Figure 1.6) The HIV-1 Gag p24 protein is an attractive HIV antigen for several reasons. The protein is present in large quantities in the virus and the amino acid sequence of Gag p24 is well conserved (Martinez-Picado et al. 2006). Upon virus infection, Gag p24 is processed and presented by MHC class I immediately after infection, even prior to virus replication start (Sacha et al. 2007). It has been shown that Gag p24 is able to elicit an immune response resulting in long-term memory

(Trumpfheller et al. 2006). Trumpfheller et al. (2006) proved that p24 was able to elicit a $CD8^+$ and $CD4^+$ T cell response in mice, and Ferre et al. (2010) showed that the immune response detected in mucosal tissues of HIV patients was dominated by a Gag-specific response. This is important because results from other studies suggest that responses of mucosal $CD8^+$ T-cells to HIV antigens are important in protection against HIV (Ferre et al. 2010) and delayed development of AIDS (Borghans et al. 2007; Schneidewind et al. 2008). Gag-specific immune responses were also associated with the immune system's ability to decrease and control the amount of active viruses in the blood (Kiepiela et al. 2007; Rolland et al. 2008).

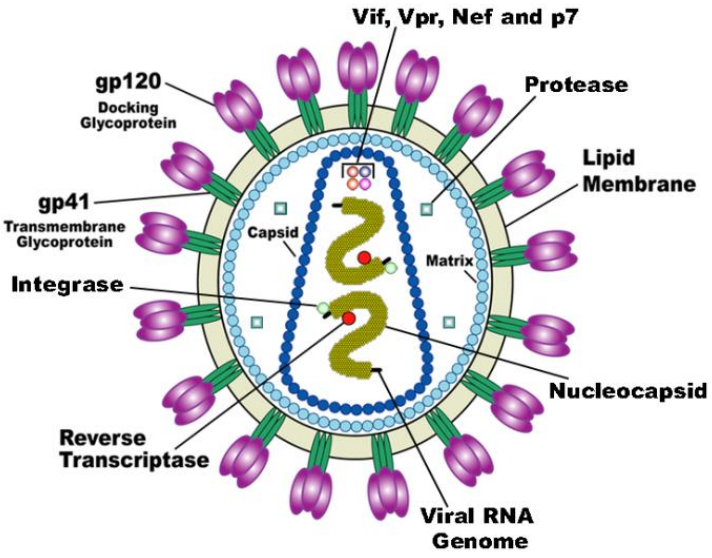


Figure 1.6: Schematic drawing of the human immunodeficiency virus (HIV). The figure is taken from US National Institute of Health (2005)

The vaccine protein was designed as a fusion between CCL17 and Gag p24 by Dr. I. Øynebråten (Centre for Immune Regulation, University of Oslo and Oslo University Hospital), and its DNA sequence was codon optimised for expression by *L. plantarum* by Dr. G. Mathiesen (Department of Chemistry, Biotechnology and Food Science, Agricultural University of Norway). The design of the fusion protein is shown in Figures 1.7 and 1.8. Hereafter, this fusion protein is referred to as HIVp24.

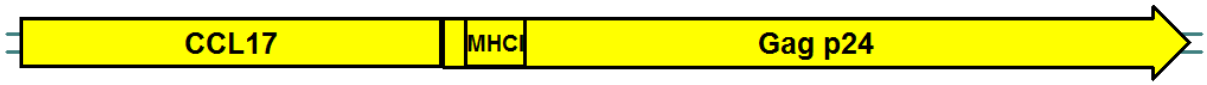


Figure 1.7: HIVp24. The figure depicts the gene encoding HIVp24, which comprises a CCL17 part and a Gag p24 part. The area labelled MHCI is the sequence encoding the part of the p24 protein presented by MHC class I in Balb/c mice.

ARATNVGRECCLDYFKGAIPIRKLVSWYKTSVECSRDAIVFLTVQGKLICADPKDKHVKKAIRLV
KNPRPGGHQAAMQMLKETINEEAAEWDRVHPVHAGPIAPGQMREPRGSDIAGTTSTLQEQIGW
MTNNPPIPVGEIYKRWIILGLNKIVRMYSPTSILDIRQGPKEPFRDYVDRFYKTLRAEQASQEVKN

Figure 1.8: Amino acid sequence of HIVp24. The region marked purple is the amino acid sequence of murine CCL17. Yellow labelled sequence is the amino acid sequence recognised by MHC class I in mice, enabling induction and evaluation of HIV-1 gag specific $CD8^+$ T cells in mice. The green labelled sequence is the sequence identified by Kaufmann et al. (2004) which is thought to be important in protection against AIDS development. The amino acids labelled red constitute a sequence binding to HLA alleles associated with good control of the virus (see text).

The HIV-1 Gag p24 sequence used in the present study is truncated to retain a total protein size compatible with bacterial expression. The expressed part was designed such that it contains epitopes known to play important roles in immune responses in mice (as the vaccine is to be tested in a mouse model), as well as epitopes important for human immune responses (as the vaccine is intended for human use). The area chosen is depicted in Figure 1.8, and contains three peptide regions proven to be important epitopes. The region marked yellow is the antigen recognised by MHC class I in mice (Mata et al. 1998; Trumpfheller et al. 2006), and is marked "*best defined CTL/$CD8^+$ T cell peptides*" in the Los Alamos-database (HIV database). Recognition of epitopes by some MHC alleles has been proven to be very important in control of HIV and control of AIDS progression, the most important alleles being HLA-B*5701 and HLA-B*5801 (Migueles & Connors 2010). The red sequence is an epitope presented through these MHC alleles. Cytotoxic $CD8^+$ T-cells recognising epitopes displayed on these MHCs show a superior ability to recognise virus infected cells despite low density of surface antigens, and show effector functions at lower antigen concentrations than low-avidity cytotoxic T-cells (Ferre et al. 2010). The green labelled sequence is an epitope identified by Kaufmann et al. (2004). They found that HIV patients eliciting a good T-cell response to this particular sequence show good protection against AIDS development.

1.9 The goals of this study

The aim of this study was to investigate expression and anchoring of HIVp24 in *L. plantarum* WCFS1 with the ultimate goal to construct a potential HIV vaccine. Expression and anchoring was performed using the inducible gene expression system pSIP, originally developed for efficient intracellular production of proteins in *L. plantarum* and *L. sakei* (Sørvig et al. 2003).

Antigen localisation in the bacteria (cytoplasmic, secreted or anchored) seems to have a high influence on the immune response. The results of previous studies indicate that antigens displayed on the bacterial cell surface have a stronger immunogenic effect than antigens that are secreted or produced in the cytoplasm (Bermudez-Humaran et al. 2004; Norton et al. 1996). But not all studies show the same results, and it is still being debated which antigen localization yields the best immune response in the case of mucosal immunisation (Wells & Mercenier 2008). Because experiments previously conducted in our lab also had suggested that surface-located antigens yield the best immune response, the focus of the present study was to investigate different anchors and anchor mechanisms. There is a lot of potential variation in "surface localization", which will affect the extent to which the antigen is displayed, which again may affect immune responses (Fredriksen et al. 2012).

The final goal was to investigate whether the engineered lactobacilli strains could stimulate an immune response against the HIV antigen. The immune responses were evaluated in a mouse model.

The work was carried out in the following four steps:

1. Initially, the ability of *L. plantarum* WCFS1 to produce the HIVp24 fusion protein linked to homologous *L. plantarum* protein anchors was evaluated using derivatives of the pSIP system (Mathiesen et al. 2004; Sørvig et al. 2003). The HIVp24 fusion protein was attached to the cell surface using anchors binding covalently to the cell wall or to the cell membrane, and an anchor binding non-covalently to the cell wall.
2. Previous studies (Maudal 2012; Sørvig et al. 2005a) had suggested that a higher plasmid copy number and higher transcription- and expression levels of the target gene could be obtained when the $pUC(GEM)_{rep}/256_{rep}$ replicons are exchanged with the $SH71_{rep}$ replicon. The exchange of replicon was performed by replacing $pUC(GEM)_{rep}/256_{rep}$ with $SH71_{rep}$ in pSIP vectors.

3. Production of the target gene in bacteria hosting plasmids derived from both pSIP vector variants (256_{rep}/pUC$(GEM)_{rep}$ or $SH71_{rep}$) was evaluated by analyzing the presence of HIVp24 in total cell extracts and on the bacterial cell surface, using immunological imaging methods.
4. Finally, the immunogenicity of the transgenic bacteria was evaluated in a mouse model. Immunological responses were monitored by analysing lymphocytes from the spleen, Peyer's patches and mesenteric lymph nodes of immunised mice in regard of cytokine production and the number of IFN-γ producing cells.

2 MATERIALS

2.1 Laboratory equipment

Laboratory equipment	Supplier
Amicon® Ultra Centrifugal Filters	Millipore
ART® Aerosol resistant pipette tips	Promega
Pipette tips, next generation tip refill	VWR
CL-Xposure™ Film	Thermo Scientific
Corex® tubes	Life Sciences
Cuvettes	
Disposable cuvettes, 1.5 ml	Brand
Electroporation cuvettes, Gene Pulser® 0.2 cm	Bio-Rad
DNA gel electrophoresis tanks	Bio-Rad
SDS-PAGE tanks	Invitrogen
Western blotting tank	Bio-Rad
Eppendorf tubes	
Regular 1.5 ml	Axygen
PCR tubes	Axygen
2 ml tubes	Axygen
Falcon 2059 Polypropylene Round Bottom tubes	Bacton Dickinson
FastPrep® tubes and lids	MP Biomedicals
Forceps	
Glass beads	Sigma
Microscope slides	Thermo Scientific
Microscope cover glass (confocal)	
Nitrile gloves	VWR
Callstar® tubes, 15 ml and 50 ml	Greiner bio-one
Petri Dishes	
Poly-l-lysine coated cover glass	
PVDF membrane (0.2 µm pore size)	Millipore
Stainless steel surgical blade	Swan-Morton
Sterile filters, 0.20 µm pore size	Starstedt
Super PAP Pen, Large tip	Chemi-Teknik as
Syringes, 10-50 ml	Plastipak
Syringe needle BD Microlance™ 3 8.8 x 40 mm	Becton Dickinson S.A
Tooth pics	
Varous glass equipment	Labsystems
Waterbaths	Julabo

Instruments	Supplier
Bio-Rad Gene Pulser II	Bio-Rad
Bio-Rad Pulse Controller plus	Bio-Rad
Centrifuges	
Biofuge pico	Heraeus
Eppendorf centrifuge 5415 R	Eppendorf
Eppendorf centrifuge 5430 R	Eppendorf
Table centrifuge Galaxy Mini	VWR
Vacuum centrifuge	Maxi dry lyo + Savant
Countess® Automated Cell Counter	Invitrogen
Electricity suppliers	
PowerPac™ Basic	Bio-Rad
PowerPac™ 300	Bio-Rad
FastPrep®-24	MP Biomedicals
ImmunoSpot®	CLT
Incubators	
Multitron eco	Infors
Incubator	Fermaks
MacsQuant® Analyser	Miltenyi Biotec
Magnet stirrer, IKA® RCT classic	VWR
MS2 Minishaker (vortex)	Tamro LAB
PCR-machines	
Mastercycler gradient	Eppendorf
PCR-machine	VWR
pH-meter	Metrohm
Photo equipment	
GelDoc machine	Bio-Rad
Qubit™ Fluorometer	Invitrogen
	Millipore
SNAP i.d. Protein Detection System	
Sterile hood	Telstar

Software	Supplier
CLC DNA Workbench 5	CLC bio
ExPASy	http://web.expasy.org/translate/ http://web.expasy.org/protparam/
MacsQuantify™ Software	Miltenyi Biotec
pDRAW32	www.acaclone.com

2.2 Chemicals

Chemicals	Supplier
Acetic acid	Sigma
Acetone	VWR
Agar (Bacto)	Saveen Werner
Ampicillin	Sigma
Calcium chloride, $CaCl_2$	SDS
Disodium hydrogen phosphate, Na_2HPO_4	Merck
Dithiothreitol (DTT)	Sigma
EDTA, $C_{10}H_{16}N_2O_8$	Merck
Erythromycin, $C_{37}H_{67}NO_{13}$	Sigma
Ethanol, C_2H_5OH	Arcus
Ethidium Bromide, EtBr	Sigma
Glucose, $C_6H_{12}O_6$	VWR
Glutaraldehyde, $OHC(CH_2)_3CHO$	Sigma
Glycerol, $C_3H_8O_3$	Merck
Glycine, $C_2H_5NO_2$	Duchefa Biochemie
Kanamycin	Sigma
LE agarose	Lonza
Magnesium chloride, $MgCl_2$	Merck
Magnesium sulphate, $MgSO_4$	Sigma
Methanol, CH_3OH	Merck
Paraformaldehyde, $HO(CH_2O)_nH$	Sigma
Phenylmethanesulfonylfluoride (PMSF)	Sigma
Polyethylen Glycol, PEG_{1450}	Aldrich
Potassium chloride, KCl	Riedel-de Haën
Potassium dihydrogen phosphate, KH_2PO_4	Merck
Sodium acetate, $NaC_2H_3O_2x3H_2O$	Sigma
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, $C_4H_{11}NO_3xHCl$	Sigma
Tween-20	Sigma

2.3 Proteins and enzymes

Protein/enzyme	Supplier
Antibodies	
mCCL17/TARC Biotinylated antibody (Goat anti-mouse IgG)	R&D systems
Rabbit anti-goat IgG (H+L)-HRP	SouthernBiotech
Alexa Fluor® 488 donkey anti-goat	Invitrogen
Bovine serum albumin (BSA)	Sigma
Lysozyme	Sigma
Mutanolysine	Sigma
Inducer peptide (SppIP)	Sigma
MagicMark™ XP Western standard	Invitrogen
Phusion High Fidelity DNA polymerase (with 5x Phusion HF buffer)	Finnzymes
Restriction buffers	
NEBuffer 1	NEB
NEBuffer 2	NEB
NEBuffer 3	NEB
NEBuffer EcoRI	NEB
Restriction enzymes	
Acc65I	NEB
AgeI	NEB
BamHI	NEB
EcoRI	NEB
HindIII	NEB
MluI	NEB
SalI	NEB
T4 DNA ligase (with T4 DNA ligase buffer	NEB
TaqDNA Polymerase Master Mix Red	VWR
Quick T4 DNA ligase (with 2x Quick ligation buffer)	NEB

2.4 DNA

DNA	Supplier
dNTP-mix	NEB/Invitrogen
GeneRuler™ 1kb DNA ladder	Fermentas

2.5 Primers

Table 2.1 shows the sequences of the primers used, whereas Table 2.2 describes the purpose of each primer.

Table 2.1: Primer sequences. Restriction sites are underlined

Name	Sequence	Restriction enzymes
0373F	CATATGTATACGGAAAACACGGGGAAAC	
Cwa2-Acc65IR	ATATAGGTACCTCAAGCACGACGGCGATAA	Acc65I
GagF	GTCGACGCTCGTGCTACT	
GagR	GAATTCTTAGTTCTTAACTTCTTGTGAAGCTTGTTC	EcoRI, HindIII
GagS-Acc65IR	ATATAGGTACCTCAGTTCTTAACTTCTTGTGAAGCTTGTTCA	Acc65I
SekF	GGCTTTTATAATATGAGATAATGCCGAC	
SH71F	ATAAGAATTCGGTACCCCGGGTT	EcoRI, Acc65I
SH71R	GCACTATCAACACACTCTTAAGTT	
SeqGagpF	ATGCGTGAACCACGTGGTTCAGATATC	
SeqRep2F	GGGGGGAAAGGAAGCGAAT	
SeqRep3F	GGCTATTAAATACTAAAGCTAGAAATTTTGGAT	
SeqRepF	CCGCGTCTTTGATCGCGTC	
SeqSH71R	AGCGCTACCGCTCGGCAAAAT	

Table 2.2: Primer descriptions

Name	Description
0373F	Forward primer for control PCR of constructs with the 0373 signal peptide
Cwa2-Acc65IR	Reverse primer used in PCR to add a restriction site after cwa2
GagF	Forward primer for HIVp24-containing sequences, used for PCR and sequencing
GagR	Reverse primer for HIVp24-containing sequences, used for PCR and sequencing and change of a restriction site from MluI to EcoRI
GagS-Acc65IR	Reverse primer used in PCR to construct a plasmid encoding secreted HIVp24
SekF	Forward primer for sequencing of all constructs in pSIP based vectors
SH71F	Forward primer for plasmids containing the SH71 $_{rep}$ replicon, used in both PCR and sequencing
SH71R	Reverse primer for sequencing of all constructs in pSIP based vectors
SeqGagpF	Forward primer for sequencing of all constructs containing HIVp24
SeqRep2F	Forward primer for sequencing of all constructs containing the $SH71_{rep}$ replicon
SeqRep3F	Forward primer for sequencing of all constructs containing the $SH71_{rep}$ replicon
SeqRepF	Forward primer for sequencing of all constructs containing the $SH71_{rep}$ replicon
SeqSH71R	Reverse primer for sequencing of all constructs containing the $SH71_{rep}$ replicon

2.6 Bacterial strains and plasmids

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

Table 2.3: Bacterial strains

Strain	Source of reference
***Escerichia coli* TOP10**	Invitrogen
***Escerichia coli* TG1**	Stratagene
***Lactobacillus plantarum* WCFS1**	(Kleerebezem et al. 2003)
***Lactococcus lactis* MG1363**	(Wegmann et al. 2007)

Table 2.4: Plasmids used or constructed in the presented work

Plasmid	Description	Source of reference
pUC57-HIVp24	Vector containing a synthetic gene encoding HIVp24: the antigen is codon optimised for *L. plantarum*.	GenScript, USA
pLp_0373OFAcwa2	pSIP401 derivative containing OFA N-terminally fused to the 0373 signal peptide and C-terminally fused a medium-length version of the LPxTG anchor from Lp_2578.	Fredriksen et al. (2010)
pLp_1261Inv	Derivative of pLp_2588sAmy, containing the lipoanchor Lp_1261 C-terminally fused to *Inv*	Fredriksen et al. (2012)
pLp_3014Inv	Derivative of pLp_2588sAmyA, harbouring the LysM-domain anchor Lp_3014 C-terminally fused to *Inv*	Fredriksen et al. (2012)
pSIP-EV	pSIP401 derivative without target gene	Fredriksen et al. (2012)
pCR®-Blunt II-TOPO	Vector for subcloning of PCR fragments	Invitrogen™
pLp_0373HIVp24cwa2	pLp_073OFAcwa2 derivative with HIVp24 instead of OFA	This work
pLp_1261HIVp24	pLp_1261Inv derivative with HIVp24 instead of *Inv*	This work
pLp_3014HIVp24	pLp_3014Inv derivative with HIVp24 instead of *Inv*	This work
pLp_0373HIVp24	pLp_3073HIVp24cwa2 derivative with the cwa2 anchor sequence removed	This work
pLp_0373NucASH71	Plasmid containing the $SH71_{rep}$ replicon and NucA N-terminally fused to the 0373 signal peptide	Kristina Maudal, 2012
pSIP411	pSIP401-derivative, harbouring GusA as target gene	Sørvig et al. (2005a)
pLp_1261HIVp24SH71	pLp_1261HIVp24 derivative with $SH71_{rep}$ replicon instead of 256 replicon	This work
pLp_3014HIVp24SH71	pLp_3014HIVp24 derivative with $SH71_{rep}$ replicon instead of 256 replicon	This work

2.7 Kits

Kit	Supplier
iBlot™ 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	
Mini Trans-Blot cell	Bio-Rad
Buffer tank	
Gel holder cassette	
Bio-Ice™ cooling unit	
Fiber pads	
Filter paper	

NucleoSpin® Extract II	Marcherey-Nagel
NucleoSpin® Extract II Columns with 2 ml collection tubes	
Binding buffer NT	
Wash buffer NT3	
Elution buffer NE	
NucleoSpin® Plasmid Miniprep Kit	Marcherey-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	
NuPAGE® Novex Bis-Tris Gels	Invitrogen
10 % Bis-Tris, 8 cm x 8 cm x 1 mm, 10 wells	
NuPAGE® LDS Sample buffer (4x)	
NuPAGE® reducing agent (10x)	
MES Running Buffer	Invitrogen
MOPS Running Buffer	
Pellet Paint® Co- Precipitant	Novagen
Pellet Paint® Co- Precipitant	
3M Sodium acetate, pH 5.2	
Qubit™ dsDNA BR Assay	Invitrogen
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)	
Restore™ Western Blot Stripping Buffer	Pierce
SNAP i.d Protein Detection System	Millipore
SNAP i.d Single Well Blot Holder	
SNAP i.d Spacer	
SNAP i.d. Blot Roller	
Filter paper	
SuperSignal® West Pico Chemiluminescent Substrate	Pierce
Luminol/Enhancer solution	
Stable Peroxide Solution	

Zero Blunt® TOPO® PCR Cloning Kit — Invitrogen
- pCR®-Blunt II-TOPO®
- dNTP mix
- Salt solution (1.2M NaCl, 0.06M $MgCl_2$)
- M13 Forward primer
- M13 Reverse primer
- OneShot TOP10 chemically Competent *E. coli* cells

Dark room reagents
- Film cassette
- Developer solution
- Fix solution

2.8 Agars and media

Medium		Supplier
BHI (Brain-Heart-Infusion)	Medium: 37 g BHI dH_2O to 1 litre Sterilised in a Certoclav for 15 min at 115 ºC. For solid medium: add 15 g agar (1.5 % (w/v) before autoclaving. After cooling to ~ 60 ºC, appropriate antibiotics were added and the medium was poured into petri dishes.	Oxoid
GM17	Medium: 37.25 g M17 dH_2O to 1 litre Sterilised in a Certoclav for 15 min at 121 ºC. After cooling to ~ 60 ºC, 12.5 ml 40 % (w/v) filter sterilised glucose was added to reach a concentration of 0,5 % For solid medium: add 15 g agar (1.5 % (w/v) before autoclaving. After cooling to ~ 60 ºC, glucose and appropriate antibiotics were added and the medium was poured into petri dishes.	
MRS (de Man, Rogosa, Sharpe)	Medium: 52 g MRS dH_2O to 1 litre Sterilised in a Certoclav for 15 min at 115 ºC For solid medium: add 15 g agar (1.5 % (v/v) before autoclaving. After cooling to ~ 60 ºC, appropriate antibiotics were added and the medium was poured into petri dishes.	Oxoid

MRSSM medium	52 g MRS 171 g sucrose (0.5 M) 2 g $MgCl_2x6H_2O$ (0.1 M) dH_2O to 1 litre Filter sterilised (0.22 µm pore size)	Oxoid
RPMI medium (Roswell Park Memorial Institute)	Culture medium for human leucocytes. Ready-made from the producer.	Gibco
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 g $MgSO_4$ 2 ml 1 M glucose dH_2O to 100 ml Filter sterilised (0.22 µm pore size)	
SGM17	GM17 (as described above) 0.5 M sucrose (filter steralised before added)	
2x YT medium	16 g Bacto™ Tryptone 10 g Bacto™ yeast extract 5 g NaCl dH_2O to 1 litre Sterilised in a Certoclav for 15 min at 115 ºC	

2.9 Buffers and solutions

Buffer/solution	Content
GTE	50 mM glucose 25 mM Tris-HCl, pH 8 10 mM EDTA, pH 8
PBS (Phosphate Buffered Saline), pH 7.4	8 g/l NaCl 0.2 g/l KCl 1.44 g/l Na_2HPO_4 0.24 g/l KH_2PO_4
TAE (Tris-acetate with EDTA) 50 x	242 g Tris base 57.1 ml Acetic acid 100 ml 0.5 M EDTA, pH 8 dH_2O to 1 litre

TBS (Tris Buffered Saline), pH 7.4	150 mM NaCl 10 mM Tris-HCl, pH 8
TE-buffer	30 mM Tris-HCl, pH 8 1 mM EDTA, pH 8
Towbin-buffer	25 mM Trisma base 192 mM glycine 20 % (v/v) methanol
TPBS	PBS 0.1 % (v/v) Tween-20
TTBS	TBS 0.1 % (v/v) Tween-20

3 METHODS

3.1 Growing of bacterial cultures

Different bacteria demand different growth conditions and nutrients. To obtain the best possible growth, all bacteria used in the present study were cultivated with the most optimal media and growth conditions. The plasmids used have an antibiotic resistance gene, and to ensure selection for recombinant bacteria only, the appropriate antibiotic was added to the growth medium. Further details are given below.

Cultivation of *Escherichia coli*

Escherichia coli cells were grown in Brain-Heart-Infusion (BHI) medium at 37 °C over night. Cells in liquid medium were incubated in a shaker heating cabinet and cells grown on solid medium were incubated without shaking. To solidify the medium, 1.5 % (w/v) of agar was added. The following antibiotic concentrations were used:

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

Cultivation of *Lactobacillus plantarum*

L. plantarum cells were grown in de Man, Rogosa, Shrape (MRS) medium at 37 °C over night. Cells in liquid medium and on solid medium were incubated without shaking. To solidify the medium, 1.5 % (w/v) of agar was added. The following antibiotic concentrations were used:

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

Cultivation of *Lactococcus lactis*

L. lactis cells were grown in GM17 medium at 30 °C over night. Cells in liquid medium and on solid medium were incubated without shaking. To solidify the medium, 1.5 % (w/v) of agar was added. The following antibiotic concentrations were used:

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

3.2 Long term storage of bacteria

For long term storage of bacteria, 1 ml of bacterial culture was mixed with 300 µl of 85 % (v/v) glycerol before storage at -80 ºC. This treatment prevents cell disruption and allows indefinite storage. To grow bacteria from a glycerol stock, a sterile toothpick was used to scrape off a small amount of frozen culture, which was used to inoculate the appropriate growth medium.

3.3 Plasmid isolation

3.3.1 Isolation of plasmids from *E. coli* TOP10

Isolation of plasmids from *E. coli* TOP10 cells was done using the NucleoSpin® Plasmid Miniprep Kit:

Materials:

NucleoSpin® Plasmid Miniprep Kit, Marcherey-Nagel (section 2.7)

Procedure:

The procedure was performed as described in the protocol included in the kit. All centrifugations were performed at room temperature.

1. 1 ml overnight culture was spun down in an eppendorf tube at 11,000 x g for 30 sec
2. Medium was discarded and the pellet resuspended in 250 µl resuspension buffer A1 w/RNase A.
3. The cells were then lysed by adding 250 µl of lysisbuffer A2 and mixed gently by inverting the tube 6-8 times, the tube was incubated for 5 minutes at room temperature.
4. 300 µl of precipitation buffer A3 was added to the clear lysate to precipitate cell components. The solution was mixed gently by inverting the tube 6-8 times.
5. To remove the cell debris and clear the lysate, the lysate was centrifuged at 11,000 x g for 5-10 min.
6. The clear lysate was applied onto a NucleoSpin® Plasmid Column in a 2 ml collection tube and centrifuged at 11,000 x g for 1 min. The DNA was now bound to the silica membrane and the flow through was discarded.

7. To remove nucleases, 500 µl of wash buffer AW (preheated to 50 °C) was run through the column, by centrifuging 1 min at 11,000 x g, and then discarded.
8. 600 µl A4 wash buffer with ethanol was run through by centrifuging for 1 min at 11,000 x g to remove salts and secondary metabolites. The flow through was discarded and the membrane was "dried" by centrifuging for 2 min at 11,000 x g to dispose of all ethanol.
9. The NucleoSpin® Plasmid Column was moved to a clean eppendorf tube and 50 µl elution buffer AE (preheated to 70 °C) was added. The column was incubated for 1 min at room temperature before centrifugation for 1 min at 11,000 x g. DNA was now eluted in the 50 µl AE and collected in the eppendorf tube. The DNA was either used directly or stored at -20 °C.

3.3.2 Isolation of plasmids from *E. coli* TG1

Isolation of plasmid from *E. coli* TG1 followed the same procedure as for *E. coli* TOP10. *E. coli* TG1 contains nucleases that degrade the plasmid after isolation. The plasmid consequently needs extra treatment post isolation. Isolated plasmid was treated with trypsin to digest the nucleases and thus avoid plasmid degradation.

Materials:

Trypsin

Plasmid DNA

Procedure:

1. 2 µl of trypsin was added to 50 µl of isolated plasmid and the reaction mixture was incubated in a water bath at 37 °C for 1.5 hours (maximum 2 hours).
2. The trypsin was removed by PCR-cleanup as described in section 3.4.3.
3. The plasmid was then used directly in digestion reactions or stored at -20 °C for later use.

3.3.3 Isolation of plasmids from *L. lactis* MG1363

Gram positive bacteria have a substantial outer layer of peptidoglycan, and thus demand a different approach for cell lysis than Gram negative bacteria. *L. lactis* MG1363 cells were therefore pretreated with lysozyme and mutanolysin to partly degrade the cell wall prior to plasmid isolation.

Materials:

GTE-buffer (section 2.9)

Lysozyme, 100 mg/ml

Mutanolysin, 5000 U/ml in 0.1 M potassium-phosphate buffer, pH 6.2. Filter sterilised.

RNase, 10 mg/ml, boiled for 10-20 minutes to eliminate DNases and then cooled for renaturation of the RNase

Procedure:

1. 10 ml of bacterial culture was centrifuged at 5,200 x g for 7 minutes to pellet the cells.
2. The supernatant was discarded and the pellet washed with 500 µl GTE-buffer by pipetting up and down and re-centrifugation. The supernatant, with buffer and medium remains, was discarded.
3. The cell pellet was re-suspended in a mixture of 120 µl lysozyme (100 mg/ml), 100 µl GTE-buffer, 25 µl RNase (10 mg/ml) and 3 µl Mutanolysin (5000 U/ml). The cell suspension was then incubated in a water bath at 37 °C for at least 1 hour and vortexed several times throughout the incubation.
4. The remaining steps were performed using the Nucleospin® Plasmid Miniprep Kit from the step where buffer A2 is added (see section 3.3.1). DNA was eluted in 30 µl elution buffer instead of 50 µl.

3.4 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique for *in vitro* amplification of DNA from minimal amounts of DNA. The technique uses oligonucleotides called primers, dNTPs and a thermo-stable DNA polymerase. The primers are designed to bind complementary to the start and end points of the DNA fragment that is to be amplified.

The reaction consists of the following three steps and is performed in a thermal cycler:

- Denaturation: the reaction solution is heated to 98 °C; consequently, the double stranded DNA is denatured to form single stranded DNA.
- Annealing: the reaction solution is cooled down to allow specific binding of the primers to the single stranded DNA.
- Extension: the temperature is risen to the optimal working temperature for the DNA polymerase to allow extension of new DNA strands from the primers along the template strand.

These steps are repeated 25-30 times depending on the amount of initial template.

PCR was used for control of and detection of recombinant bacterial colonies and for amplification of DNA fragments for construction of plasmids. Two different PCR kits were used: Phusion PCR has a very high proofreading capacity and was used for construction of plasmids. Taq PCR is more robust to set up, but has a poorer proofreading mechanism. The Taq PCR master mix contains loading buffer which allows direct loading to an agarose gel post PCR completion. Taq PCR was thus used for detection and control of recombinant bacteria. Details of the two methods are provided below.

3.4.1 Phusion PCR

1. The PCR reaction was carried out as suggested by the Phusion High Fidelity DNA polymerase supplier (Finnzymes). A standard reaction setup is shown in table 3.1.

Table 3.1: PCR-reagents of Phusion PCR

Component	Volume	Final concentration
dH_2O	To 50 µl	
5x Phusion HF buffer	10 µl	1x
10 mM dNTPs	1 µl	200 µM
Forward primer (50 pmol/µl)	1 µl	1 pmol/µl
Reverse primer (50 pmol/µl)	1 µl	1 pmol/µl
Template DNA	1 µl	~ 200 ng
Phusion DNA Polymerase	0.5 µl	

2. The reagents were mixed and placed in a thermal cycler. The cycler was programmed according to the setup shown in table 3.2.

Table 3.2: PCR-settings of Phusion PCR

Cycling step	Temperature	Time	Cycles
Initial denaturation	98 °C	30 seconds	1
Denaturation	98 °C	5 seconds	
Annealing	X °C*	10 seconds	25
Extension	72 °C	15-30 s/kb	
Final extension	72 °C	5-10 minutes	1
Storage	10 °C	Hold ∞	

* The temperature was set to 2-5 °C below the average melting point of the primers used

3. The resulting reaction mixture with the PCR product was used directly or stored at -20 °C.

3.4.2 Taq PCR

1. The PCR reaction was carried out as suggested by the supplier of the TaqDNA Polymerase Master Mix Red (VWR). A standard reaction setup is shown in table 3.3.

Table 3.3: PCR-reagents of Taq PCR

Component	Volume	Final concentration
Taq master mix Red (2x)	25 µl	1x
Forward primer (50 pmol/µl)	1 µl	1 pmol/µl
Reverse primer (50 pmol/µl)	1 µl	1 pmol/µl
Template DNA	1 µl/toothpic	
dH_2O	To 50 µl	

2. The reagents were mixed and placed in a thermal cycler. The cycler was programmed according to the setup shown in table 3.4.

Table 3.4: PCR-settings of Taq PCR

Cycling step	Temperature	Time	Cycles
Cell lysis and initial denaturation	94 °C	3 minutes	1
Denaturation	94 °C	1 minute	
Annealing	X °C*	2 minutes	30
Extension	72 °C	3 minutes	
Final extension	72 °C	10 minutes	1
Storage	10 °C	Hold ∞	

* The temperature was set to 2-5 °C below the average melting point of the primers used

3. The resulting reaction mixture with the PCR product was used directly or stored at -20 °C.

3.4.3 PCR-cleanup

PCR-products to be used directly without first being run through agarose gel electrophoresis were purified using a "PCR-cleanup"-step. For direct use, one needs to remove all the reagents of the PCR from the DNA. The cleanup procedure follows the same protocol as for Agarose gel extraction, with the NucleoSpin® Extract II kit (see section 3.5.2), starting at the step where buffer NT is added (step 1 in section 3.5.2). 100 µl NT buffer was used per 50 µl PCR reaction.

3.5 Agarose gel electrophoresis

Agarose gel electrophoresis is a technique used to separate DNA fragments according to size using an electrical current. It is also used to verify the quality and quantity of DNA obtained from a PCR reaction or from plasmid isolations. When an electrical current is applied, DNA will move through the gel towards the positive pole and fragments will be separated according to size (bp). The DNA strands are visualised by adding ethidium bromide (EtBr) to the gel solution prior to casting. By also applying a standard sample, containing DNA fragments of known size ("ladder") to one of the wells in the gel, the approximate sizes of the separated fragments can be decided. In this study, all fragments were run on 1.2 % agarose gels.

3.5.1 Agarose gel electrophoresis

Materials:

LE agarose

1xTAE buffer (section 2.9)

10 mg/ml ethidium bromide (EtBr)

Loading dye (10x)

DNA ladders

Procedure:

1. A 1 l stock solution of 1.2 % agarose gel was made by adding 12 g of LE agarose to 1 l 1xTAE buffer.
2. The mixture was autoclaved at 115 °C for 15 minutes, inverted for homogenisation and then stored at 60 °C.
3. Gels were made using 60 ml agarose solution with 1 µl of 10mg/ml EtBr.
4. The EtBr-containing agarose solution was casted in a gel rack, combs were inserted to make wells, and the gel was then set to solidify for 20 minutes.
5. After removing the combs, the gel was placed in an electrophoresis chamber and covered with 1xTAE buffer.
6. The appropriate ladder and DNA samples mixed with loading buffer were added to the wells. An electric current of 90 V was applied for 30-90 minutes.
7. The DNA fragments in the gel were visualised by UV light using a GelDoc system.

3.5.2 DNA extraction from agarose gel

DNA fragments separated by gel electrophoresis were excised from the gel using a scalpel and put in an eppendorf tube. The gel fragments were stored at -20 °C or extracted directly using the NucleoSpin® Extract II kit from Marcherey-Nagel.

Materials:

NucleoSpin® Extract II kit from Marcherey-Nagel (section 2.7)

Procedure:

1. The exact weight of the gel fragment was determined and the gel piece was solubilised by adding 200 µl NT buffer per 100 mg of agarose gel followed by incubation in a 50 °C water bath for 5-10 minutes. The mixture was vortexed 2-3 times during this incubation.
2. The solubilised gel-buffer mix was applied to a NucleoSpin® Plasmid Column in a 2 ml collection tube and centrifuged at 11,000 x g for 1 min. The DNA was now bound to the silica membrane. The flow through was discarded.
3. The column was washed by adding 600 µl of NT3 buffer followed by centrifugation for 1 min at 11,000 x g.

4. The flow through was discarded and the membrane was dried by centrifuging for 2 min at 11,000 x g to dispose of all remaining ethanol.
5. The NucleoSpin® Plasmid Column was moved to a clean eppendorf tube and 30-50 µl elution buffer NE was added. The column was incubated for 1 min at room temperature before centrifuging for 1 min at 11,000 x g. DNA was now eluted in the NE buffer and collected in the eppendorf tube. The DNA was either used directly or stored at -20 °C.

3.6 TOPO® cloning and transformation of *E. coli*

Zero Blunt ® TOPO® cloning was performed on blunt-end PCR products generated according to section 3.4.1, after extracting these products from an agarose gel according to section 3.5.2. The advantages of cloning the PCR products are that this allows long term storage of the PCR product (in a recombinant *E. coli*) and eliminates the need of constant reamplifying the template DNA. Restriction enzyme digestion of the PCR product for further cloning is also easier performed using a TOPO® vector isolated from *E. coli* than by direct digestion of a fresh PCR product. The principle of TOPO® cloning is depicted in Figure 3.1.

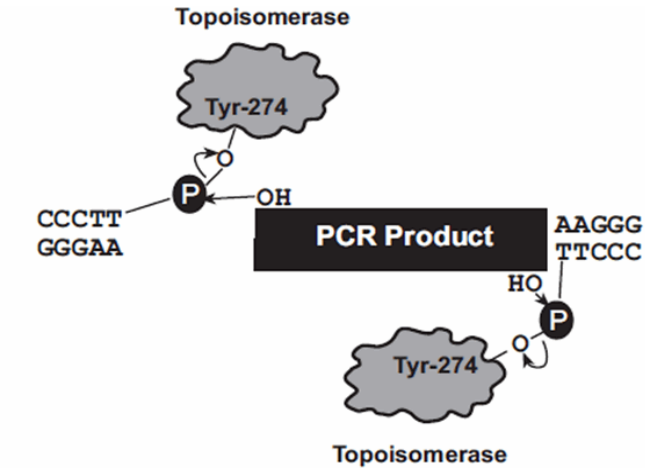


Figure 3.1: Principle of TOPO® cloning. The figure depicts the mechanism behind TOPO® cloning. The enzyme binds and cleaves the DNA strand at specific sites (Shuman 1991). It breaks the phosphodiester bond after 5'-CCCTT, and energy yielded from the reaction is stored in a covalent bond formed between the 3' phosphate of the cleaved strand and a tyrosyl residue of the enzyme. The covalent bond is broken by attack of the 5'OH of the insert DNA, which creates a new phosphodiester bond between the 3'-end of the TOPO® vector and the 5'-end of the blunt PCR product (Shuman 1994). The pCR®II-Blunt-TOPO® vector supplied by Invitrogen is already linearised with Topoisomerase I, which is covalently attached to the 3'-ends of each vector DNA strand. The figure is taken from the Zero Blunt® TOPO® PCR Cloning Kit protocol (Invitrogen™)

Zero Blunt ® TOPO® cloning was performed using the kit provided by Invitrogen, which utilises Topoisomerase I from *Vaccinia* virus.

Materials:

Fresh PCR-product

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

Sterile dH_2O

pCR®II-Blunt-TOPO®

Chemo-competent *E. coli* TOP10 cells

S.O.C.-medium

BHI agar plates with kanamycin (100 µg/ml)

Procedure:

1. Reagents were mixed in an eppendorf tube as listed in table 3.5 and incubated for 5 minutes at room temperature.

Table 3.5: Reagents in TOPO®-reaction

Reagent	Volume
Fresh PCR product	1 µl
Salt solution	1 µl
Sterile dH_2O	3 µl
pCR®II-Blunt-TOPO®	1 µl

2. 2 µl of the TOPO® cloning reaction mixture was added to 100 µl of chemo-competent *E. coli* TOP10 cells in a Falcon 2059 Polypropylene Round Bottom tube, followed by gentle mixing.
3. After incubation of the suspension on ice for 20 minutes, the cells were given a heat shock by incubation for 30 sec in a 42 °C water bath, and then moved directly back on ice.
4. After adding 250 µl of room tempered S.O.C medium, the cells were incubated in a shaker heating cabinet for 1-2 hours (200 rpm, 37 °C).
5. After incubation, 10-50 µl of the cell suspension was spread on BHI agar plates containing kanamycin; the plates were incubated over night at 37 °C.

3.7 Preparation of chemo-competent *E. coli* TOP10 cells

Chemo-competent E. coli TOP10 cells were prepared for use in TOPO® sub-cloning and cloning of ligation mixtures.

Materials:

Overnight culture of *E. coli* (TOP10)

200 ml pre-warmed 2xTY (section 2.8)

Ice-cold 0.05 M $CaCl_2$

Ice-cold 0.05 M $CaCl_2$ with 15 % (v/v) glycerol

Procedure:

All solutions, tubes and centrifuges were kept at 4 °C during cell harvest

1. ~ 5 ml of *E. coli* TOP10 overnight culture was added to 200 ml pre-warmed 2xTY, followed by incubation at 37 ºC in a shaker heating cabinet.
2. When the culture reached $OD_{600} \approx 0.5$, it was split into two aliquots using 100 ml centrifuge tubes.
3. The two cultures were incubated on ice for 10 minutes and then centrifuged at 4000 rpm for 10 minutes.
4. The supernatants were discarded and the cell pellets resuspended in 10 ml ice-cold 0.05 M $CaCl_2$ each, after which the cell suspensions were transferred to 15 ml Nunc tubes.
5. The cell suspensions were incubated on ice for 15-30 minutes and then recentrifuged.
6. The supernatants were discarded and the cell pellets were resuspended in 5 ml ice-cold 0.05 M $CaCl_2$ (with 15 % (v/v) glycerol) each, followed by incubation on ice for 5-10 minutes.
7. The cells were transferred to 1.5 ml eppendorf tubes in 200 µl aliquots and immediately frozen by placing them into a bath of dry ice and ethanol.
8. The cells were stored at – 80 ºC and used within 6 months.

3.8 Preparation of electro-competent *Lactobacillus plantarum* and *Lactococcus lactis* cells

Bacterial cells can take up DNA from the environment, but this is extra complicated for Gram positive cells due to the thick layer of peptidoglycan. To cope with this challenge, bacteria can be grown in a medium containing glycine to make the peptidoglycan layer more permeable. Glycine will replace L-alanine in the peptidoglycan during cell division and consequently make the peptidoglycan more permeable (Aukrust et al. 1995).

Preparation of electro-competent *L. plantarum* cells was done according to the protocol from Josson et al. (1989) and preparation of electro-competent *L. lactis* cells was done according to the protocol from Holo and Nes (1989), with some adjustments.

3.8.1 Preparation of electro-competent *L. plantarum* WCFS1

Materials:

MRS medium (section 2.8)

20 % glycine (w/v)

30 % PEG-1450 (w/v)

Corex tubes

Procedure:

All solutions, tubes and centrifuges were kept at 4 °C during cell handling

1. 10 ml MRS was inoculated with *L. plantarum* WCFS1 and the culture was grown over night at 37 °C.
2. The overnight culture was used to prepare a 10-fold serial dilution in MRS w/1 % glycine, resulting in 10 cultures. The cultures were incubated over night at 37 °C.
3. The OD_{600} of all cultures was measured, and the culture with $OD_{600} = 2.5 \pm 0.5$ was diluted 1:20 in 50 ml MRS w/1 % glycine. The culture was grown at 37 °C until it reached $OD_{600} = 0.7 \pm 0.07$ (logarithmic growth phase), and then placed on ice were it was kept for 10 minutes.
4. The cells were harvested by centrifuging for 10 min at 4500 rpm. The supernatant was discarded and the cell pellet resuspended in 15 ml ice-cold 30 % PEG-1450. The

culture was then transferred to a cold Corex® tube and kept on ice for 10 min before re-centrifugation.

5. The supernatant was discarded and the cell pellet resuspended in 400 µl ice-cold 30 % PEG-1450.
6. While kept in ice, the cells were applied to 1.5 ml eppendorf tubes in 40 µl aliquots and directly stored at -80 °C.

3.8.2 Preparation of electro-competent *L. lactis* MG1363

Materials:

GM17 medium (section 2.8)

0.5 M glucose

SGM17 medium (section 2.8)

SGM17 containing 1.8 % glycine (v/v)

85 % glycerol

Procedure:

All solutions, tubes and centrifuges were kept at 4 °C during handling of the cells

1. 10 ml GM17 was inoculated with *L. lactis* MG1363 and the culture was grown overnight at 30 °C.
2. The overnight culture was diluted 100 times in 10 ml GM17 medium, and incubated at 30 °C until reaching OD_{600} ~ 0.5.
3. A serial dilution of 10 ml SGM17 w/glycine (2.4-4.0 %) was set up according to table 3.6 and 100µl of the OD_{600} ~ 0.5 bacterial culture was added to each tube.

Table 3.6: Serial dilutions of SGM17 with glycine

Glycine conc. (%)	20 % glycine (µl)	SGM17 (µl)
2.4	1200	9300
2.6	1300	9200
2.8	1400	9100
3.0	1500	9000
3.2	1600	8900
3.4	1700	8800
3.6	1800	8700
3.8	1900	8600
4.0	2000	8500

4. All cultures were incubated over night at 30 °C.
5. The OD_{600} of all cultures was measured and the culture with the highest glycine content and OD_{600} = 0.3-0.4 was selected. The culture was kept on ice for 5 minutes before the cells were harvested by centrifuging for 4 minutes at 5500 x g.
6. The supernatant was discarded and the cell pellet washed 3 times in 15 ml ice-cold 0.05 M sucrose. The cells were centrifuged at 5500 x g for 2 min between every wash.
7. The cell pellet was resuspended in 350 µl ice-cold 0.5 M sucrose w/10 % glycerol, the resulting cell suspension was transferred to 1.5 ml eppendorf tubes in 40 µl aliquots, and the cells were stored at -80 °C.

3.9 Digestion with restriction endonucleases and ligation of DNA fragments

Restriction endonucleases are enzymes that are able to recognise specific nucleotide sequences and break the sugar-phosphate backbone of DNA strands. The enzymes are used to make specific cuts in the DNA and thus create linear DNA strands with known cohesive or blunt ends. Cohesive ends allow highly specific rejoining of DNA strands through ligases. Each restriction enzyme recognises different nucleotide sequences and has different buffer requirements. The enzyme's optimal reaction environment needs to be fulfilled for the enzyme to have 100 % activity. Hence, the right buffer giving correct salt-, pH- and temperature conditions needs to be used. Some enzymes require additional cofactors for optimal functionality, such as BSA.

3.9.1 Digestion with restriction endonucleases

Materials:

Restriction enzymes	Listed in section 2.3
NEBuffers (10x)	Listed in section 2.3
Cofactor BSA (10x)	Used in some reactions
dH_2O	
Insert and vector	

Procedure:

Two separate reactions were set up, one to prepare the insert and one to prepare the vector. DNA, the appropriate buffers and, if required, BSA, were mixed and dH_2O was added to a total volume of 50 µl. The volume used of the DNA-containing solution varied from 20-40 µl, depending on the experiment. The reactions were incubated in a water bath for 2-3 hours at 37 °C.

Some double digestion reactions required to use enzymes that needed different buffers. Such digestions were carried out in two separate steps, using only one enzyme at the time. In between the two steps, a PCR-cleanup (as described in section 3.4.3) was performed to remove buffer and enzyme.

The digested DNA fragments were separated by gel electrophoreses as described in section 3.5.1 and the desired DNA fragments were purified from the gel for further use as described in section 3.5.2. If large amounts of DNA were required, several reactions of both vector and insert were set up with maximum volume of DNA. Fragments from the multiple reactions were combined and concentrated during DNA extraction from agarose gels.

3.9.2 Precipitation and concentration of DNA

Some strains of bacteria yield very little DNA through plasmid isolation. It is therefore necessary to precipitate or concentrate DNA from several plasmid isolations to obtain enough and sufficiently concentrated DNA for further use in e.g. transformation or digestion reactions.

3.9.2.1 Pellet Paint®

Materials:

Pellet Paint® Co- Precipitant from Novagen (section 2.7)

Procedure:

1. All reagents were brought to room temperature, and each reagent was inverted several times to obtain uniform suspensions.
2. 2 µl of Pellet Paint® suspension was added to the DNA sample (independent of sample volume).

3. 0.1 volumes of 3 M NaAcetate was added to the sample and the sample was mixed briefly.
4. After adding 2 volumes of 96 % EtOH to the sample, it was vortexed for 5-10 seconds and incubated for 2 minutes at room temperature.
5. The sample was centrifuged for 5 minutes at 16,000 x g until a red pellet was visible, and the supernatant was carefully aspirated.
6. The red pellet was washed with 500 µl 70 % EtOH, briefly vortexed and recentrifuged.
7. The EtOH was removed and the pellet dried for 5 minutes in a vacuum centrifuge.
8. The pellet was dissolved in 20-40 µl elution buffer (NucleoSpin® AE or NE buffer) and used directly or stored at -20 °C.

3.9.2.2 Alternative concentration methods

After we found that precipitation by Pellet Paint® had a high failure rate, DNA being lost, alternative methods, such as concentration by vacuum drying or PCR-cleanup, were investigated.

- For concentration by vacuum drying, samples were mixed and run in a vacuum centrifuge until the desired volume was reached.
- For concentration by PCR-cleanup, the samples were mixed and treated as if they were PCR reaction mixtures using NucleoSpin® Plasmid Columns for concentration as described in section 3.4.3.

3.9.3 Measurement of DNA concentration (Quant-iT™ dsDNA BR Assay)

Materials:

QubitTM dsDNA BR Assay kit from Invitrogen (see section 2.7)

Procedure:

Preparation of the samples

1. A 1:200 working solution was made by diluting Quant-iT dsDNA BR reagent in Quant-iT dsDNA BR buffer.

2. Standard 1 and 2 for calibration of the Qubit™ fluorometer were made by diluting 10 µl of each standard in 190 µl working solution.
3. The DNA samples were prepared for measurement by adding 2 µl DNA sample to 198 µl of working solution.
4. All tubes were vortexed and incubated for 2 minutes at room temperature.
5. The Qubit™ flourometer was calibrated using standards 1 and 2 after which the absorbance and DNA concentrations of the DNA samples were measured.
6. The volumes of insert and vector solutions needed to get a 3:1 molar ratio in the subsequent ligation reactions. The amount needed of insert and vector to obtain a 3:1 molar ratio was calculated using the ”Ligation Calculator” of Düsseldorf University (http://www.insilico.uni-duesseldorf.de/Lig_Input.html).

3.9.4 Ligation of DNA fragments

Ligation of digested DNA fragments was performed using commercially available DNA ligases. Ligases are enzymes capable of repairing single- and double stranded breaks in a DNA strand, both blunt and cohesive ends, at the cost of ATP. The enzyme forms a phosphodiester bond between the 5’phosphate and 3’hydroxyl ends and thus ligates the two strands together.

3.9.4.1 Quick ligation

The Quick ligation protocol was performed when the DNA used in the reaction was isolated from *E. coli* TOP10, and the ligation reaction was to be transformed into *E. coli* TOP10.

Materials:

Insert- and vector fragments from restriction enzyme digestions (in separate tubes)
dH_2O
2x Quick Ligation Reaction Buffer
Quick T4 DNA Ligase

Procedure:

1. 50 ng of vector and 3-fold molar excess of insert were added to an eppendorf tube. The volume was adjusted to 10 µl using dH_2O. The appropriate volumes needed of

each vector and insert reaction to obtain the correct amount of DNA was calculated using procedure 3.9.3.

2. 10 µl of 2x Quick Ligation Reaction Buffer and 1 µl Quick T4 DNA Ligase were added to the DNA solution followed by thorough mixing.
3. The solution was briefly centrifuged in a table centrifuge to collect all fluid in the bottom of the tube and then incubated at room temperature for 5 minutes.
4. The ligation reaction mixture was then stored on ice prior to transformation or stored at -20 ºC for long term storage.

3.9.4.2 Overnight ligation

The Overnight ligation protocol was performed when the DNA used in the reaction was isolated from *E. coli* TG1 or *L. lactis* MG1363, and the ligation reaction was to be transformed into *L. lactis* MG1363. The amount of plasmid obtained through plasmid isolations from *E. coli* TG1 and *L. lactis* MG1363 varied greatly, something which had to be considered for every overnight ligation reaction.

Materials:

Insert- and vector fragments from restriction enzyme digestions (in separate tubes)
dH_2O
T4 Ligase
T4 Ligase Buffer

Procedure:

1. The ligation reaction was set up with a 3:1 molar ratio of insert and vector. The appropriate volumes needed of each vector and insert reaction to obtain the correct amount of DNA was calculated using procedure 3.9.3. Vector and insert solutions were added to a 1.5 ml eppendorf tube.
2. The volume of the reaction mixture was adjusted to 17 µl using dH_2O.
3. 2 µl T4 Ligase Buffer and 1 µl T4 Ligase were added to the solution and mixed thoroughly.
4. The solution was briefly centrifuged in a table centrifuge to collect all fluid in the bottom of the tube and then incubated over night at 16 ºC (in a PCR-machine or in a water bath in the cold room).

3.9.5 Drop dialysis

Drop dialysis was performed on ligation mixtures that were to be transformed into bacteria through electroporation. Through drop dialysis, salts from the ligation reaction are removed through dialysis across a membrane.

Materials:

Small, circular dialysis membrane

TE-buffer (section 2.9)

Ligation reaction mixture

Procedure:

1. TE buffer was poured in a petri dish and a dialysis membrane was placed onto the TE-buffer surface. Care was taken to make sure the membrane surface remained absolutely dry.
2. The ligation reaction mixture was applied carefully to the dialysis membrane and incubated for 30 minutes at room temperature to allow diffusion of salt ions across the membrane and into the TE-buffer.
3. The ligation reaction mixture was carefully pipetted off the membrane and transferred to a clean eppendorf tube, and then used directly for electroporation of *L. lactis*.

3.10 Transformation of chemo-competent *E. coli* TOP10 cells

Chemo-competent *E. coli* TOP10 cells were used in TOPO® cloning and for transformation of ligation mixes from Quick ligation (described in section 3.9.4.1)

Materials:

Chemo-competent *E. coli* TOP10

Ligation reaction mixture

S.O.C.-medium (section 2.8)

Falcon 2059 Polypropylene Round Bottom tubes (14 ml)

BHI agar plates with erythromycin (200 µg/ml)

Procedure:

1. 100 µl chemo-competent *E. coli* TOP10 culture and the whole ligation reaction mixture were added to a round-bottomed Falcon tube and incubated on ice for 30 minutes.
2. The tube was given a heat shock for 30 seconds using a 42 °C water bath and then moved directly back on ice.
3. 500 µl of room tempered S.O.C medium was added, and the tube incubated in a shaker heating cabinet for 1-2 hours (200 rpm, 37 °C).
4. After incubation, 100-150 µl of cell culture was spread on BHI agar plates containing erythromycin and the plates were incubated over night at 37 °C.

3.11 Electroporation of electro-competent *L. plantarum* WCFS1 and *Lc. lactis* MG1363 cells

Electroporation is a widely used technique for transforming Gram positive bacterial cells. When DNA is exposed to an electric field, DNA will move towards the positive pole. Simultaneously, the electric field can create transient pores in the plasma membrane of the bacterial cells. These pores allow the moving DNA to penetrate the permeable cell wall and plasma membrane and enter the cell (Fromm et al. 1985; Zerbib et al. 1985). Not all cells are transformed through this procedure; thus, recombinant cells need to be selected by spreading the cell suspension on agar plates containing erythromycin.

Below follows a general procedure for electroporation of *L. plantarum* WCFS1 and *L. lactis* MG1363. Details about growth media and instrument settings are shown in Table 3.7.

Materials:

Electro-competent cells

Plasmid DNA/ligated mixture suspension

MRSSM/SGM17 medium (section 2.8)

Agar plates with antibiotics

Procedure:

1. 40 µl of electro-competent cell culture was thawed on ice and 5 µl plasmid solution (*L. plantarum*), or 20 µl desalted ligation mix (*L. lactis*) was added. The cell suspension was immediately transferred to an ice-cold 2 mm electroporation cuvette. The cell suspension was mixed in the cuvette by gently tapping to avoid bubbles.
2. The parameters for electroporation were adjusted as shown in Table 3.7. The cuvette was placed in the electroporation handle and given the tension pulse.
3. Appropriate culture medium (Table 3.7) was added to the cuvette and mixed with the bacterial cell suspension. The suspension was then transferred to a sterile eppendorf tube and incubated for 2-4 hours at the appropriate temperature (see Table 3.7).
4. 100-150 µl of cell suspension was spread on agar plates with the appropriate growth medium, antibiotics, incubation time and temperature (see Table 3.7).

Table 3.7: Instrument settings, media and growth conditions during and after electroporation

Strain	Instrument settings	Transformation medium and incubation temperature post electroporation	Agar plates and incubation conditions
L. plantarum	Capacitance: 25 µF Volt: 1.5 kV Resistance: 400 ohms	500 µl ice cold MRSSM 2-4 hours at 37 °C	MRS with 10 µg/ml erythromycin 24-48 hours at 37 °C
L. lactis	Capacitance: 25 µF Volt: 2.0 kV Resistance: 200 ohms	700 µl preheated SGM17 2-4 hours at 30 °C	GM17 with 10 µg/ml erythromycin 48-72 hours at 30 °C

3.12 DNA sequencing of plasmids

Relevant parts of recombinant plasmids were sequenced to verify the sequence of the inserted fragments and to detect potential mutations. The sequencing was performed based on the technique developed by F. Sanger et al. (1977), using ddNTPs as specific chain-terminating inhibitors. The ddNTPs are colour coded by different fluorochromes for A, T, C and G, allowing specific detection. DNA polymerase will randomly add dNTPs and ddNTPs to the DNA strand. The ddNTPs lack both the 2'- and 3'-OH group; hence no further dNTPs can bind, and the elongation reaction is stopped every time a ddNTP is added. The reaction results in DNA strands of varying lengths ending in a colour coded ddNTP. The strands are separated according to size, only varying in length by one nucleotide, and identity of the terminal

ddNTPs can be detected by a laser. The detected order of ddNTPs yields the sequences of the template which may be analysed using computer software.

1. DNAsamples were prepared in eppendorf tubes by mixing reagents according to Table 3.8.

Table 3.8: Reagents in sequencing reaction

Component	Volume
Plasmid DNA	10 % of elution volume (or ~ 300 ng)
Primer (10 pmol/µl)	2.5 µl
dH_2O	To 11 µl

2. The eppendorf tubes were labelled and sent to GATC biotech (http://www.gatc-biotech.com/en/index.html) for sequencing.
3. Analysis of the sequences was performed using CLC DNA Workbench 5.

3.13 Cultivation and harvesting of recombinant *L. plantarum* for analysis of gene products

Materials:

50 ml preheated MRS medium (Section 2.8)

Erythromycin

Inducing peptide SppIP (IP673)

Ice-cold 1xPBS

Protease inhibitor (PMSF)

MP FastPrep® tubes with glass beads

Procedure:

1. 10 ml cultures were inoculated (MRS, 5 µg/ml erythromycin) and grown over night at 37 ºC. 50 ml portions of sterile MRS medium was pre-heated over night at 37 ºC.
2. Erythromycin was added to all the 50 ml portions of pre-heated 37 ºC MRS medium.
3. 700-1000 µl of the overnight cultures were diluted in pre-heated 50 ml MRS medium until OD_{600} ~ 0.15.

4. The cells were incubated at 37 ºC until OD_{600} ~ 0.30 was reached. OD_{600} was measured at 60 and 90 minutes after incubation start. Extra care was taken at all times to keep shaking of the media to a minimum.
5. At OD_{600} ~ 0.30 (0.27-0.34), the cultures were induced by adding SppIP pheromone to a final concentration of 25 ng/ml.
6. The cultures were then incubated for 1-3.5 hours at 37 ºC.
7. The cells were moved to ice, the OD_{600} was measured, and 20 µg/ml PMSF (final concentration) was added to each culture.
8. The cells were harvested by centrifuging at 5500 x g for 3 minutes. Supernatants were disposed of or transferred to separate tubes and stored at -20 °C, depending on the experiment. The cells were washed with 10 ml ice-cold 1xPBS.
9. The cells were recentrifuged and the supernatant was discarded. The cells were used directly or stored at -20 ºC.
10. For cell lysis and preparation of total cell extract, the cell pellet was resuspended in 1 ml PBS and added to MP FastPrep® tubes with glass beads.
11. The cells were lysed in a MP FastPrep® machine which was operated at 6.5 m/s for 45 seconds; subsequently the lysates were centrifuged at 16,100 x g for 30 seconds.
12. Supernatants were moved to clean eppendorf tubes and recentrifuged; the tubes with pellet were discarded.
13. The supernatants were again transferred to clean eppendorf tubes and stored on ice for direct use in further experiments or stored at -20 °C for later use.

3.14 Precipitation and concentration of proteins

3.14.1 TCA precipitation

The procedure was performed to precipitate culture supernatant for use in Western blot analyses.

Materials:

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

6M NaOH

20 mg/ml Sodium Deoxycholate

Ice-cold acetone

50 mM NaOH

Procedure:

1. The pH of the culture supernatant was adjusted to pH 7 using 6M NaOH and pH paper. Sodium Deoxycholate was added to a concentration of 0.2 mg/ml and the sample was kept on ice for 30 minutes.
2. One volume of 100 % (w/v) TCA was added to 4 volumes of supernatant. The sample was then vortexed and kept on ice for 20 min.
3. The sample was centrifuged at 16,100xg for 5 minutes at 4 ºC to generate a pellet of the precipitated proteins.
4. The supernatant was discarded and the protein pellet washed in 200 µl acetone (to remove remaining TCA). The pellet was then vortexed and re-centrifuged.
5. The pellet was dried in a vacuum centrifuge for 5 minutes.
6. The pellet was prepared for direct use in SDS-PAGE (section 3.15) by dissolving the pellet in 50 mM NaOH and adding sample buffer and reducing agent. The sample was boiled at 100 ºC for 7 minutes and then loaded on the gel.

3.14.2 Concentration through Amicon® Ultra-0.5 (10,000 MWCO) centrifugal filters

This procedure was used to concentrate crude total cell extracts with a low concentration of HIVp24.

1. 500 µl of crude total cell extract was applied to the filter tube and centrifuged at 14,000 x g for 5-15 minutes at 4 °C until the desired volume (20-80 µl) was reached.
2. The filter tube was inverted in a clean collection tube and centrifuged for 1 minute at 6,000 x g at 4 °C to recover the concentrated cell extract. The cell extract was then moved to a clean eppendorf tube and used directly or stored at -20 °C.

3.15 Gel electrophoresis of proteins using the NuPAGE® Electrophoresis system

Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) electrophoresis is a commonly used technique to separate proteins according to size. Protein samples are mixed with a detergent (in this study lithium dodecyl sulphate, LDS), buffer and a reducing agent, dithiothreitol (DTT), to ensure full denaturation and a uniform net negative charge of all

proteins in the sample. LDS disrupts secondary and non-disulfide-associated structures (same as SDS) while DTT ensures reduction of all disulfide bonds and maintain protein samples in a reduced state during electrophoresis.

When an electrical charge is applied, the proteins will move through the gel towards the positive pole and be separated according to size (molecular weight). By comparison with a standard sample containing proteins of known size ("ladder"), the size of other proteins can be determined. Protein bands may be visualized by direct staining or, in this study, by Western blotting.

Materials:
PBS
NuPAGE® Novex Bis-Tris Gels kit from Invitrogen (section 2.7)
MOPS/MES running buffer
MagicMarkTM XP Western standard ("ladder")

Procedure:

1. Each protein sample was prepared according to Table 3.9 and boiled for 7 minutes at 100 °C prior to application onto the gel. The remaining lysates were stored at -20 °C.

Table 3.9: Reagents for SDS-PAGE

Reagent	Volume
Sample*	X µl
NuPAGE® LDS Sample buffer (4x)	7 µl
NuPAGE® reducing agent (10x)	3 µl
dH_2O	To 30 µl

* To allow approximate comparisons of protein content, sample volume was adjusted according to culture OD_{600} measured at time of harvest. A maximum of 20 µl were added per sample

2. A 10 % Bis-Tris gel (8 cm x 8 cm x 1 mm, 10 wells) was placed in the gel chamber and secured with a gel tension wedge. The inner chamber was filled with new running buffer and the outer chamber with used running buffer
3. 1 µl of MagicMarkTM XP Western standard ladder was added to the first well, and protein samples were added to the following wells.

4. The electrophoresis was conducted by applying an electrical potential of 200 V for 35 minutes (MES buffer) or 50 minutes (MOPS buffer). The buffer used depended on the experiment.

3.16 Western blot analysis

Western blot analysis is a technique used for specific detection of proteins in a mixed sample. The protein samples are first separated according to size through gel electrophoresis (SDS-PAGE). Then the proteins are transferred from the gel to a membrane using an electrical current. Once the proteins are attached to the membrane, the membrane can be immunostained, using specific primary antibodies that only bind to the protein of interest. A secondary antibody conjugated to a detection system is then used to bind to the primary antibody, allowing visualisation of the protein of interest. A schematic overview is shown in Figure 3.2. A widely used detection system, based on chemiluminescence, uses horse-raddish peroxidise conjugated to the secondary antibody. The enzyme utilises hydrogen peroxidise as a substrate, and the enzymatic reaction yields a detectable chemiluminescent signal.

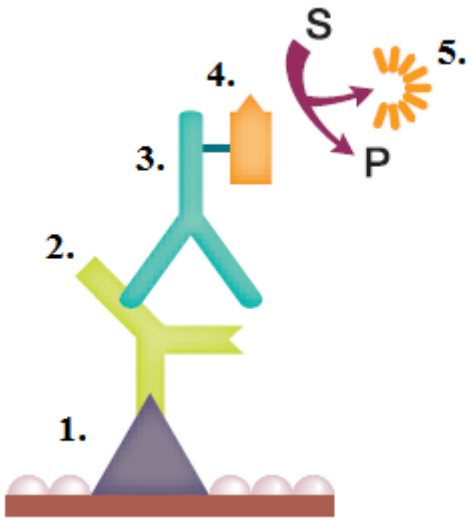


Figure 3.2. Principle of immunodetection in Western blotting. The primary antibody (2) binds to a specific epitope of the protein (1) attached to a membrane. A secondary antibody (3) binds to the primary antibody. An enzyme attached to the secondary antibody (4) catalyses a chemical reaction yielding chemiluminescence (5). The figure is taken from the Bio-Rad blotting guide (3rd edition).

Two different methods of blotting were used. For a "quick and dirty"-approach, the iBLOT™ Dry Bloting System was used. For proper blots, tank blotting using Mini Trans-Blot Cell System was performed.

3.16.1 Blotting with the iBLOT™ Dry Bloting System

In a dry blotting system, the top and bottom stacks of the blotting assembly contain buffer gels delivering all the buffer necessary for the procedure. The components are assembled in the iBLOT™ Gel transfer device as shown in Figure 3.2, after which an electrical current is applied. The electrical current transports the proteins from the gel and onto the membrane. This system allows blotting to be performed in only 7-10 minutes.

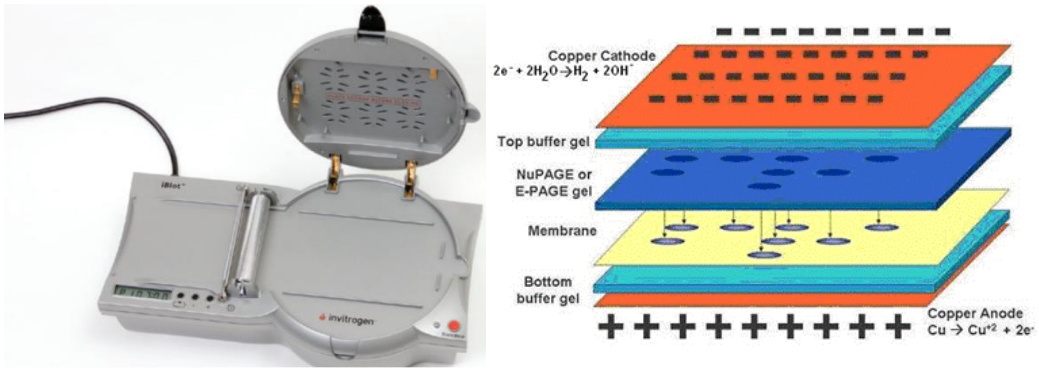


Figure 3.3: Assembly of components in the iBLOT™ Gel Transfer Stack. The components are layered from the bottom and up (as shown in the right picture). The bottom part contains a tray containing the copper anode, a buffer-gel layer and a PVDF membrane. A NuPAGE® gel is placed over the bottom tray and covered with a wet filter paper (not in the picture). A top stack containing a buffer-gel and the copper cathode is then added to complete the assembly. The gel transfer stack is assembled on the iBlot™ Gel Transfer Device (the device to the left). The figure is taken from the iBLOT™ Dry Bloting System user manual (Invitrogen™)

Materials:

iBlot™ Blotting system kit from Invitrogen (section 2.7)

Procedure:

1. The NuPAGE® Bis-Tris gel was washed in dH_2O for 5 minutes
2. The bottom anode stack was placed in its aligned place in the iBlot™ Gel Transfer Device and a blot roller was used to remove any air bobbles between the buffer-gel layer and the membrane.

3. The gel and the wet filter paper were placed on top of the bottom anode stack and a blot roller was used to remove any air bobbles.
4. The cathode stack was placed on top of the filter paper, copper-electrode facing up, and a blot roller was used to remove any air bobbles.
5. The disposable sponge was placed in the lid of the transfer device and the lid was closed.
6. The blot machine was set to program P2 (23 V) for 8 min. When finished, the gel was disposed of and the membrane was immediately moved to a tray containing TBS.

3.16.2 Blotting with the Tank transfer system Mini Trans-Blot Cell

Blotting in a tank transfer system requires more time, but yields cleaner blots with less background.

Materials:

Mini Trans-Blot cell kit from Bio-Rad (section 2.7)

Towbin buffer (section 2.9)

Methanol

PVDF membrane

Procedure:

1. The NuPAGE® Bis-Tris gel was washed in dH_2O for 15 minutes.
2. A piece of PVDF membrane was cut to approximately the same size as the gel.
3. The membrane was briefly soaked in methanol before both gel and membrane were soaked in Towbin buffer for 15 minutes.
4. Filter paper and filter pads were soaked briefly in Towbin buffer.
5. Filter paper, filter pads, membrane and gel were assembled as illustrated in Figure 3.4.

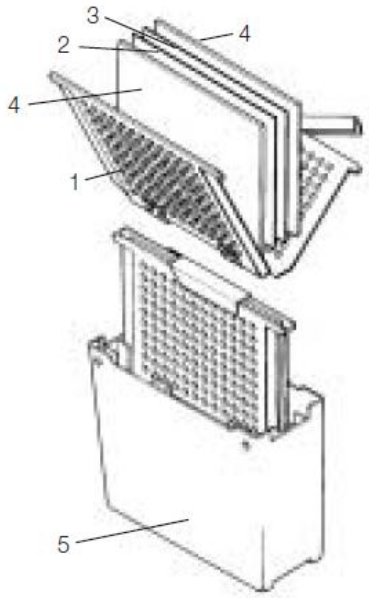


Figure 3.4: Assembly of the blotting sandwich. The cassette (1) (black side facing the front, white side facing the back) holds the gel (2) and membrane (3) while fiber pads and filter paper (4) on both sides provide complete contact within the gel sandwich. The gel cassette is inserted vertically in the buffer tank (5). Figure and text is taken from the Bio-Rad blotting guide (3rd edition).

6. The black side of the gel cassette was inserted facing the black side of the buffer tank.
7. An ice unit (for cooling) and a magnet (for buffer stirring) were added and the tank was filled with Towbin buffer.
8. A lid with electrodes was placed on top of the tank, and the tank was placed on a magnetic stirrer.
9. Transfer was achieved by applying power (60 V) for 60 minutes.
10. When finished, the gel and membrane were placed in trays containing dH_2O and TBS, respectively.

3.16.3 Hybridization with antibodies

Several different protocols exist for hybridization with antibodies. Use of the SNAP i.d. Protein Detection System (Figure 3.5) allows a much more rapid and easy antibody hybridisation than the traditional methods. All western blots were therefore hybridised using the SNAP i.d. Protein Detection System.

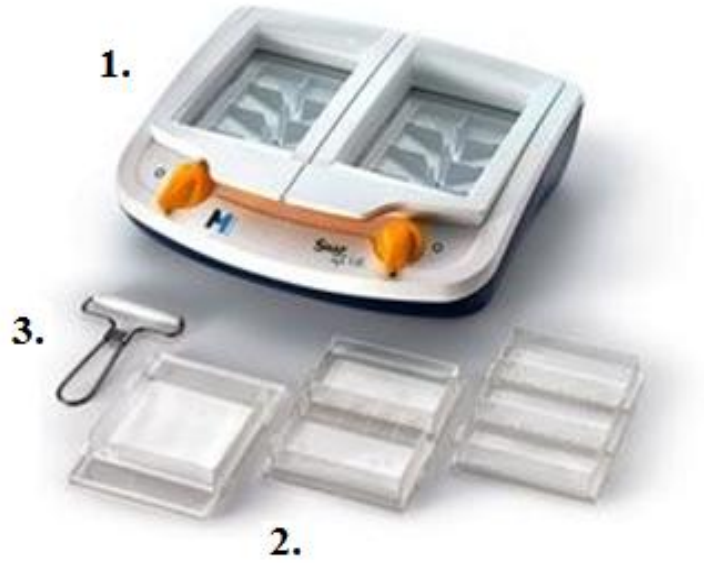


Figure 3.5: Illustration of the SNAP i.d. Protein Detection System. The figure illustrates all components in the SNAP i.d. Protein Detection System. The system (1) contains two blot holder chambers, each controllable with yellow vacuum switches. Different blot holders (2) can be used according to blot size and antibody hybridisation composition. The blot roller (3) is used to remove air bobbles between the blot holder surface and the blotted membrane. A description of how the device is used is given in the text. The figure is taken from the SNAP i.d. Protein Detection System protocol (Millipore).

Materials:

TTBS (section 2.9)

TTBS w/0.5 % BSA

Blocking solution (TTBS w/1 % BSA)

Primary antibody (section 2.3): mCCL17/TARC, 1.125 µg/ml

Secondary antibody (section 2.3): Rabbit anti-goat IgG (H+L)-HRP, 1:12,000 dilution

SNAP i.d. Protein Detection System

Procedure:

1. The blot holder was wetted with dH_2O and the blotted membrane (stored in TBS) was carefully placed on it, face down.
2. Wetted filter paper was placed on top of the membrane and excess fluid and air bubbles were removed using the blot roller.
3. The blot holder was closed and placed in the blot holder chamber of the SNAP i.d. system (protein side of the membrane was now facing up).

4. 30 ml of blocking solution was added to the well and immediately removed by vacuum.
5. The vacuum was turned off and 3 ml of TTBS w/0.5 % BSA containing primary antibody was added to the well and incubated for 10 minutes. Care was taken that the whole membrane was covered with antibody solution.
6. The antibody solution was removed by vacuum, and without turning the vacuum off, the blot was washed 3 times with 10 ml TTBS.
7. The vacuum was turned off and 3 ml of TTBS w/0.5 % BSA containing secondary antibody was added to the well and incubated for 10 minutes. Care was taken that the whole membrane was covered with antibody solution.
8. The antibody solution was removed by vacuum, and without turning the vacuum off, the blot was washed 3 times with 10 ml TTBS.
9. The blot holder was removed from the SNAP i.d. system. The filter paper was removed and discarded and the membrane placed in a tray and incubated with chemiluminescent detection solution as described in section 3.16.4.

3.16.4 Chemiluminescent detection of proteins

Materials:

SuperSignal® West Pico Chemiluminescent Substrate kit from Pierce (section 2.7)

CL-Xposure™ Film

Stripping buffer

1xTBS

Procedure:

1. A 1:1 ratio of Luminol/enhancer and peroxide solution was prepared (10+10 ml).
2. The blotted membrane was placed in a tray and covered with the Luminol/enhancer-peroxide solution.
3. The membrane was incubated for 5 minutes on a shaker at room temperature.
4. After incubation, the membrane was covered with plastic foil and placed in a film cassette, protein side facing up.
5. The cassette was moved directly to the dark room.

6. CL-Xposure™ Film was cut to fit the membrane and placed on top of the membrane. The cassette was closed and the film exposed for ~1 minute to 10 minutes depending on the intensity of the chemiluminescent signal.
7. The film was placed in a tray containing developer solution for 2 minutes.
8. The film was placed in a tray containing fix solution for 2 minutes.
9. The film was rinsed in water for 1 minute, air-dried and computer scanned.
10. If the antibody hybridisation was unsuccessful, antibodies were removed from the membrane by immersing the membrane in stripping buffer for 10-15 minutes followed by washing 2 times with TBS. Subsequently, a new hybridisation was started.

3.17 Detection of surface-located antigen using Alexa Fluor® 488-labelled secondary antibody

Surface located HIVp24 antigen can be detected using the same principle of immunostaining as described in section 3.16 (Western blot analysis). Cells are incubated with primary and secondary antibodies to detect antigens on the cell surface. The secondary antibody is conjugated with a fluorochrome which emits light when excited by a laser beam. The Alexa Fluor® 488-labelled secondary antibody used in this study contains a fluorochrome that emits light with a wavelength of 519 nm when excited with a laser of 488 nm. The presence of the desired protein on the bacterial surface will be indicated by a fluorescent signal form the excited fluorochrome.

In the present study, two different approaches for detection of surface-located antigens were used: detection through flow cytometry and detection through confocal microscopy.

Through flow cytometry, the properties of each individual cell can be measured. The cells pass a laser beam one at the time, and the light emitted is detected by individual detectors. The detectors register forward and side scatter, indicative of cell size and granularity, respectively, and emitted fluorescence is also registered (Rahman 2006). Fluorescence measured by the flow cytometer is often depicted using single dimension histograms, where relative fluorescence (x-axis) is plotted against the relative number of events (number of particles counted by the flow cytometer) (y-axis). How far the peak of the histogram is shifted to the right illustrates the strength of the fluorescent signal detected. An illustration of the flow cytometer and is principles is given in Figure 3.6.

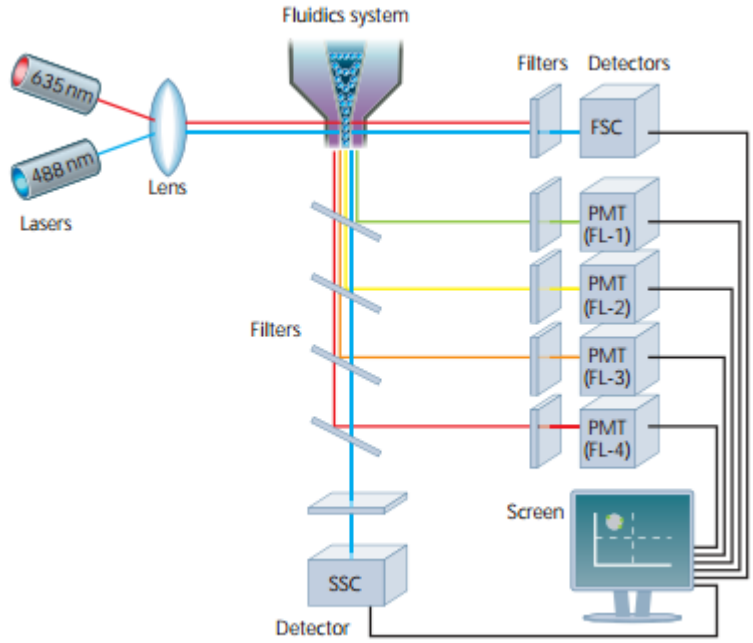


Figure 3.6: Schematic overview of a typical Flow cytometer setup. The cells enter the cytometer through the fluidic system and are probed by a laser beam. The scattered light and fluorescence are detected by several detectors and analysed by a computer.

Confocal microscopy follows many of the same principles as the flow cytometer, except that all takes place inside a normal light microscope. The laser probes cells on a microscope slide and fluorescent light emitted from the fluorochromes is detected. By the use of a pinhole, only focused light will be detected, and one can observe the sample in layers. By adjusting the size of the pinhole one can decide the thickness of the observable layers.

3.17.1 Staining of surface antigens for flow cytometry analysis

Materials:

PBS (section 2.9)

PBS with 1 % (w/v) BSA

Anti-mouseCCL17/TARC Biotinylated polyclonal goat anti-mouse primary antibody (section 2.3), 1.5 µg/ml in PBS with 1 % (w/v) BSA

Alexa Fluor® 488 labelled donkey anti-goat secondary antibody (section 2.3), 1:3,000 dilution in PBS with 1 % (w/v) BSA

Procedure:

The cells used were harvested according to the procedure described in section 3.13 (step 1-9) time and stored at – 80 ºC in 20 % glycerol. If fresh cells were used, the procedure was followed from step 2, and only one wash was performed.

All centrifugation steps were performed at 4 ºC

1. The cells were thawed in a 30 ºC water bath for 2 minutes, then put directly on ice.
2. The cells were centrifuged at 6,000 x g for 2 minutes, the supernatant was discarded, and the pellet was washed twice with 1 ml ice-cold 1xPBS to remove all traces of glycerol (the pellet was resuspended in 1 ml 1xPBS for each wash).
3. The pellet was then resuspended in 500 µl 1xPBS containing 1 % BSA and the appropriate amount of primary antibody, and incubated 45 minutes on ice.
4. The cells were washed 3 times with 1 ml ice-cold 1xPBS (as step 2).
5. The pellet was then resuspended in 500 µl 1xPBS containing 1 % BSA and the appropriate amount of secondary antibody, and incubated on ice in the dark for 30 minutes.
6. The cells were washed 4 times with 1 ml ice-cold 1xPBS (as step 2) and extra care was taken to make sure the cells were exposed to light as little as possible.
7. After the last washing step, all the supernatant was discarded. The cells were resuspended in 100 µl 1xPBS right before analysis.

The cells were analysed in a MacsQuant® Analyzer (Miltenyi Biotec) and the results were visualised using MacsQuantify™ Software. The software used pre-set settings for optimal detection of Alexa Fluor® 488, and the sample uptake volume was set to 7 µl.

3.17.2 Staining of surface antigen for confocal microscopy

Materials:

Glass slides

Cover slides (normal, or special for confocal microscopy)

1xPBS w/ 0.01 % Tween (wash solution)

1xPBS w/ 0.01 % Tween and 5 % BSA (w/v) (block solution)

2 % Paraform aldehyde (PFA) in PBS (v/v)

Primary antibody solution (1.5 µg/ml P.A. in 1xPBS w/ 0.01 % Tween)

Secondary antibody solution (1:3000 dilution of S.A. in 1xPBS w/ 0.01 % Tween)

Procedure:

The cells used were harvested according to the procedure described in 3.13 (steps 1-9) and stored at – 80 ºC in 20 % glycerol. If fresh cells were used, the procedure was followed from step 2, and only one wash was performed.

1. The cells were thawed in a 30 ºC water bath for 2 minutes, then put directly on ice.
2. The cells were centrifuged at 6000 x g for 2 minutes at 4º C, the supernatant was discarded, and the pellet was washed twice with 1 ml ice-cold 1xPBS to remove all traces of glycerol (the pellet was resuspended in 1 ml 1xPBS for each wash).
3. The cells were then fixed by resuspending the pellet in 1.5 ml of 2 % PFA in PBS (v/v).
4. The samples were incubated for 1 hour at 4 ºC.
5. After incubation, the cells were centrifuged at 4º C and the pellet washed 3 times with ice-cold PBS.
6. After the last wash, the pellet was resuspended in 250 µl-2 ml PBS depending on pellet size.
7. Glass slides were then prepared, one glass slide per sample, by drawing a square on the plates with a Super PAP PEN. The square defines an area surrounded by a hydrophobic border to which all reagents and samples are added. By marking an area with the PAP PEN, one minimises the use of reagents.
8. 70 µl of each sample was added to its own glass slide (inside the square) and the glass slides were set to dry at 37 ºC.
9. When all liquid had evaporated from the slides, the samples were blocked by adding 200 µl of block solution to each slide. They were then incubated in a humid chamber at room temperature for 60 minutes.
10. The block solution was pipetted off and 200 µl of primary antibody solution was added.
11. The slides were incubated in a humid chamber at 4 ºC over night.
12. After incubation, the slides were washed 4 times with 200 µl 1xPBS w/ 0.01 % Tween (1xPBS w/ 0.01 % Tween was added and then pipetted off the glass slides and into a beaker). Extra care was taken not to wash the bacteria off the slides.
13. The slides were then incubated in a humid chamber for 1 hour at room temperature with 200 µl of secondary antibody solution.

14. The secondary antibody solution was then pipetted off and the slides were washed as in step 12.
15. While the slides were still moist/wet, a cover glass was put over the sample and the slides were either taken straight to the microscope or sealed with nail polish and stored at 4 ºC in the dark.

The cells were visualised in a Zeiss LSM 700 Confocal Microscope (Carl Zeiss) and viewed and recorded using Zen software (Carl Zeiss).

3.18 Preparation of *L. plantarum* strains for scanning electron microscopy (SEM)

SEM of the bacteria is performed under high vacuum, and thus demands appropriate preparations of the bacteria to endure such treatment. The bacteria were induced and harvested, then fixed, dehydrated, critical point dried (CPD) and gold-palladium coated as described below.

Materials:
PBS (section 2.9)
Fix-solution (1.25 % (v/v) glutar aldehyde and 2 % paraform aldehyde in 0.05 M PIPES buffer)
Small, crcular cover glasses
Poly-l-lysine solution (1 mg/ml)
0.05 M PIPES buffer, pH 7
70 % EtOH
90 % EtOH
96 % EtOH
100 % EtOH

Procedure:

1. Bacterial strains were grown and induced according to the protocol described in section 3.13 (steps 1-9).
2. 1 ml of each strain was harvested in eppendorf tubes by centrifuging 1 min at 11,000 x g; the cells were washed once in 1 ml PBS and recentrifuged.

3. The pellets were resuspended in 1 ml fix-solution and incubated at 4 °C over night
4. Small cover glasses were coated with poly-l-lysine by giving them a static electrical charge with filter paper before suspending in 1 mg/ml poly-l-lysine solution for 20 seconds (one cover glass per recombinant strain).
5. The coated cover glasses were air dried while the bacterial suspensions were pipetted into flat-bottomed glass cylinders.
6. One poly-l-lysine-covered cover glass was added to each bacterial suspension (care was taken to make sure it sank to the bottom of the cylinder) and left for 1 hour to let bacteria cover the cover glass.
7. The bacterial suspensions were removed from the cylinders and the cover glasses were rinsed 3 times with 0.05 M PIPES buffer, pH 7. The bacteria-covered cover glasses were incubated for 10 minutes each time.
8. The samples were dehydrated by step by step replacing all H_2O with EtOH. The PIPES buffer was removed from the cylinders and 70 % EtOH was added. The bacteria-covered cover glasses were incubated for 10 minutes.
9. The 70 % EtOH was removed from the cylinders and 90 % EtOH was added. The bacteria-covered cover glasses were incubated for 10 minutes.
10. The 90 % EtOH was removed from the cylinders and 96 % EtOH was added. The bacteria-covered cover glasses were incubated for 10 minutes.
11. The 96 % EtOH was removed from the cylinders and 100 % EtOH was added four times. The bacteria-covered cover glasses were incubated for 10 minutes each time. Extra care was taken to never let the bacteria-covered cover glasses dry out between each step.
12. The sample holder for the critical point drier was completely covered in 100 % EtOH and one bacteria-covered cover glass was moved into each compartment. The lid was closed and the sample holder was installed in the critical point drier, where all EtOH was to be exchanged with CO_2. The purpose of critical point drying is to remove all liquid (especially H_2O) from the sample without disrupting any structures, something which is required for the use of high-vacuum scanning electron microscopy.
13. When all fluid EtOH was exchanged with CO_2, the cover glasses were attached to SEM-bolts and coated with gold-palladium. Gold-palladium coating increases the electron-density of the sample, which increases the resolution and allows the use of higher magnification. After gold-palladium coating the samples were stored or installed in the microscope.

3.19 Effects of the HIVp24 expressing strains on mice

The vaccine properties of *L. plantarum* strains harbouring different plasmids for anchoring of HIVp24 were tested in mice. Inbred female 10-week-old BALB/c mice were purchased from Taconic (Bomholt, Denmark). Animal care was in accordance with national legislation and institutional guidelines, and the experimental protocol was accepted by the local ethical committee. The set up of the mouse trial was identical to Kajikawa et al. (2012), where the mice were immunised by 1 dose per day on days 1-3, 15-17 and 29-31. The mice were euthanized during days 43-47. The strains chosen for mouse trials are listed in Table 3.10, where each treatment group consisted of 5 mice.

Each dose given to the mice consisted of $2x10^9$ cells, where $1x10^9$ cells were live bacteria and $1x10^9$ cells were heat-killed bacteria. Group five (see Table 3.10) consisted of a mix between cells of groups 2-4. I.e. the sample contained $0.33x10^9$ live bacteria and $0.33x10^9$ heat-killed bacteria from each of three strains.

Table 3.10: Groups used during the mouse trials

Group	Bacterium	Plasmid	Characteristic
1	*L. plantarum*	pSIP-EV	Empty vector, negative control
2	*L. plantarum*	pLp_0373HIVp24cwa2	Cell wall anchored HIVp24
3	*L. plantarum*	pLp_1261HIVp24	Lipoprotein anchored HIVp24
4	*L. plantarum*	pLp_3014HIVp24	LysM domain anchored HIVp24
5	*L. plantarum*	Mix of groups 2, 3, 4	
NaCl	-	-	Extra negative control. No cells, only NaCl

3.19.1 Cultivation and harvesting of *L. plantarum* strains for mouse trials

1. Prior to harvesting cells for mouse trials, it was necessary to determine the number of live cells/colony forming units per millilitre (CFU/ml) at time of harvest
2. Cell cultures of all 4 strains were grown and induced as described in section 3.13 (steps 1-9). Cells harbouring plasmids pSIP-EV, pLp_1261HIVp24 and pLp_3014HIVp24 were harvested at 3.5 hours after induction. Cells harbouring pLp_0373HIVp24cwa2 were harvested 1 hour after induction. To analyze CFU/ml, a 10 x serial dilution was made for each culture and 100 µl of each dilution was spread on agar plates. The plates were incubated for 2 days at 37 °C and the colonies counted to determine CFU/ml. The strains harbouring the different plasmids had shown characteristic and reproducible growth through all experiments, yielding very

reproducible OD_{600}-values (which can be seen in the Appendix, Figure A.4). These data were used to determine the CFU/ml. OD_{600}-values and CFU counted were used to determine the amount of cell culture needed from each strain to harvest the right amount of cells.

3. The harvest was performed according to section 3.13 (steps 1-9), based on the CFU/ml-values found in step 2 (data not shown). The cells of groups 1, 3 and 4 were harvested 3.5 hours after induction and the strains of group 2 was harvested 1 hour after induction. 1 ml culture from each strain was used for a new CFU-count to ensure the correct amount of cells was harvested.
4. During harvest, cell suspensions were separated into aliquots of $6x10^9$ cells per 500 µl PBS and added to 2 ml eppendorf tubes.
5. Half of the tubes were kept on ice while the cells of the other half were heat killed as described in section 3.19.2.
6. Extra cells were harvested from each strain for Western blots, to confirm HIVp24 production.

3.19.2 Heat-kill and storage of cells

A literature search had been conducted to investigate the best conditions for heat-killing of *L. plantarum* cells (Chuang et al. 2007; Kajikawa et al. 2010; Maeda et al. 2009; Vintini & Medina 2011). As this yielded no consistent results, an experiment was conducted to identify the temperature and incubation time which would kill the cells without cell lysis. We estimated that cell lysis would be avoided if we found the lowest temperature and shortest exposure time needed to kill the cells. Such conditions were found to be 70 °C for 5 minutes (data not shown).

Procedure:

1. Cells had been grown, harvested and washed according to protocol 3.19.1.
2. The cells for heat-kill were incubated for 5 minutes in a 70 ºC water bath, and moved directly back on ice.
3. The heat-killed cells were added to the live cell suspensions (the cells kept on ice). The cells were stored in 20 % glycerol at -80 °C until administration to mice.

3.19.3 Administration to mice

Steps 1-4 in the protocol described below were primarily performed by the personnel at the animal clinic at Rikshospitalet, with some assistance from the author of this thesis on the first day of immunisation.

1. On an administration day, relevant bacterial samples prepared as described in section 3.19.1 and 3.19.2 and containing a mixture of live and heat-killed cells were thawed by incubation in a 30 °C water bath for 2 minutes, after which the cells were immediately placed on ice. The cells were washed twice with 1.5 ml ice-cold PBS using centrifugation at 6,000 x g for 3 minutes at 4 °C. The supernatant was discarded each time.
2. Each immunisation group consisted of five mice. Every bacterial cell sample contained enough cells for 6 mice (one extra in case of trouble).
3. Each bacterial cell pellet was resuspended in PBS to reach a cell suspension of 1200 µl, meaning that the dose volume was 200 µl. Before resuspending the cell pellet, it was necessary to account for an increase in volume, thus the volume of the cell pellet was measured using a pipette and PBS was added to reach a total volume of 1200 µl.
4. The mice were given oral immunisations by ball-tipped syringes for oral gavage inoculation on days 1-3 and 29-31 and vaginal immunisations on days 15-17, i.e. nine immunizations in total.

3.19.4 Harvesting of mice

The steps were primarily performed by the personnel at the animal clinic at Rikshospitalet with some assistance from the author of this thesis on the first day of harvest.

1. The mice were euthanized during week 6 (days 43-47). The mice were given anaesthesia by injection of 200 µl Midazolam (Dormicum) mixed with Fentanyl/Fluanison (Hypnorm) prior to euthanizing.
2. 1 ml blood and vaginal fluids were harvested before the mice were euthanized by neck pull. After death, spleen, Peyer's patches and mesenteric lymph nodes were harvested.
3. All organs were stored in RPMI on ice until further treatment.
4. Spleen, Peyer's patches and mesenteric lymph nodes were homogenised by mashing them through a sieve and into Nunc tubes containing 10 ml RMPI. The homogenates were centrifuged at 11,000 x g for 7 minutes. Supernatants were discarded.

5. The spleen pellet was resuspended in 5 ml ACT (140 mM NH_4Cl in Tris-Base, pH 7.6) and incubated for 5 minutes in ice to lyse erythrocytes. After incubation, 5 ml PBS was added. Cells from mesenteric lymph nodes and Peyer's patches were resuspended in 3 ml RPMI, no ACT was added.
6. Execution of ELISPOT and multiplex analyses requires that all wells that are to be compared to each other contain the same number of cells. Cells of all organs were thus counted using Invitrogen's Countess® Automated Cell Counter to determine live cells/ml.
7. The samples of all organs were recentrifuged and resuspended in an appropriate volume of RPMI for ELISPOT and multiplex analysis so that each well with spleen cells contained $3x10^6$ cells, each well with cells from Peyer's patches contained $1.5x10^6$ cells and each well with cells from mesenteric lymph nodes contained $0.75x10^6$ cells.

3.19.5 Enzyme-linked immunosorbent spot (ELISPOT)

Enzyme-linked immunosorbent spot (ELISPOT) is a technique used to determine the number of cytokine producing cells in a sample, and to detect cells with specific and unspecific reactivity towards a certain antigen. ELISPOT is the most sensitive analysis method for detection of cytokines, and was in this study used for detecting IFN-γ producing cells. IFN-γ is a proinflammatory cytokine which promotes systemic inflammation. A high amount of IFN-γ producing cells thus indicates an immune response.

The analysis was performed by Dr. I. Øynebråten and colleagues at the Centre for Immune Regulation, University of Oslo. In short, the bottom of 96-well plates was covered with nitrocellulose membrane and coated with monoclonal anti-IFN-γ antibodies. The wells were washed and lymphocytes isolated from spleen or Peyer's patches were added to the wells. Each cell sample was analysed in parallel, one parallel to be stimulated by Gag p24-peptides and one parallel left unstimulated. The immunogenic p24 peptide was added to the wells at a concentration of 2 µg/ml. The plate was then set to incubate at 37 °C for 27 hours before final analysis. After incubation, the cells were washed off and biotinylated anti-IFN-γ antibodies were added to bind to the IFN-γ produced by the lymphocytes and immobilized on the plate by the anti- IFN-γ antibodies on the bottom membrane. The wells were washed to dispose of excess anti-IFN-γ antibodies. Subsequently, streptavidin-conjugated phosphatase was added

and through its specific interaction with biotin, streptavidin will only bind to the plate at positions were biotinylated anti-IFN-γ antibodies are bound. The plates were washed one more time to remove excess streptavidin-conjugated phosphatase, and phosphatase substrate was added to the wells. Due to the alakaline phosphatase reaction, dyed precipitates will form where biotinylated anti-IFN-γ antibodies are bound, resulting in concentric circles around the places where the IFN-γ producing cells resided. Thus, each cell producing IFN-γ would result in a coloured circle in the bottom of the wells. Using an ELISPOT reader named "Immunospot" (from CLT Europe GmbH), the number of circles (cells) were counted for each sample and cell numbers of peptide-stimulated cells and non-stimulated cells were compared.

3.19.6 Multiplex cytokine quantification

Analysis of total cytokine production from immune cells harvested from mice can be performed through multiplex cytokine quantification using Luminex technology. The technique utilizes multiplex beads which are dyed with fluorescent dyes and coated with antibodies for detection of different cytokines. In short, vaginal fluids and lymphocytes harvested from the spleen, Peyer's patches and mesenteric lymph nodes of immunised mice were added to wells of 96-well-plates, and beads were added to each well. Cytokines produced by the lymphocytes bind to the antibodies coupled to the different beads. After incubation, the beads are collected from the wells and run through a Luminex-100 instrument. The Luminex-100 instrument uses flow cytometric technologies for classification of the beads, as well as detection of phycoerythrin emission due to cytokine presence, to determine the composition of cytokines present in the different samples. The analysis was performed by Dr. I. Øynebråten and colleagues at Centre for Immune Regulation, University of Oslo.

4 RESULTS

4.1 Anchoring of HIVp24 in *L. plantarum* WCFS1

The aim of the study was to clone, express and anchor the fusion protein HIVp24 in *L. plantarum* WCFS1 using the pSIP system developed by Sørvig and colleagues (2003; Sørvig et al. 2005a) and use *L. plantarum* as a vaccine delivery vector. *L. plantarum* WCFS1 has GRAS status and is believed to have probiotic and adjuvant effects, and is thus regarded as a good candidate for the construction of a safe, "food grade" vaccine. In this study, we focused on anchoring heterologous proteins to the bacterial surface. The anchor sequences used parts of homologous proteins of *L. plantarum* that contain an anchoring motif that attaches the protein to the cell wall or cell membrane of the bacterium. Three different anchors were selected based on previous work performed by Lasse Fredriksen (2010; 2012) and Rannei Tjåland (2011): a covalent cell wall anchor (LPxTG-type), a lipoanchor (for covalent coupling to the cell membrane) and a LysM-type anchor (for non-covalent coupling to the cell wall). After successful construction of the recombinant strains, their ability to elicit a HIV specific immune response was to be tested in a mouse model. The use of a bacterial vector for delivery of HIVp24 had not been tested prior to this study.

4.1.1 Construction of a plasmid for cell-wall anchoring of HIVp24

For covalent cell wall anchoring of HIVp24, the HIVp24-encoding DNA sequence was fused to the C-terminal part of a gene derived from *L. plantarum* named *Lp_2578*. The *Lp_2578* gene product contains an LPxTG-motif that will become linked to the cell wall by the sortase enzyme. Fredriksen et al. (2010) created tree anchors based on Lp_2578 (cwa1-3) with different lengths. These three anchors all contain the C-terminal part of Lp_2578 (i.e. the part with the LPxTG anchor) and contain varying parts of the upstream sequence (i.e. upstream of the LPxTG). The truncated anchor, consisting of 194 amino acids (cwa2), was chosen for this study because it according to Fredriksen et al. (2012) seems to have the most optimal anchor length for surface display of heterologous proteins in *L. plantarum*. According to Tjåland (2011), strains expressing heterologous proteins fused to cwa2 show higher growth rates after induction of expression with SppIP, compared to cwa1 and cwa3. HIVp24 was fused to cwa2 as shown in Figure 4.1. The cassette used for cloning was originally constructed by Fredriksen et al (2010) to allow easy exchange of the signal peptide, target protein and anchor by insertion of unique restriction enzyme sites at crucial positions in the sequence. Figure 4.1

also shows the complete amino acid sequence of Lp_2578 and describes the anchor sequence of all three anchors.

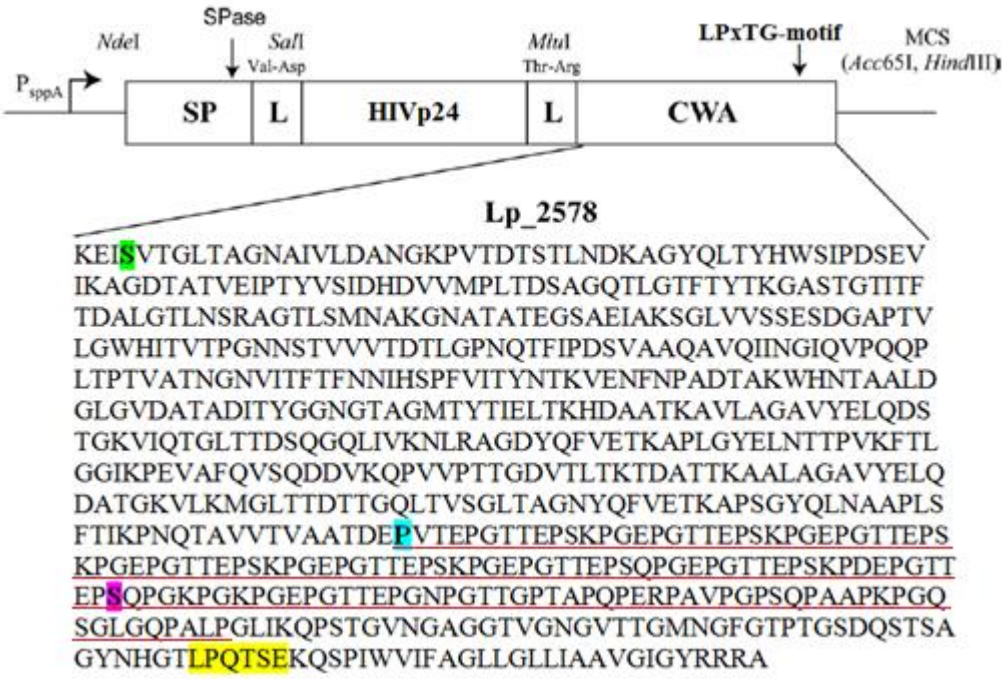


Figure 4.1: A schematic outline of the SP-HIVp24cwa2 cassette. The figure schematically shows how the signal peptide (SP), the target protein (HIVp24) and the cell wall anchor (cwa2) are organised relative to each other. It also depicts the linker areas (L) and multiple cloning site (MCS) containing the restriction sites that may be used for exchange of the different components. The amino acid sequence represents the original sequence of Lp_2578, without the signal peptide. The yellow box area marks the actual consensus sequence of the LPxTG-motif in *L. plantarum* (i.e. LPQTxE) and the underlined sequence marks the hydrophobic, proline-rich motif. Cwa1, the longest anchor, consists of almost the entire part of Lp_2578 (everything downstream of the green S). Cwa3, the shortest anchor, consists of only the last 128 aa, starting from the pink S. The cwa2 anchor sequence (the anchor used in this study) consists of the last 194 aa, starting at the blue P. The P also marks the point where HIVp24 and cwa2 are fused together. The figure is taken from (Fredriksen et al. 2010) and slightly edited to fit the present study.

The construction of the plasmid for production of cell-wall anchored HIVp24 is shown in Figure 4.2. The plasmids pUC57-HIVp24 and pLp_0373sOFAcwa2 (all plasmids used in this study are listed in Table 2.4) were digested with restriction enzymes SalI and MluI. The 588 bp fragment of pUC57-HIVp24 was ligated (section 3.9.4.1) into the 6381 bp fragment from pLp_0373sOFAcwa2, giving the plasmid pLp_0373HIVp24cwa2. The resulting plasmid was transformed into chemo-competent *E. coli* TOP10 before final transformation into electro-competent *L. plantarum* WCFS1.

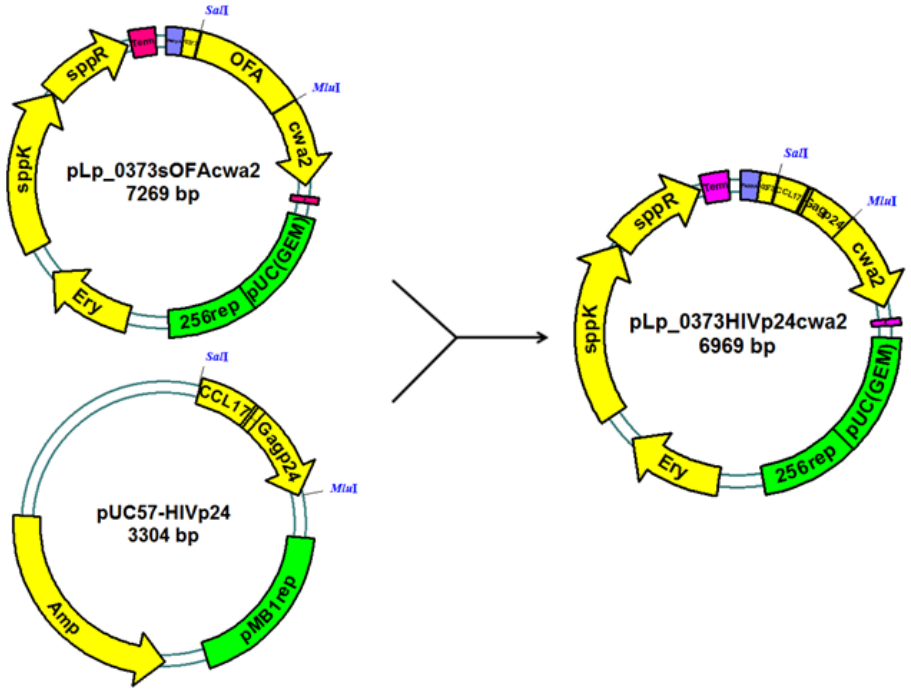


Figure 4.2: Cloning strategy for construction of pLp_0373HIVp24cwa2 for cell wall anchoring of HIVp24. The erythromycin resistance gene (*Ery*), the histidine protein kinase gene (*sppK*), the response regulator (*sppR*), the signal peptide (0373) and the target gene (*HIVp24-cwa2*) are all shown in yellow. The terminators (*Term* and *pepT*) are shown in pink and the P_{sppA} promoter is shown in blue. The replicons are shown in green.

4.1.2 Construction of a plasmid for membrane-anchoring of HIVp24

For covalent anchoring to the cell membrane, the HIVp24 DNA sequence was fused to a truncated version of the *L. plantarum* lipoprotein Lp_1261. The full length lipoprotein is an oligopeptide ABC transporter that contains an N-terminal lipid-modified cystein residue that binds to the outer part of the cell plasma membrane (Hutchings et al. 2009). The truncated Lp_1261 lipoanchor consists of the first 75 amino acids of the primary gene product and was chosen based on results from Fredriksen et al. (2012). The first 22 residues comprise the leader peptide and the Cysteine appears at postion 23 (as predicted by Prosite). The lipoanchor was truncated right upstream of the ABC transporter domain (predicted in Pfam). HIVp24 was coupled to Lp_1261 as shown in Figure 4.3. The cassette was originally constructed by Fredriksen et al (2012) to allow for easy exchange of both anchor and target protein.

The plasmid for production of covalent membrane anchored HIVp24 was constructed as shown in Figure 4.4. The MluI site present in the original HIVp24 fragment (see e.g. Fig. 4.2) had to be changed to EcoRI to match the restriction site in the starting vector, pLp_1261Inv.

For this purpose, HIVp24 was PCR amplified from template pLp_0373HIVp24cwa2 with primers GagF and GagR introducing the restriction sites SalI and EcoRI (all primers used in this study are listed in table 2.1 and described in table 2.2). The resulting 597 bp fragment was subcloned into a TOPO® vector (as described in section 3.6). The fragment was then excised from the vector using SalI and EcoRI and Quick ligated (section 3.9.4.1) with the 5849 bp fragment formed by digesting pLp_1261Inv with the same restriction enzymes, giving the plasmid pLp_1261HIVp24. pLp_1261HIVp24 was then transformed into chemo-competent *E. coli* TOP10 before final transformation into electro-competent *L. plantarum* WCFS1.

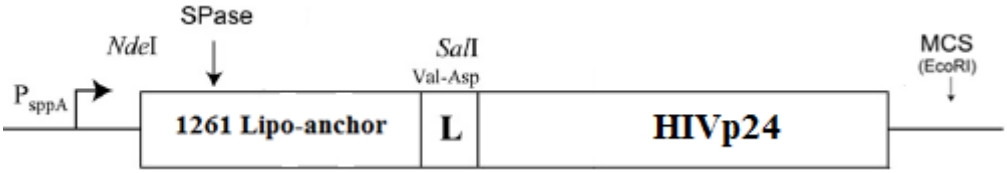


Figure 4.3: A schematic outline of the 1261-HIVp24 cassette. The figure schematically shows how the signal peptide (SP), cell membrane anchor (Lp_1261) and the target protein (HIVp24) are placed relative to each other. It also depicts the linker area (L) and the multiple cloning site (MCS) containing the restriction sites that may be used for exchange of the different components.

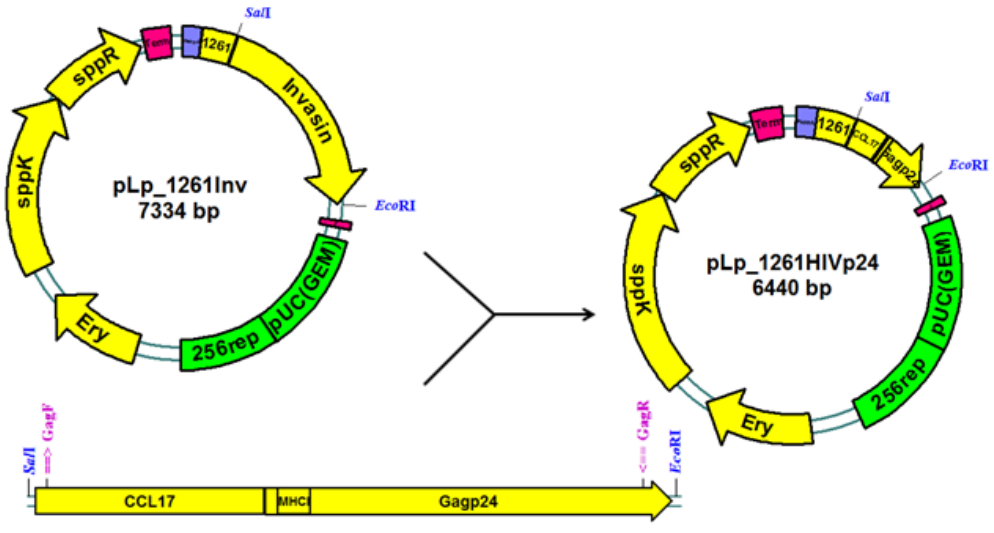


Figure 4.4: Cloning strategy for construction of pLp_1261HIVp24 for membrane anchoring of HIVp24. The invasin gene in pLp_1261Inv is replaced by PCR amplified HIVp24. Colour coding is as in Figure 4.2.

4.1.3 Construction of a plasmid for non-covalent cell wall anchoring of HIVp24

For noncovalent N-terminal anchoring to the cell wall, the HIVp24 DNA sequence was fused to a gene derived from *L. plantarum* named *Lp_3014*. The *Lp_3014* gene product is a cell surface protein, putatively a transglycosylase, with one N-terminal LysM anchoring motif that binds to the peptidoglycan in the cell wall (Fredriksen et al. 2012; Zhou et al. 2010). The Lp_3014 LysM anchor consists of 204 amino acids where the LysM domain appears at residues 32-74 and the transglycosylase domain at residues 132-196. The protein was selected based on results from Fredriksen et al. (2012). Fredriksen et al observed that a strain expressing truncated invasin fused to this LysM-containing protein showed good growth after induction by SppIP, and they could detect invasin on the cell surface. HIVp24 was coupled to the C-terminal part of Lp_3014 as shown in Figure 4.5. The cassette was originally constructed by Fredriksen et al (2012) to allow easy exchange of anchor and target protein.

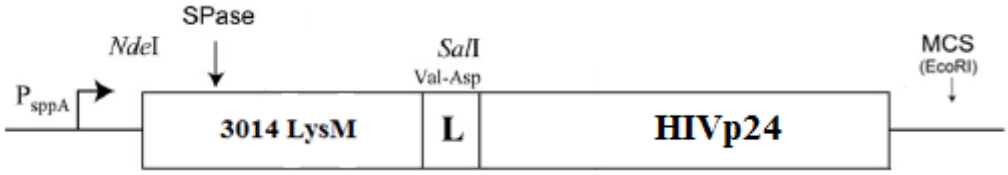


Figure 4.5: A schematic outline of the 3014-HIVp24 cassette. The figure schematically shows how the signal peptide (SP), the 204 amino acid LysM domain anchor (Lp_3014) and the target protein (HIVp24) are placed relative to each other. It also depicts the linker area (L) and the multiple cloning site (MCS) containing the restriction sites that may be used for exchange of the different components.

The plasmid for production of non-covalently membrane-anchored HIVp24 was constructed as shown in Figure 4.6. PCR amplified HIVp24 (using the same primers as for construction of pLp_1261HIVp24) was subcloned into a TOPO® vector. The HIVp24 gene fragment was excised from the TOPO® vector using SalI and EcoRI and ligated (section 3.9.4.1) with the 6221 bp fragment formed by digesting pLp_3014Inv with the same restriction enzymes, giving the plasmid pLp_3014HIVp24. pLp_3014HIVp24 was then transformed into chemo-competent *E. coli* TOP10 before final transformation into electro-competent *L. plantarum* WCFS1.

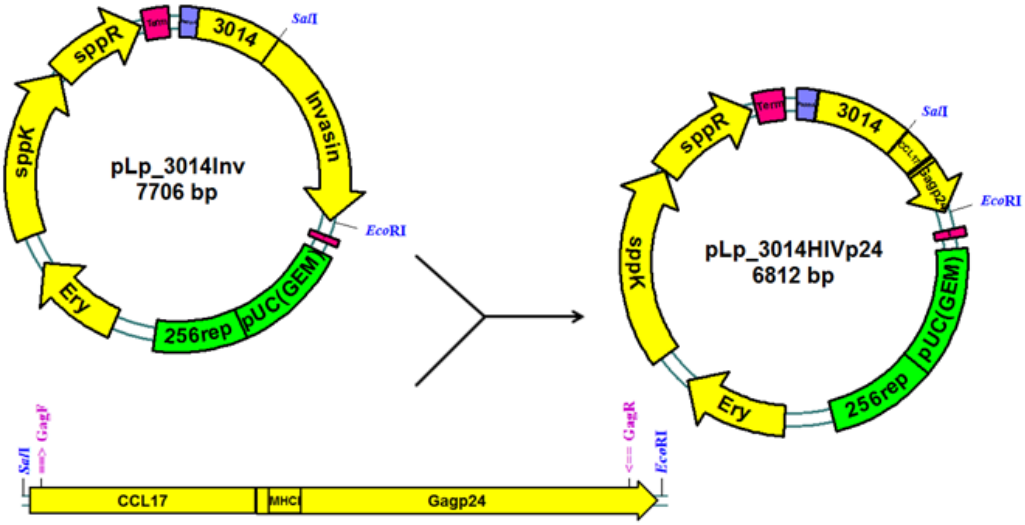


Figure 4.6: Cloning strategy for construction of pLp_30141HIVp24 for non-covalent cell wall anchoring of HIVp24. The invasin gene in pLp_3014Inv is replaced by PCR amplified HIVp24. Colour coding is as in Figure 4.2.

4.1.4 Construction of a plasmid for secretion of HIVp24

A secreted version of HIVp24 was also constructed, primarily for use as a control in the various analyses of recombinant strains described below. The signal peptide used was the same as that used for covalent cell wall anchoring (0373). The plasmid was constructed as shown in Figure 4.7.

HIVp24 with the signal peptide 0373 was PCR amplified from template pLp_0373HIVp24cwa2 with primers SekF and GagS-Acc65IR introducing restriction sites AgeI and Acc65I. The resulting 990 bp fragment was digested directly (without TOPO® subcloning) using AgeI and Acc65I and ligated (section 3.9.4.1) with the 5448 bp fragment formed by digesting pLp_1261HIVp24 with the same restriction enzymes, giving the plasmid pLp_0373HIVp24. pLp_0373HIVp24 was further transformed into chemo-competent *E. coli* TOP10 before final transformation into electro-competent *L. plantarum* WCFS1.

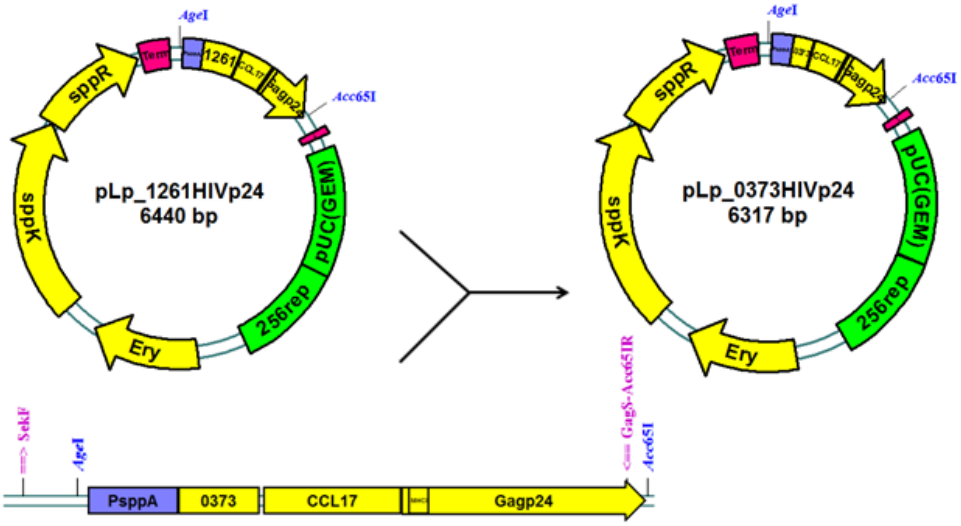


Figure 4.7: Cloning strategy for construction of pLp_0373HIVp24 for secretion of HIVp24. The promoter (P_{sppA}), the lipoprotein and HIVp24 in pLp_1261HIVp24 is replaced by PCR amplified promoter (P_{sppA}), signal peptide (0373) and HIVp24. Colour coding is as in Figure 4.2.

The relevant parts of the recombinant plasmids constructed in section 4.1 were all sequenced to verify the sequence of the inserted fragments and to detect potential mutations. Ensuring that no mutations had occurred was especially important for the strains that were to be tested in a mouse model. The sequencing was performed as described in section 3.12 using primers from in Table 2.1. Table 4.1 lists the different primers used to sequence the different plasmids.

Table 4.1: List of primers used for sequencing of relevant parts of the recombinant plasmids

Plasmid	Forward primer	Reverse primer
pLp_0373HIVp24cwa2	SekF, SeqGagpF	
pLp_1261HIVp24	GagF	GagR
pLp_3014HIVp24	GagF	GagR
pLp_0373HIVp24	SekF, SeqGagpF	GagR

4.2 Change of replicon from pUC(GEM)$_{rep}$/256$_{rep}$ to SH71$_{rep}$

In the development of a mucosal vaccine, it is desirable to investigate production HIVp24 in other LABs that might yield a different immunomodulating effect than *L. plantarum* WCFS1. The 256$_{rep}$ replicon is not working in most other LABs, and hence needs to be exchanged with another replicon such as SH71$_{rep}$. Kristina Maudal (2012) showed that replacing the replicons pUC(GEM)$_{rep}$ and 256$_{rep}$ with SH71$_{rep}$ also may have an effect in *L. plantarum* WCFS1. The replicon change increased the plasmid copy number in *L. plantarum* and resulted in higher expression of the target gene. We wanted to investigate the effect of a replicon change on

production and surface display of HIVp24 in *L. plantarum* WCFS1. The use of a different vaccine delivery vector than *L. plantarum* WCFS1 was not investigated in the present study.

The $SH71_{rep}$ replicon does not work in *E. coli* TOP10 and is generally unstable in *E. coli*, and the combined presence of the $SH71_{rep}$ replicon with the $pUC(GEM)_{rep}$ replicon makes the $pUC(GEM)_{rep}$ replicon very unstable (Personal comment Dr. G. Mathiesen). It is therefore impossible to keep both replicons in the same plasmid. Therefore, *E. coli* cannot be used as a subcloning host for plasmids containing this replicon, so subcloning was done in *Lc. lactis* MG1363. The replicons were successfully exchanged in pLp_1261HIVp24 and pLp_3014HIVp24, whereas attempts to exchange the replicons in pLp_0373HIVp24 and pLp_0373HIVp24cwa2 were not successful.

A plasmid containing the $SH71_{rep}$ replicon was initially isolated from *Lc. lactis* MG1363 carrying pSIP0373NucASH71. However, this approach yielded very small amounts of plasmid and it was difficult to get a clean product without "smear" on the gel. In an alternative approach pSIP411 (harbouring the $SH71_{rep}$ replicon) was isolated from *E. coli* TG1. This proved to be the better alternative as the procedure, after treatment with trypsin and PCR-cleanup to remove nucleases (for further details see section 3.3.2), resulted in a clean plasmid in sufficient amounts. Gel pictures showing digestions of plasmids isolated from *E. coli* TG1 and *Lc. lactis*, and illustrating the rationale behind the choice to use plasmid isolated form *E. coli* TG1, can be seen in the Appendix, Figure A.1.

Plasmids for construction of membrane-anchored and LysM anchored HIVp24 with exchanged replicon were constructed as illustrated in Figure 4.8. The plasmids pLp_1261HIVp24 and pLp_3014HIVp24 (section 4.1) were digested with Acc65I and BamHI. pSIP411 was digested using the same enzymes, and the 2002 bp fragment containing $SH71_{rep}$ was saved as an insert. Inserts and vectors were ligated together through overnight ligation (section 3.9.4.2) to create the plasmids pLp_1261HIVp24SH71 and pLp_3014HIVp24SH71. The resulting plasmids were transformed into electro-competent *Lc. lactis* MG1363 before final transformation into electro-competent *L. plantarum* WCFS1.

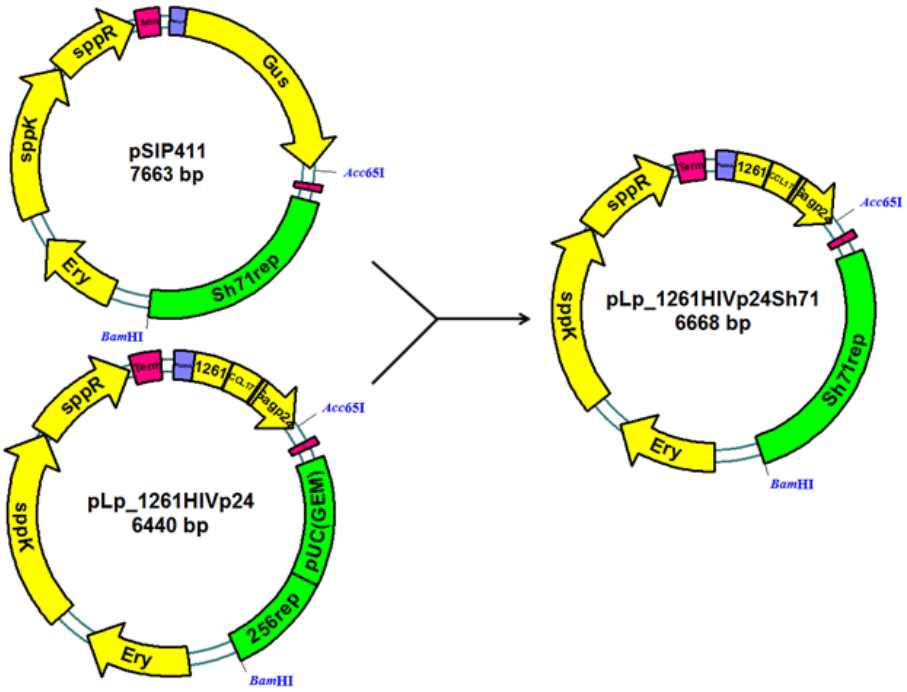


Figure 4.8: Cloning strategy for replacing the pUC(GEM)$_{rep}$/256$_{rep}$ replicons with the SH71$_{rep}$ replicon. The plasmid containing HIVp24 was used as a vector (here exemplified by pLp_1261HIVp24). The pUC(GEM)$_{rep}$/256$_{rep}$ fragment in this vector was replaced by SH71$_{rep}$ from pSIP411. Colour coding is as in Figure 4.2: The erythromycin resistance gene (*Ery*), the histidine protein kinase gene (*sppK*), the response regulator (*sppR*), the signal peptide (0373) and the target gene (*HIVp24-cwa2*) are all shown in yellow. The terminators (*Term* and *pepT*) are shown in pink and the P_{sppA} promoter is shown in blue. The replicons are shown in green.

Great troubles were experienced during the clonings of these plasmids. The relevant parts of the recombinant plasmids pLp_1261HIVp24SH71 and pLp_3014HIVp24SH71 were therefore sequenced, as well as controlled by PCR, to verify the presence of both the sequence encoding HIVp24 and the SH71$_{rep}$ replicon. The sequencing was performed as described in section 3.12 using primers from in Table 2.1. Table 4.2 lists the different primers used to sequence the two plasmids.

Table 4.2: List of primers used for sequencing of relevant parts of the recombinant plasmids

Plasmid	Forward primers	Reverse primers
pLp_1261HIVp24SH71	SekF, SeqRep2F	GagR, Sh71R
pLp_3014HIVp24SH71	SekF, SeqRep2F	GagR, Sh71R

Several attempts were made to change the replicon for the plasmids pLp_0373HIVp24 and pLp_0373HIVp24cwa2, following the strategy depicted in Fig. 4.9. Direct exchange of the replicons as described above (Fig. 4.8) was impossible due to the presence of interfering restriction enzyme sites. Therefore, the pSIP411 plasmid containing $SH71_{rep}$ was used as a vector and attempts were made to exchange the *Gus* gene in pSIP411 (Fig. 4.9) with PCR-amplified HIVp24. The cassette containing the 0373 signal peptide and HIVp24 was PCR amplified with and without cwa2 from template pLp_0373HIVp24cwa2 using reverse primers Cwa2-Acc65IR and GagS-Acc65IR, respectively, and forward primer SekF, introducing restriction sites AgeI and Acc65I for cloning. pSIP411 was digested with the same restriction enzymes, resulting in a 5681 bp vector. Cloning of PCR amplified HIVp24 into the vector was first attempted through direct digestion, then through TOPO® subcloning, but both strategies failed. The cloning attempts had to be stopped due to time limitations.

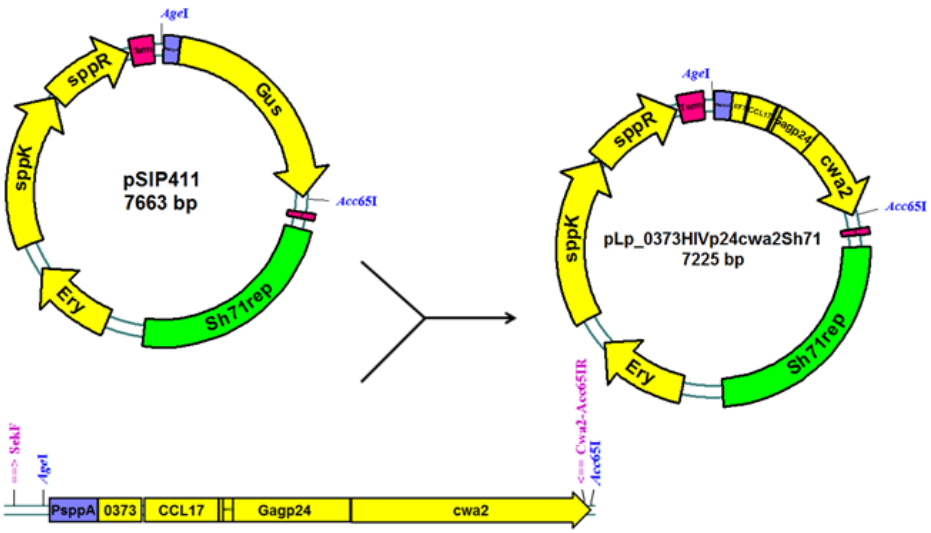


Figure 4.9: Cloning strategy for replacing the beta-glucuronidase (Gus) gene with HIVp24 in pSIP411 to create a pSIP plasmid with HIVp24 and $SH71_{rep}$. pSIP411 was kept as a vector and the beta-glucuronidase (Gus) gene was replaced by PCR amplified HIVp24. Colour coding as in Figure 4.8: The erythromycin resistance gene (*Ery*), the histidine protein kinase gene (*sppK*), the response regulator (*sppR*), the signal peptide (0373) and the target gene (*HIVp24-cwa2*) are all shown in yellow. The terminators (*Term* and *pepT*) are shown in pink and the P_{sppA} promoter is shown in blue. The replicons are shown in green.

4.3 Growth curves for *L. plantarum* strains harbouring plasmids for secretion and anchoring of HIVp24

After the cloning efforts described in sections 4.1 and 4.2 in total seven recombinant *L. plantarum* strains were available for further work. To check for possible effects of heterologous gene expression on bacterial fitness, growth curves were recorded for all strains. The cells were grown in 100 ml cultures of MRS containing erythromycin. Gene expression was induced at OD_{600} ~ 0.3 (as described in section 3.13) and the OD_{600} was measured at time intervals of 0.5-1 hour from 0 to 10 hours after induction.

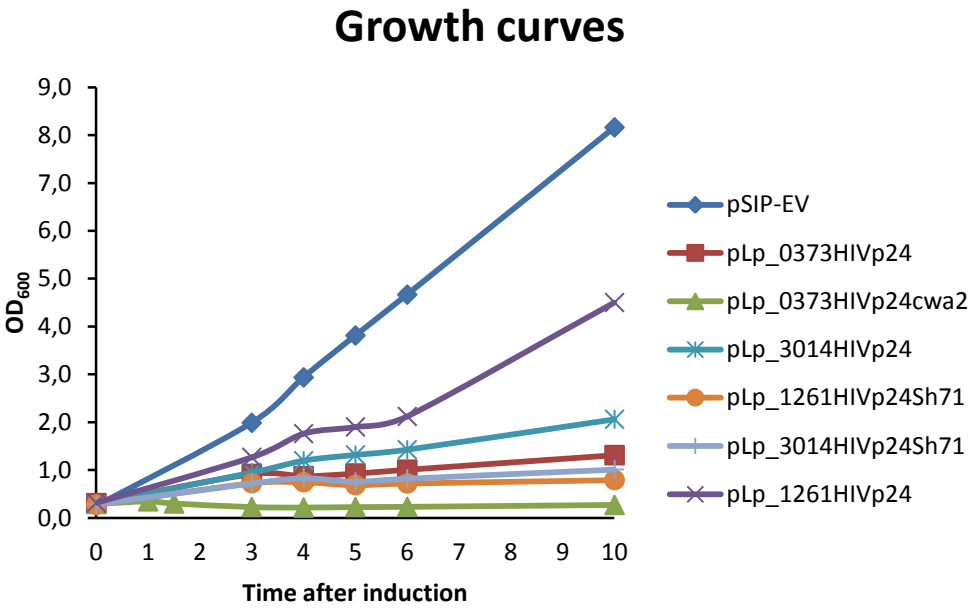


Figure 4.10: Growth curves of strains harbouring different plasmids for secretion or anchoring of HIVp24. All cultures were induced with SppIP. The cells were grown for a total of 10 hours after induction. pSIP-EV refers to a strain carrying an "empty vector", i.e. a strain that will not respond to induction by overexpressing a heterologous protein.

As shown in Figure 4.10, the strains showed large variation in their growth rates. Cells carrying the empty vector (pSIP-EV) had the highest growth rate. The other strains showed clearly reduced growth. The strain harbouring pLp_0373HIVp24cwa2 did not seem to grow from 1 hour post induction. The strain harbouring pLp_1261HIVp24 had the highest growth rate and reached the highest OD_{600} of all the strains producing HIVp24.

4.4 Detection of HIVp24 production in *L. plantarum* WCFS1 using Western blot analysis

4.4.1 Detection of HIVp24 in crude total cell extracts

To investigate whether the HIVp24 protein was produced in the different *L. plantarum* strains, all strains were grown, induced and harvested as described in section 3.13 and the total cell extracts were analysed by Western blotting. In short, the recombinant strains were cultivated in 50 ml erythromycin-containing MRS medium at 37 °C without shaking. The cultures were then grown until OD_{600} ~ 0.30 and after adding 25 ng/ml of SppIP the cultures were and grown for another 3.5 hours. Because the strain harbouring pLp_0373HIVp24cwa2 hardly grows post induction, this strain was harvested 1 hour post induction.

The HIVp24 fusion protein was detected using SDS-PAGE and Western blot analysis (using the Tank transfer system) performed as described in sections 3.15 and 3.16. Primary antibody mCCL17/TARC and secondary antibody Rabbit anti-goat IgG (H+L)-HRP were used for antibody hybridisation. To allow approximate comparisons of the amounts of proteins produced in the different strains, sample volumes loaded on the gel were varied according to the OD_{600} measured at the time of harvest. The Western blot (Figure 4.11) clearly shows that all *L. plantarum* containing the HIVp24 gene were able to produce the HIVp24 protein. The calculated theoretical molecular weight (MW) of the produced HIVp24 variants matches the bands seen on the blot for all constructs.

In lane 4 of Figure 4.11, displaying cell extract from the strain harbouring the plasmid for cell wall anchoring of HIVp24, a very weak band can be seen at ~49.0 kDa, i.e. the expected size, while the most dominant band has a size of about 60 kDa. As cwa2 is covalently bound to the cell wall, the 60 kDa band can be cwa2 covalently bound to parts of the cell wall. The Smears/bands below the arrow-marked bands in lanes 6-9 of Figure 4.11 are possibly degradation products of HIVp24, as they are not present in the negative control (Fig. 4.11, lane 2).

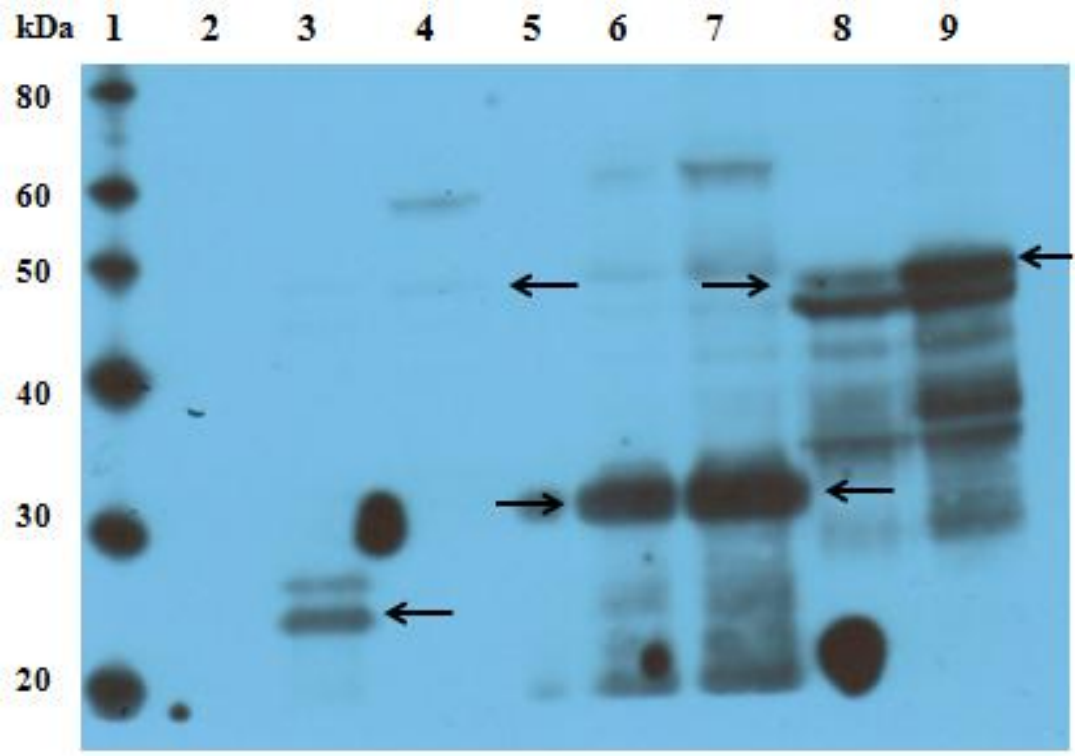


Figure 4.11: Western blot analysis of crude total cell extracts from strains harbouring plasmids for production of HIVp24 with different anchors. The numbered lanes show extracts of the different induced strains; theoretical molecular masses of the HIVp24 protein are given between parentheses and marked by arrows on the blot. 1, Magic Mark protein ladder with sizes indicated in kDA; 2, pSIP-EV (negative control); 3, pLp_0373HIVp24 (27.0 kDa for the unprocessed protein and 22.0 kDa for the processed protein); 4, pLp_0373HIVp24cwa2 (49.0 kDa); 5, Empty; 6, pLp_1261HIVp24 (30.3 kDa); 7, pLp_1261HIVp24SH71 (30.3 kDa); 8, pLp_3014HIVp24 (43.5 kDa), 9. pLp_3014HIVp24SH71 (43.5 kDa). The volume loaded on the gel for each strain was adjusted according to theOD_{600} measured at the time of harvest. This allows approximate comparison of the amounts of protein produced in the different strains. The cell extract of pLp_0373HIVp24cwa2 (lane 4) was concentrated 5x prior to gel electrophoresis.

4.4.2 The effect of changing the replicon

Figure 4.12 shows the results of additional analysis done to investigate the effect of a change in replicon on production of HIVp24. The figure shows part of the blot shown in Fig. 4.11 (right) and another blot run at a later time under exactly the same conditions, using another set of cell extracts (left). The figure shows that strains carrying constructs with the $SH71_{rep}$ replicon gave slightly higher production of HIVp24 than strains harbouring plasmids with the $pUC(GEM)_{rep}/256_{rep}$ replicons.

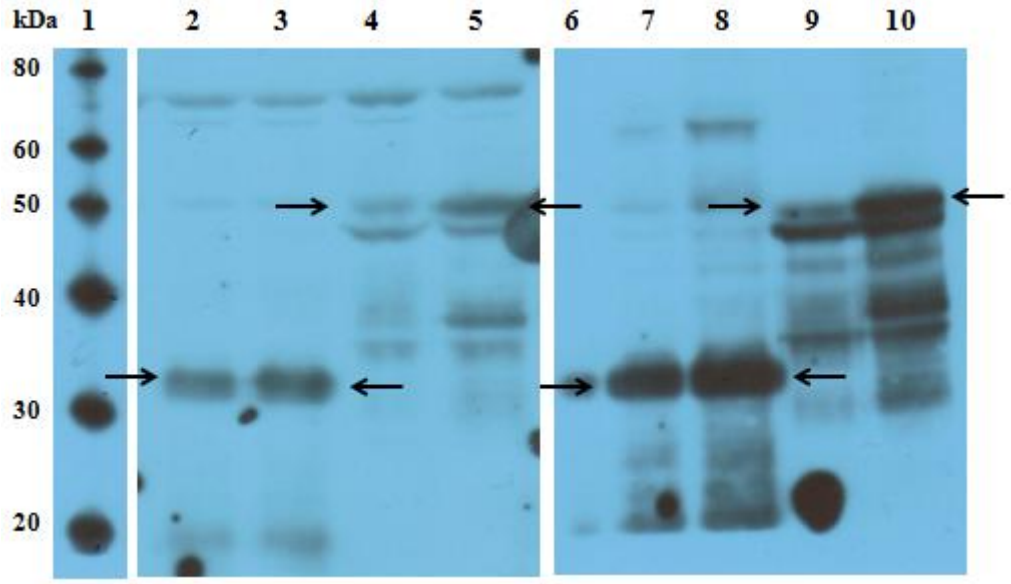


Figure 4.12: Western blot analysis of crude total cell extract from strains harbouring plasmids with different replicon. The figure is a mosaic of 2 parallel blots. The numbered lanes show extracts from the different induced strains; theoretical molecular masses of the HIVp24 protein are given between parentheses and marked by arrows on the blot. 1, Magic Mark protein ladder with sizes indicated in kDa; 2, pLp_1261HIVp24 (30.3 kDa); 3, pLp_1261HIVp24SH71 (30.3 kDa); 4, pLp_3014HIVp24 (43.5 kDa); 5, pLp_3014HIVp24 (43.5 kDa); 6, Empty; 7, pLp_1261HIVp24 (30.3 kDa); 8, pLp_1261HIVp24SH71 (30.3 kDa); 9, pLp_3014HIVp24 (43.5 kDa); 10, pLp_3014HIVp24SH71 (43.5 kDa). The volume loaded on the gel for each strain was adjusted according to the OD_{600} measured at the time of harvest. This allows approximate comparison of the amounts of protein produced in the different strains.

4.4.3 Production of HIVp24 in a time course experiment

To select conditions that would potentially yield maximal display of HIVp24, production of the antigen was monitored over time for selected strains.

100 ml cultures of the strains harbouring pLp0373HIVp24cwa2, pLp_1261HIVp24 and pLp_3014HIVp24 were grown and expression of HIVp24 was induced as described in section 3.13. Subsequently, a 10 ml sample was harvested every 30 minutes and OD_{600} was measured. The last sample was taken at 3.5 hours post induction.

When preparing cells for Western blotting, cells from 50 ml culture is harvested and the cells are lysed in 1 ml PBS (see section 3.13). In this experiment, only 10 ml culture was harvested, and thus to compensate for the lower amount of cells, the cells were resuspended in only 500 µl PBS prior to lysis. To allow approximate comparison of the amount of protein produced per cell at the different time intervals, the volumes loaded onto the SDS-PAGE gel from each sample were adapted to the OD_{600} measured at the time of harvest. The results are shown in figures 4.13-4.14.

Prior to this analysis all extracts from the strain harbouring pLp_0373HIVp24cwa2 were concentrated using Millipore Amicon® Ultra Centrifugal Filters from 500 µl to ~80 µl. Several attempts were made to produce a sensible blot, but none were successful (blots not shown). The first blot showed some bands, but it was not of good enough quality to be properly interpreted. Further attempts yielded no bands, possibly as a result of protein degradation during freezing and thawing of the samples.

The results for pLp_1261HIVp24 (Figure 4.13; lipo-anchored HIVp24) show that the 30.3 kDa HIVp24 protein appears about 1.5 hours after induction. The production increases with time and seems to reach a peak 3 hours post induction, as indicated by a vertical arrow in Figure 4.13.

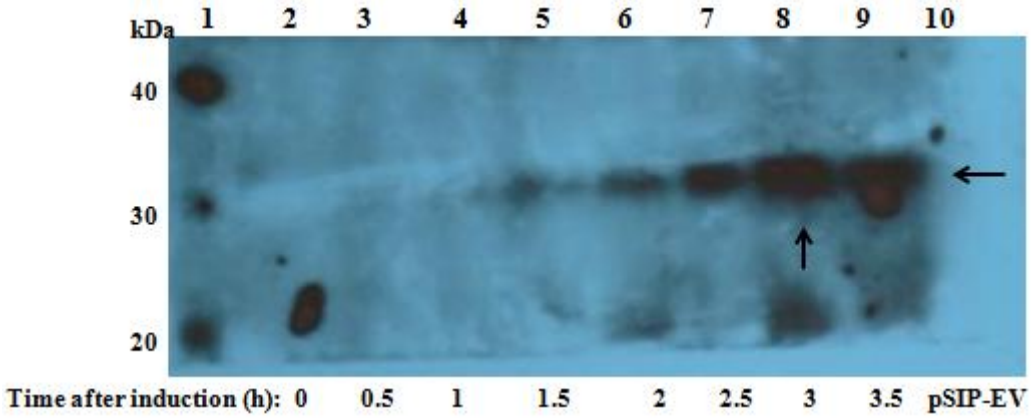


Figure 4.13: Western blot analysis of production of membrane anchored HIVp24 over time. The blot shows crude total cell extracts from an induced *L. plantarum* strain harbouring pLp_1261HIVp24. The lane labelling is as follows: 1, MagicMark protein ladder with sizes indicated in kDa; lanes 2-9, crude total cell extract from induced *L. plantarum* strain harbouring pLp_1261HIVp24 harvested at 0-3.5 hours after induction (time points are given below the lanes), 10. pSIP-EV harvested 3.5 hours post induction. The volume loaded on the gel for each sample was adapted to the OD_{600} measured at the time of harvest, in attempt to load approximately similar amounts of "cells" in each lane; this allows approximate comparison of the amounts of protein produced per cell at the different time intervals.

Figure 4.14 shows a Western blot analysis of expression of LysM-anchored HIVp24 in a time course experiment. Interestingly, different from the results seen for pLp_1261HIVp24 (Figure 4.13), Figure 4.14 shows that that production of the 43.5 kDa Lp_3014-fused HIVp24 starts earlier, prior to 0.5 hours post induction. The production reaches a peak at 1-1.5 hours post induction and then declines before reaching a seemingly stable level. Possible degradation products appear about 1 hour post induction. Notably, Figure 4.14 shows two bands that are

very close to each other. These bands probably represent the LysM-HIVp24 protein without (i.e. SPase processed) and with its signal peptide.

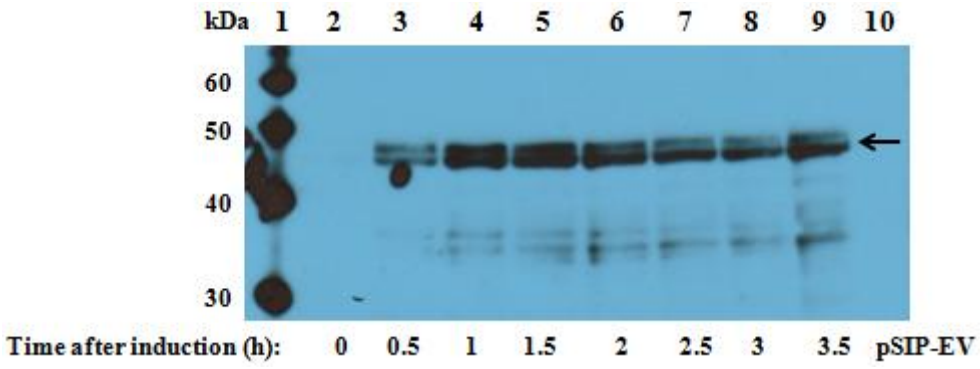


Figure 4.14: Western blot analysis of production of non-covalent membrane anchored HIVp24 over time. The blot shows crude total cell extracts from an induced *L. plantarum* strain harbouring pLp_3014HIVp24. The lane labelling is as follows: 1. MagicMark protein ladder with sizes indicated in kDa; lanes 2-9, crude total cell extracts from induced *L. plantarum* strain harbouring pLp_3014HIVp24 harvested at 0-3.5 hours after induction (time points are given below the lanes). 10. pSIP-EV harvested 3.5 hours post induction. The volume loaded on the gel for each sample was adapted to the OD_{600} measured at the time of harvest, in attempt to load approximately similar amounts of "cells" in each lane; this allows approximate comparison of the amounts of protein produced per cell at the different time intervals.

For practical reasons it is best to use the same harvest times for flow cytometry analysis and mouse trials on as many strains as possible. Based on the results shown in figures 4.13 and 4.14, it was decided to harvest cells at 3.5 hours post induction. It is estimated that at that time, the strains harbouring plasmid for expression of Lp_1261HIVp24 and Lp_3014HIVp24 will both have a high amount of HIVp24 presented on the cell surface. Peak production of cwa2-fused HIVp24 could not be determined, as no functional blots were produced. As the growth curve produced in section 4.3 (Figure 4.10) shows a decrease in growth at 1 hour post induction, it was decided to harvest this strain at 1 hour post induction.

4.4.4 Detection of HIVp24 in culture supernatants

Culture supernatants collected during cell harvesting (section 4.4.1) were also analyzed for the presence of HIVp24. Preparations and visualisation of the blot were performed as described in section 4.1.1. Before the analysis, 1 ml of culture supernatant of all strains (2 ml for the strain harbouring cwa2-anchored HIVp24) was concentrated using TCA precipitation (see section 3.14.1). The result is shown in Figure 4.15.

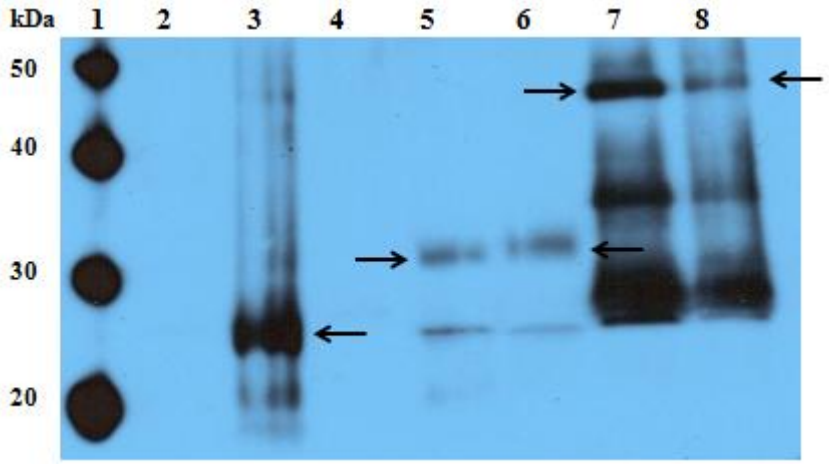


Figure 4.15: Western blot analysis of supernatants from strains harbouring different plasmids for secretion and anchoring of HIVp24. All samples were harvested at 3.5 hours post induction, except the strain harbouring pLp0373HIVp24cwa2, where supernatant was harvested at 1 hour after induction. The numbered lanes show extracts the different induced strains; theoretical molecular masses of the HIVp24 protein are given between parentheses and marked by arrows on the blot. 1, Magic Mark protein ladder with sizes indicated in kDa; 2, pSIP-EV (negative control); 3, Secreted HIVp24 with no signal peptide (22.0 kDa); 4, pLp_0373HIVp24cwa2 (49.0 kDa); 5, pLp_1261HIVp24 (30.3 kDa); 6, pLp_1261HIVp24SH71 (30.3 kDa); 7, pLp_3014HIVp24 (43.5 kDa); 8, pLp_3014HIVp24SH71 (43.5 kDa).

As expected, HIVp24 was detected in the supernatant of the strain harbouring a plasmid for secretion of HIVp24 (Fig. 4.15, lane 3), whereas no HIVp24 was detected in the negative control (Fig. 4.15, lane 2). No HIVp24 could be detected in supernatant of the strain producing cwa2-fused HIVp24 (Fig. 4.15, lane 4), and very little HIVp24 was detected in the supernatant of strains with Lp_1261-fused HIVp24 (lipo-anchor). However, supernatants from the strains harbouring Lp_3014-fused HIVp24 (Fig. 4.15, lanes 7 and 8) had considerable amounts of HIVp24 in the supernatant, and also showed a lot of degraded protein products.

4.5 Detection of surface located HIVp24 in *L. plantarum* WCFS1 by immunostaining

Prior to mouse trials it is essential to confirm the presence of the antigen on the surface of the bacteria. Surface localization of the HIVp24 protein was analyzed using immunostaining followed by flow cytometry and confocal microscopy. Immunostaining was performed using the HIVp24-specific mCCL17/TARC goat anti-mouse IgG primary antibody and an Alexa Fluor® 488-conjugated secondary antibody. The samples for flow cytometry and confocal microscopy were prepared as described in section 3.17.1 and section 3.17.2, respectively.

4.5.1 Detection of surface located HIVp24 on *L. plantarum* WCFS1 by flow cytometry

For flow cytometry analysis, the cells were grown, harvested and immunostained as described in section 3.17.1. All strains were harvested at 3.5 hours post induction, and an additional harvest was done at 1 hour post induction for the strain producing HIVp24 fused to cwa2. Figure 4.16 shows the results as single dimension histograms, one for each tested strain, whereas the most important strains are compared in Fig. 4.17. The more the peak is shifted to the right, along the x-axis, the stronger the signal (which indicates more accessible HIVp24 at the surface). The y-axis indicates the relative number of cells detected.

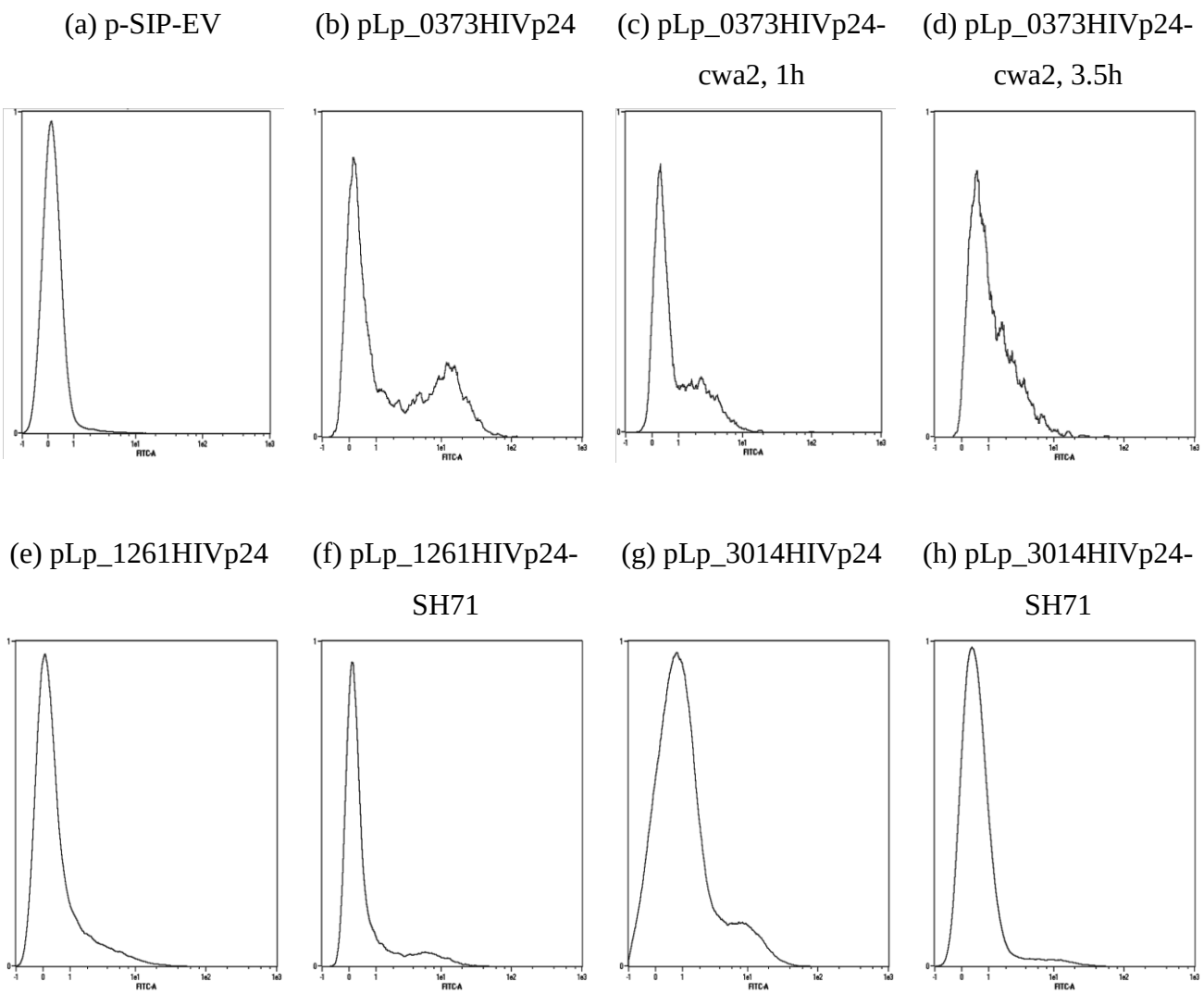
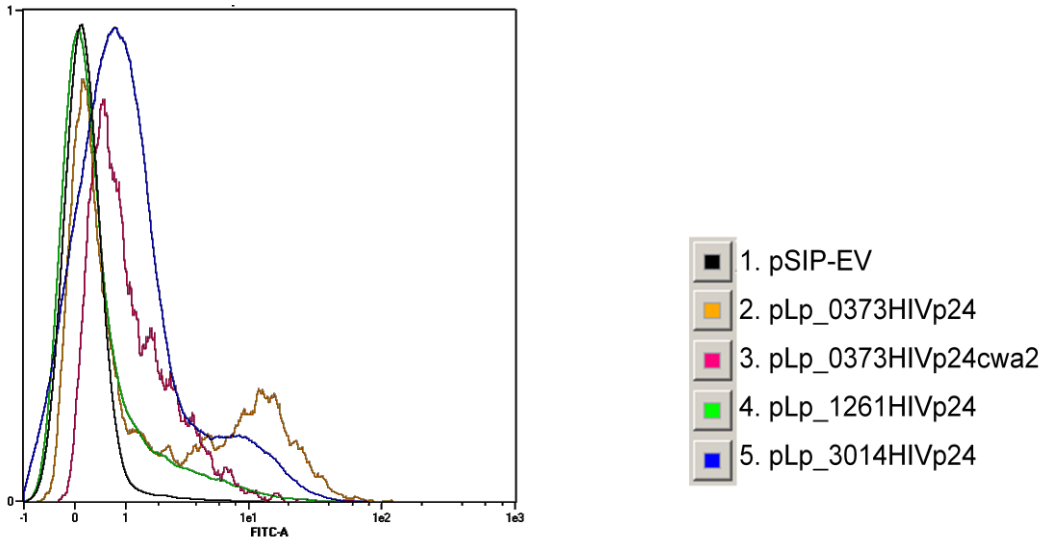


Figure 4.16: Flow cytometry analysis of Alexa Fluor® 488-stained *L. plantarum* strains harbouring various plasmids for anchoring and secretion of HIVp24. The figure shows single dimension histograms of all the strains where relative fluorescence (x-axis) is plotted against the relative number of events (y-axis). The cells were immunostained using the HIVp24 specific mCCL17/TARC goat anti-mouse IgG primary antibody and Alexa Fluor® 488 donkey anti-goat secondary antibody.

Figure 4.17a shows a single dimension histogram containing signal graphs from five of the strains. Figure 4.17b depicts the median values given from the flow cytometer as a bar chart, for all eight tested samples. The median value gives the overall horizontal displacement of the cell populations along the x-axis of the histograms. The length of the bar illustrates the amount of displacement of the histogram peak along the x-axis. The bar chart allows easier comparison of the fluorescent signal given from each strain than comparison through the single dimension histogram (Figure 4.17a).

(a)



(b)

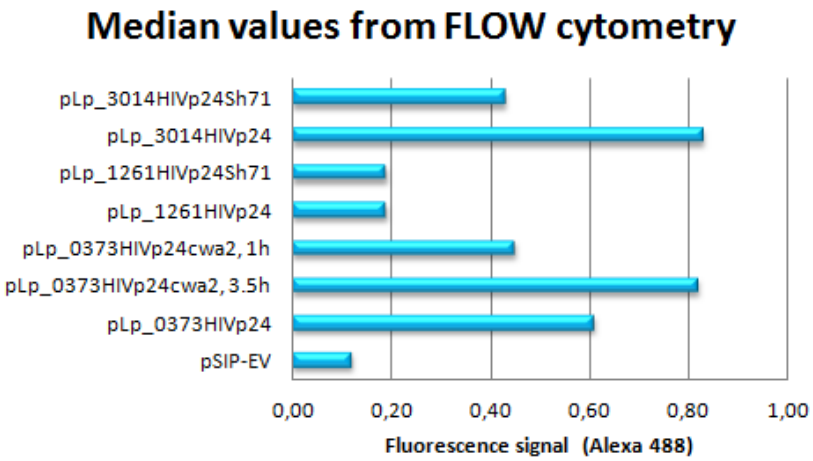


Figure 4.17: Flow cytometry analysis of Alexa Fluor® 488-stained *L. plantarum* strains harbouring various plasmids for anchoring and secretion of HIVp24. (a) Results for selected strains in one single dimension histogram, allowing a comparison of the signals depicted individually in Fig. 4.16. The colour scheme is shown to the right of the histogram. (b) Horizontal bar chart illustrating the median values obtained for each sample. The median value illustrates the average displacement along the x-axis of the histogram for the cell populations.

The negative control, pSIP-EV, gave no fluorescent signal, which indicates that HIVp24 indeed must be present when a fluorescent signal is detected. The majority of cells from the strains harbouring Lp_1261HIVp24 also yielded no signal, but the "shoulder" indicates some cells with fluorescent signals. For the strains harbouring cwa2- and Lp_3014-fused HIVp24, close to all cells elicited a fluorescent signal, indicating presence of HIVp24 on the surface of close to all the cells. The strain with Lp_3014-fused HIVp24 showed the strongest signal, indicating that this strain has the highest amount of available HIVp24 on the surface. The strain harbouring a plasmid for secretion of HIVp24 (pLp_0373HIVp24) showed two populations of cells (two peaks). The largest peak has the same displacement as the negative control, while the smaller peak has the greatest displacement of all the strains. This indicates no presence of HIVp24 on the surface of most of the cells, while some cells have a great amount of HIVp24 present on the cell surface.

The flow cytometry analysis indicated that all strains have some cells with HIVp24 antigen present on the cell surface. Further tests were done to select the best strains/conditions for further work.

Figure 4.18a shows a comparison of the signal from each harvest time used for the pLp_0373HIVp24-cwa2 construct (1 hour and 3.5 hours). A slightly stronger signal can be detected at 3.5 hours than 1 hour post induction. Although the signal obtained at 1 hour post induction is weaker than the signal given at 3.5 hours after induction, the signal obtained at one hour after induction is still strong enough to indicate presence of HIVp24 antigen on the cell surface of the bacteria. Considering the observed growth arrest one hour after induction (Fig. 4.10), it was decided to harvest the strain producing cwa2-anchored HIVp24 at one hour post induction for the mouse trials.

Figures 4.18b&c show a comparison of signals detected for the strains producing Lp_1261-fused HIVp24 and Lp_3014-fused HIVp24 from plasmids with or without the $SH71_{rep}$-replicon. There is close to no difference between the two strains producing Lp_1261-fused HIVp24. Surprisingly, cells producing Lp_3014-fused HIVp24 from the plasmid with the 256_{rep} replicon show a stronger and wider signal than the cells producing Lp_3014-fused HIVp24 from the plasmid with the $SH71_{rep}$ replicon. It was concluded from these results that the change of replicon had no detectable positive effect on the amount of antigen present on the cell surface of the bacteria.

(a) Harvest comparison Cwa2 (b) Replicon comparison Lp_1261 (c) Replicon comparison Lp_3014

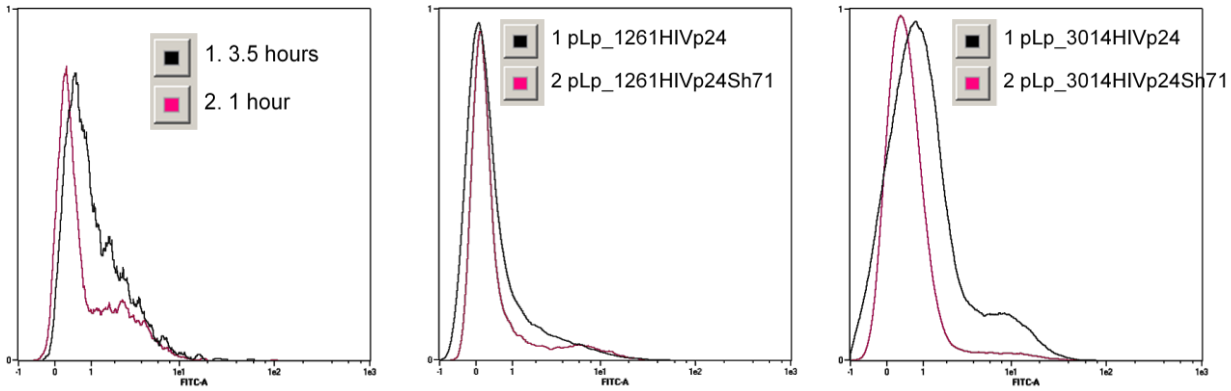


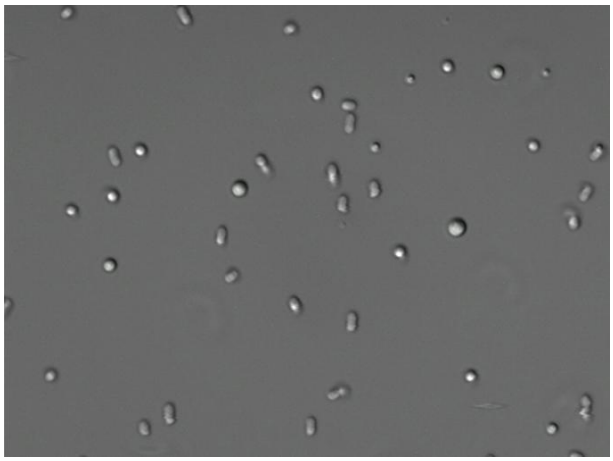
Figure 4.18: Flow cytometry analysis of Alexa Fluor® 488-stained *L. plantarum* strains harbouring various plasmids for anchoring and secretion of HIVp24. The figure shows comparisons of the results presented in figure 4.16. (a) Comparison for the strain harbouring pLp_0373HIVp24cwa2 harvested at 1 hour and 3.5 hours after induction. (b) Comparison of strains harbouring pLp_1261HIVp24-encoding plasmids with different replicons. (c) Comparison of strains harbouring pLp_3014HIVp24-encoding plasmids with different replicons.

The strain harbouring the plasmid pLp_1261HIVp24 showed a high production of Lp_1261-fused HIVp24 (seen on Western blots, Figure 4.11), but, according to the flow cytometry results, very little HIVp24 was located on the surface of the bacteria. In this strain, HIVp24 is supposed to be covalently bound to the plasma membrane, and one explanation for the weak signal can be that the antigen is buried within the cell wall, unavailable for the antibodies used in the immunostaining. An experiment was conducted to investigate this. Induced and harvested cells were treated with lysozyme for 30 minutes at 37 °C prior to immunostaining to partly break down the cell wall, which could make the possibly hidden antigen more accessible for the antibody. The cells were subsequently analysed by flow cytometry. Lysozyme treatment yielded no change in signal; the data are shown in the Appendix, Figure A.5.

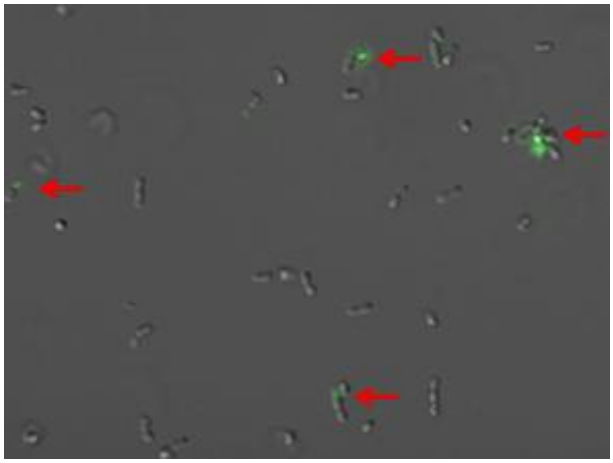
4.5.2 Detection of surface located HIVp24 on *L. plantarum* WCFS1 by confocal microscopy

A different way of detecting surface located HIVp24 in cells producing HIVp24 antigen is through confocal microscopy. The cells were stained with the same antibodies as in the flow cytometry analysis, following the protocol described in section 3.17.2. The results from this experiment correlate well with the results found through flow cytometry analysis. No signal

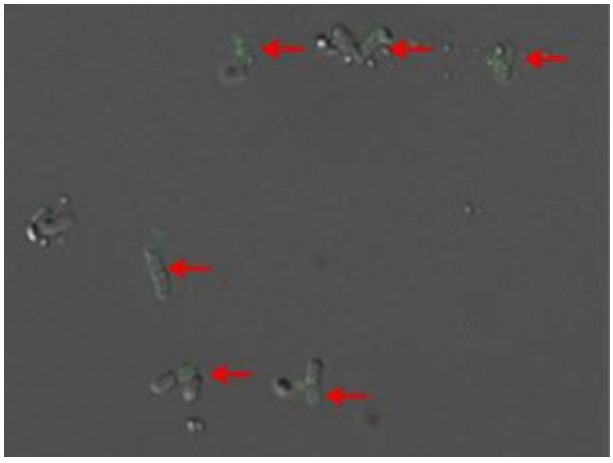
was detected for the negative control (pSIP-EV, Fig 4.19a). For the strains producing Lp_1261-fused HIVp24 (Fig. 4.19d-e) and secreted HIVp24 (Fig. 4.19b), only a few cells showed a fluorescent signal. Close to all the cells producing cwa2- and Lp_3014-fused HIVp24 (Fig. 4.19c and 4.19f-g, respectively) gave a fluorescent signal, and cells producing Lp_3014-fused HIVp24 show the strongest signals. An additional observation made from these micrographs is that all the cells producing cwa2-anchored HIVp24 seemed deformed (Fig. 4.19c). This was investigated closer through scanning electron microscopy (presented in section 4.6).



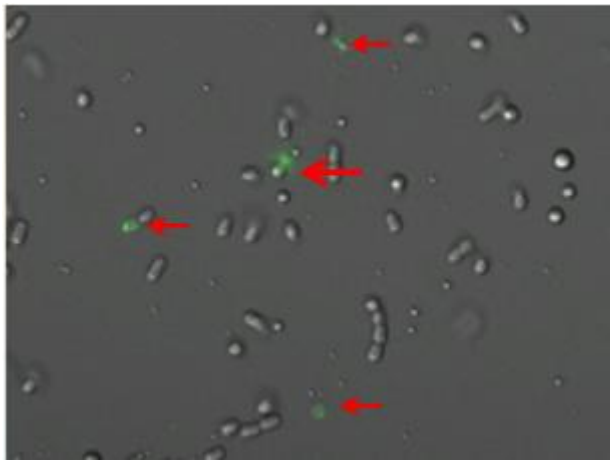
(a) pSIP-EV



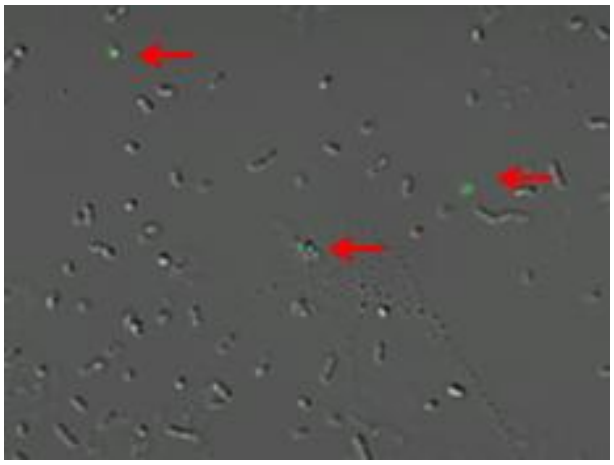
(b) pLp_0373HIVp24



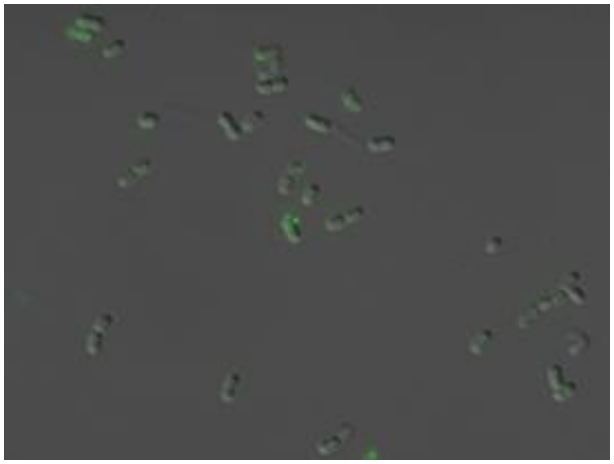
(c) pLp_0373HIVp24cwa2, 3.5 hrs



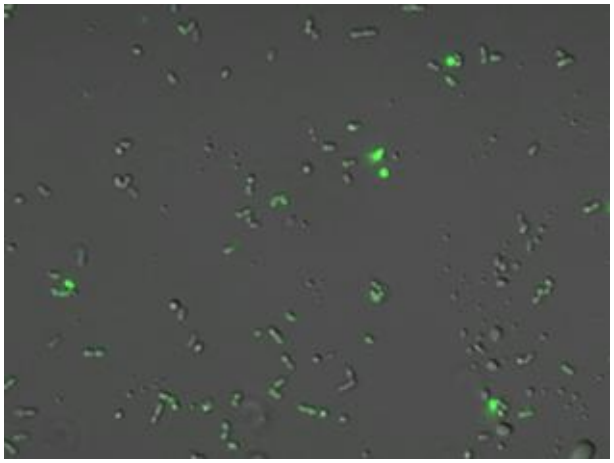
(d) pLp_1261HIVp24



(e) pLp_1261HIVp24SH71



(f) pLp_3014HIVp24



(g) pLp_3014HIVp24SH71

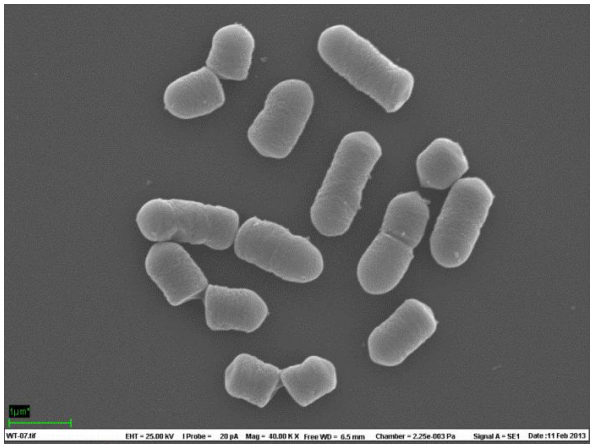
Figure 4.19: Confocal microscopy of *L. plantarum* strains harbouring plasmids for anchoring or secretion of HIVp24. The figure shows confocal microscopy micrographs of all the strains, (a)-(g). The cells were harvested 3.5 hours post induction and immunostained using the HIVp24 specific mCCL17/TARC goat anti-mouse IgG primary antibody and Alexa Fluor® 488 donkey anti-goat secondary antibody as described in section 3.17.2. (a) displays pSIP-EV, negative control. For images (a)-(e), the cells eliciting fluorescence are marked by arrows. In images (f)-(g), all cells show fluorescence and are thus not marked by arrows.

4.6 Visualisation of *L. plantarum* using scanning electron microscopy

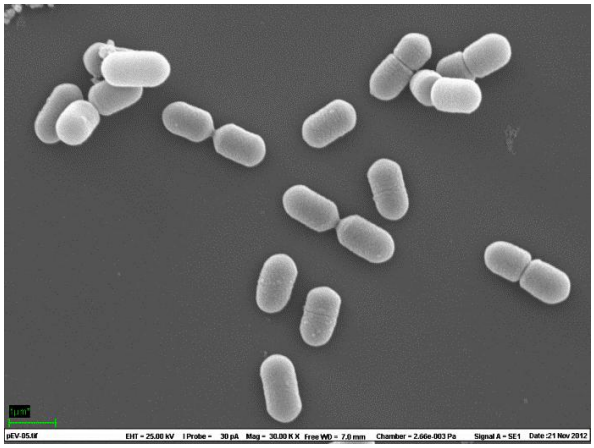
The confocal micrographs of the strains producing cwa2-anchored HIVp24 showed what looked like disrupted cells. To closer investigate these changes, the strains were examined by scanning electron microscopy (SEM). The SEM was also performed to search for general changes in the bacterial surface due to HIVp24 production that might change the immunomodulatory effects.

One to three millilitre of bacterial culture from each of the strains was harvested from bacteria grown and induced as described in section 4.4.1. The strain harbouring

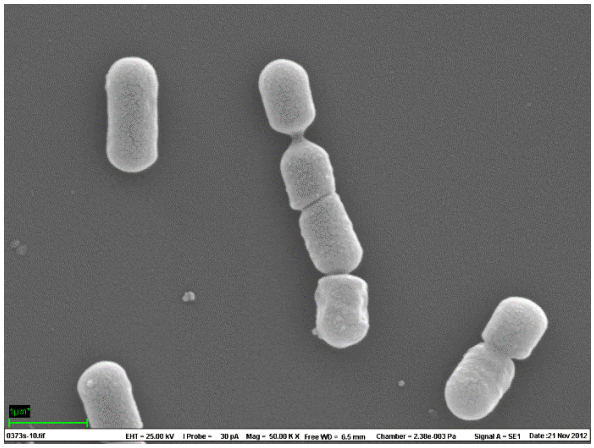
pLp_0373HIVp24cwa2 was harvested at both 1 hour and 3.5 hours post induction. In addition, *L. plantarum* with no plasmid (wild type, WT) was grown over night and harvested without induction. The cells were then fixed, dehydrated, critical point dried (CDP) and gold-palladium coated as described in section 3.18. The results of the subsequent SEM analyses are shown in Figure 4.20.



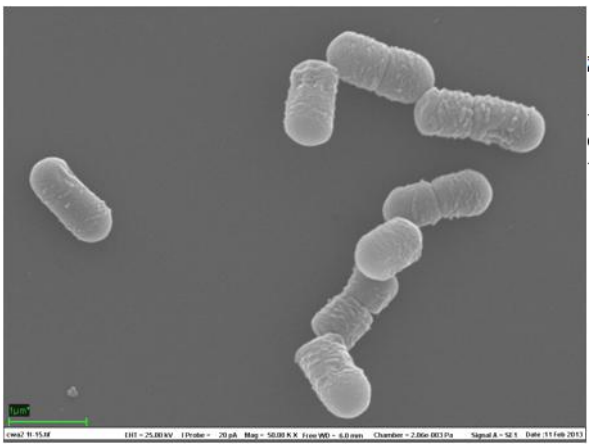
(a) *L. plantarum*, WT



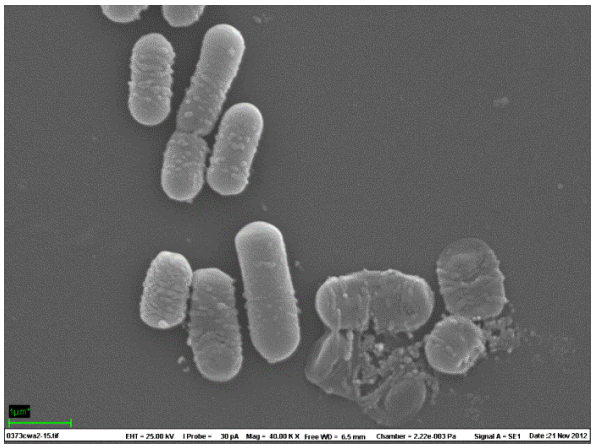
(b) pSIP-EV



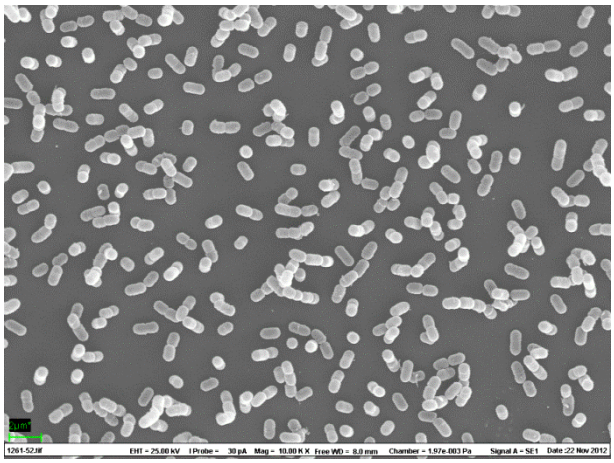
(c) pLp_0373HIVp24



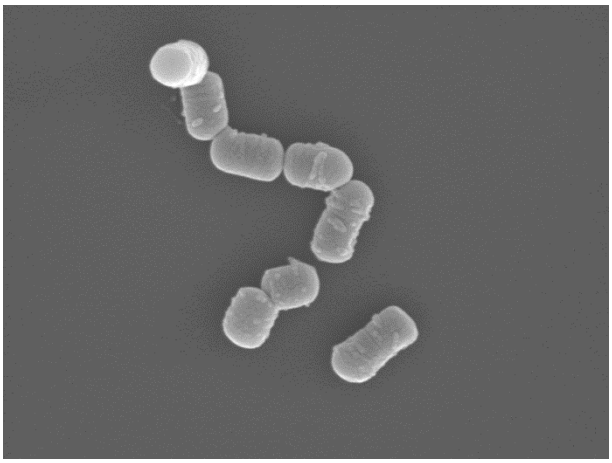
(d) pLp_0373HIVp24cwa2, 1 hr



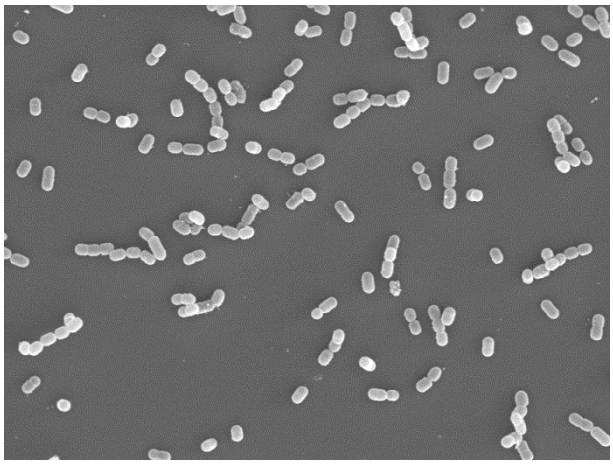
(e) pLp_0373HIVp24cwa2, 3.5 hrs



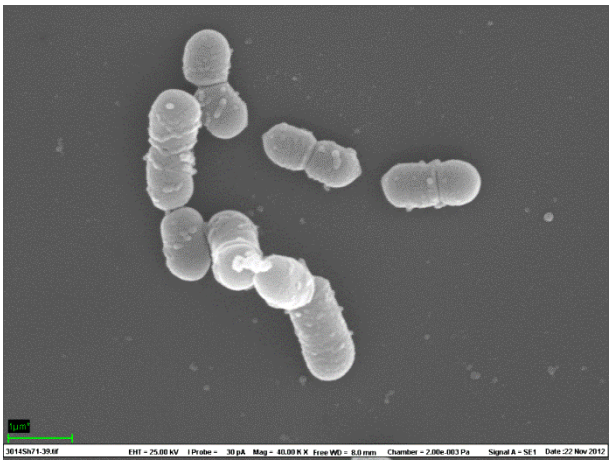
(f) pLp_1261HIVp24



(g) pLp_1261HIVp24SH71



(h) pLp_3014HIVp24



(i) pLp_3014HIVp24SH71

Figure 4.20: Scanning electron micrographs of *L. plantarum* WT cells and *L. plantarum* cells harbouring plasmids for secretion or anchoring of HIVp24.

From the micrographs presented in Figure 4.20 (Fig. 4.20a-i) it is seen that the cells of the wild type (WT) (Fig. 4.20a), the cells harbouring pSIP-EV (Fig. 4.20b) and the cells secreting HIVp24 (c) all look very similar. They have a smooth surface and appear mainly as single or double rods. The strain harbouring pLp_0373HIVp24cwa2 (Fig. 4.20d-e) also mainly appears as single or double rods, but interestingly the cells have a very blistery surface. The blisters are present in cells harvested 1 hour and 3.5 hours post induction. As suggested by the OD_{600}-measurements, showing that the OD_{600} declines from 1 hour post induction, there are more lysed cells visible in the cell population harvested 3.5 hours post induction than in the population harvested at 1 hour post induction; the cells in culture go into lysis about 1 hour after induction. The strains harbouring pLp_1261HIVp24 (f-g) and pLp_3014HIVp24 (h-i) show some blisters, but not to the same extent as the strain harbouring pLp_0373HIVp24cwa2. The cells producing lipo- and LysM-anchored HIVp24 also appear

much more in chains of 4 or more bacteria than the control strains. The strains producing LysM-anchored HIVp24 appear even more in chains than the strains producing lipoanchored HIVp24. The strains harbouring plasmids with the $SH71_{rep}$-replicon (g)+(i) show the same results as the corresponding strains with the other replicon (f)+(h).

Van Baarlen and colleagues (2009) found that cells harvested from different growth stages had dissimilar immunomodulating effects, which could be a result of varying surface patterns presented in the different growth stages. The SEM micrographs show bacteria with varying characteristics and surface patterns depending on the anchor protein. Although there are several problems with growing and harvesting the strain producing HIVp24cwa2, it is still a very interesting candidate for vaccine delivery due to its unique surface pattern. The strain was thus included in further, despite its shortcomings.

4.7 Detection of surface-located HIVp24 fusion protein in strains subjected to conditions met during mouse trials

4.7.1 Detection of surface-located HIVp24 fusion protein in strains stored at -80 ºC in 20 % glycerol

One must be able to store and transport induced and harvested cells without losing antigen presence on the surface if the recombinant bacteria are to be used as a vaccine. The mouse trials also depend on the possibility to store induced bacteria without losing antigen on the surface, as it for practical reasons is impossible to immunise the mice with freshly harvested cells.

Antigen presence after storage in glycerol was investigated through a flow cytometry experiment where freshly induced and harvested cells, cells frozen one night and cells frozen for 2 weeks were analyzed. The results are presented in Figure 4.21, and prove no negative effects on antigen presence by storing at -80 ºC in 20 % glycerol. Storage seems to rather have a positive effect on antigen presence for the strains harbouring the Lp_3014HIVp24 fusion protein. Experiments were also conducted to investigate the bacteria's ability to survive the storage, but the results were inconclusive. Experiments conducted by a fellow master student, L. Øverland, showed that the bacteria survive storage without losing surface signal (Pers. com., L. Øverland).

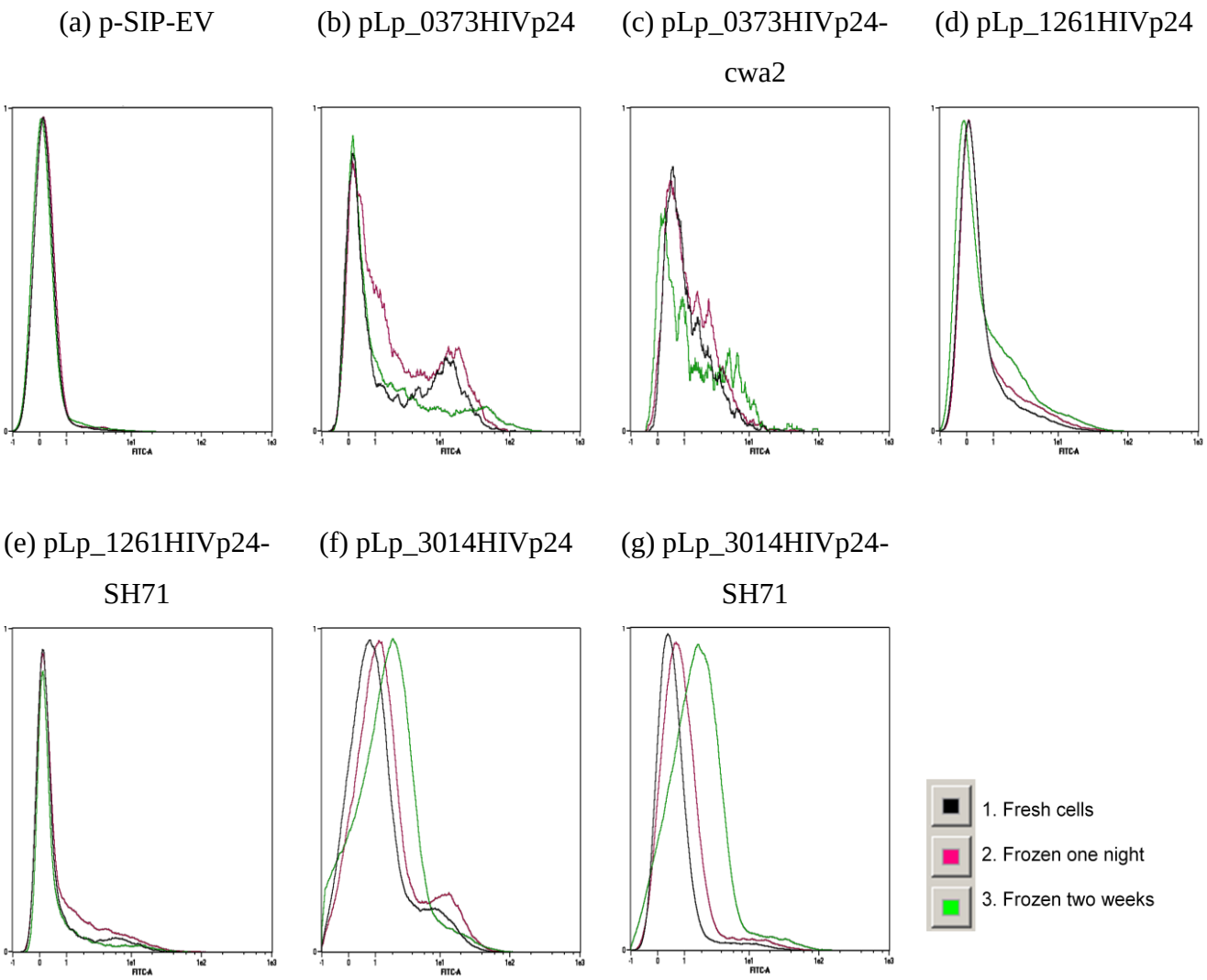


Figure 4.21: Flow cytometry analysis of Alexa Fluor® 488-stained *L. plantarum* strains harbouring various plasmids for anchoring and secretion of HIVp24 – comparing fresh cells with stored cells. The figures show single dimension histograms of all the strains where relative fluorescence (x-axis) is plotted against the relative number of events (y-axis). The cells were immunostained using the HIVp24 specific mCCL17/TARC goat anti-mouse IgG primary antibody and Alexa Fluor® 488 donkey anti-goat secondary antibody. The colour scheme is shown to the right of the last histogram.

4.7.2 Detection of surface-located HIVp24 in strains subjected to conditions met in the mouse stomach

In order to function as an oral vaccine, the antigen presented on the cell surface must remain intact during passage through the GI-tract, for the cells to allow an interaction with the intestinal immune system. It is widely accepted that *L. plantarum* can survive the passage of the stomach (Lebeer et al. 2008; Marco et al. 2009; van Bokhorst-van de Veen et al. 2012; Vesa et al. 2000), but it is still important to test whether the HIVp24 antigen displayed on the cell's surface can withstand the acidic environment in the mouse stomach. The strain harbouring Lp_3014-fused HIVp24 was chosen for the experiment as it showed the strongest

signal in flow cytometry analysis, meaning that it provides the most sensitive system for detecting a possible decline. The stomach of fed mice holds pH 3 (McConnell et al. 2008). The cells were induced, harvested, stored in glycerol and thawed, as would be done for mouse trials. The bacteria were then exposed to PBS made acidic by HCl to pH 3, 37 °C for 5, 20 and 60 minutes and for 2 seconds (bacteria submerged in the acid, then instantly removed). After exposure to acid, the cells were washed and stained for flow. The result is depicted in Figure 4.22.

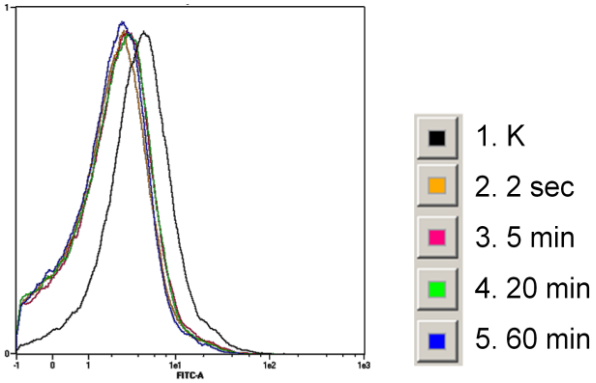


Figure 4.22: Flow cytometry analysis of Alexa Fluor® 488-stained *L. plantarum* strain harbouring a plasmid for noncovalent cell wall anchoring of HIVp24 (pLp_3014HIVp24) subjected to conditions met in the mouse stomach. The figure shows a single dimension histogram of cells incubated at pH 3.0. Relative fluorescence (x-axis) is plotted against the relative number of events (y-axis). The cells were immunostained by using the HIVp24 specific mCCL17/TARC goat anti-mouse IgG primary antibody and Alexa Fluor® 488 donkey anti-goat secondary antibody. The colour scheme is shown to the right of the histogram. K = control, that is, no exposure to acid.

The results presented in Figure 4.22 show a slight negative change in signal after contact with acid. But after the first contact is made, there is no change in signal for at least one hour.

4.8 Effects of the HIVp24 expressing strains on mice

4.8.1 Detection of cytokines through ELISPOT and multiplex cytokine quantification analyses

Interferon gamma (IFN-γ) is a proinflammatory cytokine which promotes systemic inflammation. High detectable levels of this cytokine thus indicate an immune response to the vaccine. An ELISPOT assay detecting IFN-γ producing cells was performed on cells from

Peyer's patches (PP) and spleen of all mice as briefly described in section 3.19.5. Cells from PP yielded no results (data not shown), while data from spleen cells are shown in Figure 4.23.

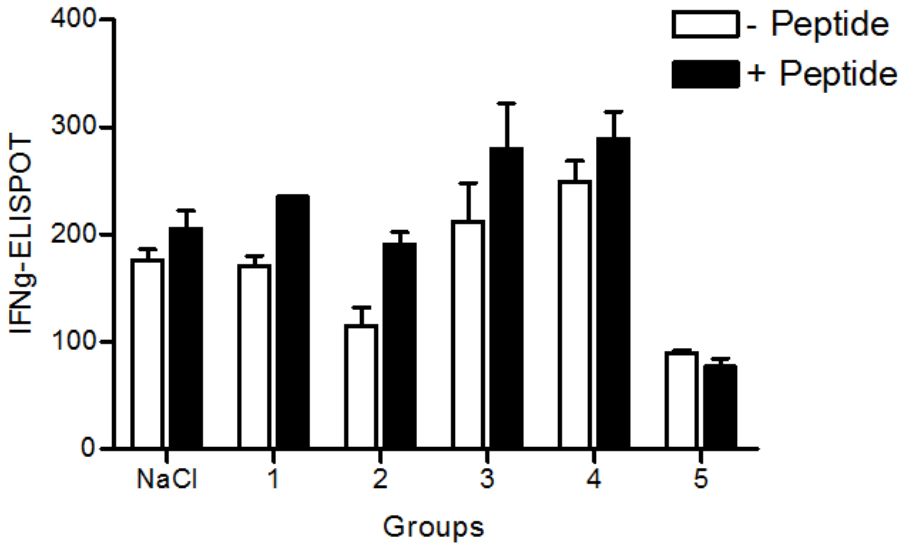


Figure 4.23: IFN-γ-ELISPOT of lymphocytes harvested from the spleens of immunised mice. The cells were harvested from the spleen of mice immunised with HIVp24 expressing *L. plantarum* WCFS1. Numbers along the x-axis indicate the different groups (5 mice per group) immunized with different strains: 1, pSIP-EV (negative control); 2, pLp_0373HIVp24cwa2; 3, pLp_1261HIVp24; 4, pLp_3014HIVp24; 5, Mix of groups 2-4. NaCl indicates a group that received sterile buffer. The y-axis gives the number of IFN-γ-producing cells detected per sample. The cells were analyzed without and with stimulation by Gag p24 peptide. Black and white columns represent responses with and without peptide stimulus, respectively.

Results from the ELISPOT assay shown in Figure 4.23 show no significant increase in cells producing IFN-γ after immunisation with HIVp24 displaying *L. plantarum* strains. A tendency towards an increased response could maybe be seen for groups 3 and 4, but the response was not antigen specific. Only group 2 displayed an antigen specific response, but the overall response was too weak to be considered a positive result. Surprisingly, group 5 displays a much lower amount of IFN-γ producing cells than the other groups.

Multiplex cytokine quantification analysis was also performed, but the results were not ready at the time of writing of this thesis.

Both ELISPOT and multiplex cytokine quantification analyses were performed by Dr. I. Øynebråten and colleagues at Centre for Immune Regulation, University of Oslo and Oslo University Hospital.

5 DISCUSSION

5.1 Production and anchoring of HIVp24 in *L. plantarum* WCFS1

In this thesis, a fusion protein called HIVp24, consisting of a chemokine and an HIV antigen, was expressed in *L. plantarum* WCFS1 to investigate the possibility of creating an HIV vaccine able to elicit an immune response through the mucosal immune system. HIVp24 had not been expressed in any bacteria prior to this study. The three different selected anchors, representing three different anchor mechanisms, were used to investigate which anchor mechanism would give the most optimal antigen display of HIVp24 on the cell surface, and thus potentially yield the most optimal immune response when given to a host organism.

HIVp24 was cloned into pSIP vectors harbouring the different anchors to allow expression and anchoring of the antigen. Three different anchors were selected: a cell wall anchor (cwa2), a lipoanchor (Lp_1261) and a LysM-domain anchor (Lp_3014). The LPxTG-anchor attaches the HIVp24 fusion protein covalently to the cell wall, and the lipoanchor covalently binds the HIVp24 fusion protein to the cell membrane. The LysM-domain anchor associates the antigen non-covalently to the cell wall. The lipo- and LysM-domain anchors attach the HIVp24 antigen N-terminally to the cell surface and the LPxTG-anchor attaches the HIVp24 antigen C-terminally to the cell surface. All three anchors have previously been successfully used to anchor heterologous proteins to the cell surface of *L. plantarum* WCFS1 by Fredriksen and colleagues; the lipo- and LysM anchors were used to anchor the protein invasin to the cell surface (Fredriksen et al. 2012), and the LPxTG-anchor was used to anchor human oncofetal antigen to the cell surface (Fredriksen et al. 2010). Tjåland (2011) investigated the use of the lipoanchor and cwa2-anchor for surface display of a tuberculosis antigen. In all these previous studies, done in our lab, the heterologous protein was successfully detected on the cell surface.

It is believed that a higher antigen count on the cell surface is preferable when constructing a LAB-based vaccine (Wells & Mercenier 2008). Therefore, as part of this study, we also investigated the effect of introducing a high-copy number replicon in the expression vectors. Change of replicon was accomplished for the plasmids encoding lipo- and LysM-anchored HIVp24. Introduction of the $SH71_{rep}$ replicon in plasmids encoding secreted and covalently cell wall anchored HIVp24 was unsuccessful. Figure 4.10 shows that the strain harbouring pLp_0373HIVp24cwa2 almost stops to grow upon induction. A putative increased production

level of HIVp24 resulting from change of replicon would probably induce even further stress on the bacteria.

The growth curves of the induced HIVp24-producing strains clearly showed the retarded growth (to various degrees), indicating induced stress. The strain harbouring pLp_1261HIVp24 had the highest growth rate (after pSIP-EV); production of lipoanchored HIVp24 was thus best tolerated by the bacteria. Interestingly, the strain producing lipoanchored HIVp24 also has the highest production level of HIVp24 (as seen on the Western blots in Figure 4.11), but has the lowest amount of HIVp24 displayed on the cell surface (seen in Figure 4.17). Both Tjåland (2011) and Fredriksen et al. (2010) detected low growth rates for strains over-rxpressing cwa2-fused heterologous proteins, but never a decline in growth as seen in the present thesis. This indicates that the growth stress not is due to high secretion of the anchor sequence alone, but also to stress caused by production and secretion of the HIVp24 fusion protein. A lowered growth rate was also detected in the strain that secreted HIVp24, which also implies that growth stress is more due to passage of HIVp24 through the plasma membrane than to over-expression and presence of the different protein anchors in the cell envelope. The strain secreting HIVp24 actually showed more growth stress than the strains harbouring plasmids for LysM- and lipoanchored HIVp24, judging from the growth curve in Figure 4.10. This underlines the importance of correct match of signal peptide, anchor, target protein and host strain, as pointed out in several other studies (Brockmeier et al. 2006; Caspers et al. 2010; Dieye et al. 2001; Mathiesen et al. 2008; Perez-Martinez et al. 1992; Slos et al. 1998; Sørvig et al. 2005a). After changing the replicon, the strains harbouring plasmids with the $SH71_{rep}$ replicon (pLp_1261HIVp24SH71 and pLp_3014HIVp24SH71) showed reduced growth compared to the strains harbouring the same anchors but with the $pUC(GEM)_{rep}/256_{rep}$ replicons (pLp_1261HIVp24 and pLp_3014HIVp24). As previously discussed, a change of replicon can increase plasmid copy number as well as increase expression of the target gene (Maudal 2012; Sørvig et al. 2005a). The reduction in growth rate could be due to increased stress due to an increase in HIVp24 production. Increased HIVp24production was confirmed through Western blot analysis (see figures 4.11 and 4.12)

The bacterium's ability to express and anchor HIVp24 is crucial to all experiments in this thesis. Western blot analysis was thus conducted to confirm presence of HIVp24 fusion

protein in the total cell extract. The Western blot, shown in Figure 4.11, confirms that all strains were able to express the fusion protein. The strains harbouring plasmids pLp_1261HIVp24 and pLp_3014HIVp24 (Fig. 411, lanes 6-9) show a high degree of degradation products. As a protease inhibitor was added to the media immediately post cell harvest, the protein degradation was most likely due to intracellular protease activity during growth, prior to harvesting. Kleerebezem et al. (2003) found 19 predicted proteases in the *L. plantarum* WCFS1 genome, and cytoplasmic degradation of heterologous proteins has previously been described in experiments conducted in *Lc. lactis* and *Bacillus subtilis* (Bolhuis et al. 1999; Le Loir et al. 2005; Lulko et al. 2007; Miyoshi et al. 2002).

As expected, HIVp24 was found in the supernatant of the strain containing the vector constructed for secretion of HIVp24 (Figure 4.15, lane 3), while no HIVp24 was detected in the supernatant of the negative control (Figure 4.15, lane 2). HIVp24cwa2 is covalently anchored to the cell wall, and should thus in theory not be detected in the supernatant, as indeed was observed (Figure 4.15, lane 4). However, covalent cell wall anchored heterologous proteins have been detected in the supernatant in previous studies, a phenomenon which is ascribed to cell wall shedding (Brinster et al. 2007; Dieye et al. 2001; Fredriksen et al. 2010; Tjåland 2011), and one would thus expect the presence of some HIVp24cwa2 in the supernatant. The strain harbouring cwa2-anchored HIVp24 show OD_{600}-values much below what is detected from the other HIVp24 producing strains. Lack of HIVp24cwa2 found in the culture supernatant is thus most likely due to presence of protein under the detectable level, rather than a stronger cell wall affinity. The strains harbouring lipoanchored HIVp24 showed some protein in the supernatant (Figure 4.15, lanes 5 and 6), which correlates with results from previous studies (Antelmann et al. 2001; Bøhle et al. 2011; Tjalsma et al. 2008). Antelmann et al. (2001), Bøhle et al. (2011) and Tjalsma et al. (2008) found lipoproteins normally covalently liked to cell membrane in the culture supernatant, and concluded this was due to passive release of anchored lipoproteins, also termed natural shedding. A lot of LysM-anchored HIVp24 was found in the supernatant of the 3014HIVp24 producing strains (Figure 4.15, lanes 7 and 8). LysM is not covalently anchored to the cell wall, and one may speculate that this protein much easier detaches from the cell surface (Tjalsma et al. 2008), in addition to release caused by normal cell wall shedding (Sanchez et al. 2010). Large quantities of proteins containing LysM-domains in the supernatant have been detected in studies performed by Turner et al. (2004) and Sanchez et al. (2009). The supernatants of cells producing HIVp24 fused to Lp_1261 and Lp_3014 also showed some

degradation products, especially in the supernatants of the cells producing LysM-anchored HIVp24 (Fig. 4.15, lanes 7 and 8). The degradation is most likely due to membrane bound proteases or proteases released into the supernatant.

Immunostaining of the bacterial surface was performed using HIVp24 specific goat primary antibody and goat specific Alexa Fluor® 488 secondary antibody to detect surface located HIVp24. Results of the immunostaining were evaluated through flow cytometry and confocal microscopy. The results from flow cytometry and confocal microscopy (see Figures 4.16-4.19) correlated well. No signal was detected for the negative control (pSIP-EV). The strains producing Lp_1261HIVp24 and secreted HIVp24 showed very few cells eliciting a fluorescent signal. Close to all the cells producing cwa2- and Lp_3014-fused HIVp24 elicited a fluorescent signal, and cells producing Lp_3014-fused HIVp24 elicited the strongest signal. The width of the histogram obtained in flow cytometry indicates the difference in signal elicited amongst each cell in the analysed population. A narrow histogram indicates that all the cells elicit approximately the same amount of signal, and vice versa. Lack of signal indicates absence of the antigen on the surface of the bacteria, or presence of a hidden epitope unable to bind the primary antibody. All populations of cells in this thesis that were induced through the pSIP system and analysed through flow cytometry, including the cells harbouring pSIP-EV, show broad histograms. The broad histogram could be a result of variance within each culture regarding how well each cell responds to protein expression through the pSIP system. A large variation in the response to induction using the pSIP system was also detected by Tjåland (2011) and Øverland (2013) (Pers. com.). Within the same population of cells, there may be large variations in phenotypes (Ingham et al. 2008). Thus bacterial cells harbouring the same vector can respond differently to stimuli and yield varying results although they receive the exact same treatment. Further investigations regarding induction efficiency and population diversity can be performed using green fluorescent protein as a target gene and analysis through flow cytometry or confocal microscopy.

The strain producing HIVp24cwa2 showed very low content of HIVp24 in the total cell extract, but showed presence of HIVp24 on the surface of almost all cells. The strains producing Lp_3014HIVp24 show more HIVp24 in the total cell extract and also more HIVp24 on the cell surface. When viewed in a confocal microscope, cells of both strains show a very even distribution of fluorescent signal across the entire surface of the cell, which

correlates with results found by Fredriksen et al. (2012). For the strain producing heterologous protein fused to Lp_1261, Fredriksen et al. (2012) found the signal to be concentrated around the septum. This could not be verified for the cells producing Lp_1261HIVp24, as very low amounts of signal were detected.

A high amount of Lp_1261HIVp24 protein was found in the total cell extract in Figure 4.11, but very little HIVp24 was detected on the cell surface through flow cytometry and confocal microscopy. The strains producing Lp_1261HIVp24 had a much higher intracellular production than the strains producing Lp_3014HIVp24 and HIVp24cwa2, yet the strains producing Lp_3014HIVp24 and HIVp24cwa23014 elicit a much stronger fluorescent signal than the strains producing Lp_1261HIVp24. Judging from expression levels, the strains producing Lp_1261HIVp24 should elicit the strongest fluorescent signal from the cell surface. Also, Fredriksen et al. (2012), (Tjåland 2011) and fellow master student L. Øverland (Pers. com.) detected fluorescent signals from the surface of the cells producing a heterologous protein fused to Lp_1261. It was thus reasonable to expect high levels of Lp_1261-fused HIVp24 on the surface of the bacteria. To investigate whether the lack of signal was because the fusion protein was hidden in the cell wall and thus unavailable to the antibodies, or whether it was due to absence of HIVp24, a new flow cytometry experiment was conducted where the strain producing Lp_1261-fused HIVp24 was treated with lysozyme to partly degrade the cell wall prior to analysis. Treatment with lysozyme did not reveal any hidden epitopes (data shown in the Appendix, Figure A.5), leading to the conclusion that the lack of signal is most likely due to absence of anchored HIVp24 on the cell surface.

In the time harvest experiment of the strain producing Lp_1261HIVp24, shown in the Appendix (Figure A.3), one can see a band at ~ 20kDa which appears already at about 1-1.5 hours post induction. Mathiesen et al. (2008) have suggested that too high production of a gene product can lead to clogging of the secretion machinery. Lack of HIVp24 on the cell surface could thus be due to low secretion efficiency because the Sec translocase machinery is clogged. This underlines the importance of a correct match of signal peptide, anchor, target protein and host strain.

Le Loir et al. (2005) stated that heterologous protein precursors which are not efficiently secreted are subject to intracellular protease activity. Also, too high expression levels of heterologous proteins for secretion can lead to protein misfolding (Sarvas et al. 2004).

Misfolded proteins targeted for secretion can form aggregates and become stuck in the plasma membrane, unable to be secreted. Such proteins become the target of membrane bound or cytoplasmic proteases (Bolhuis et al. 1999; Sarvas et al. 2004). Both membrane bound and cytoplasmic proteases have been identified in *L. plantarum* WCFS1 (Bove et al. 2012; Kleerebezem et al. 2003). Some Lp_1261HIVp24 was detected in the culture supernatant, and it is possible that presence of this protein not is due to natural shedding alone, but also to protease-digested membrane-bound HIVp24, resulting in Lp_1261HIVp24 being removed from the cell surface and release into the culture supernatant. In conclusion, there is a large possibility that expression of Lp_1261HIVp24 is too high, leading to degradation of most of the fusion protein before it reaches the cell surface, or digestion of surface-anchored HIVp24. Thus very low amounts of HIVp24 may be present on the cell surface. The immunostaining indeed revealed some cells eliciting a fluorescent signal. These could be cells displaying HIVp24 temporarily on the cell surface before it is digested off by proteases.

The strain harbouring a plasmid for secretion of HIVp24 (pLp_0373HIVp24) showed two populations of cells (two peaks) in the flow cytometry analysis. The largest peak had the same displacement as the negative control, while the smaller peak had the greatest displacement of all the strains. This indicates the absence of HIVp24 on the surface of most of the cells, while some cells may have a large amount of HIVp24 present on the cell surface. Detected HIVp24 is most likely due to the protein being temporarily stuck in the membrane prior to signal peptidase cleavage and release into the cell media, as described by Driessen and Nouwen (2008). Because of the type of signal detected, immunostaining can only verify presence of HIVp24 on the cell surface, not accurately confirm permanent anchoring.

The growth curve of the strains harbouring plasmids with the $SH71_{rep}$ replicon displayed more growth stress than the strains harbouring plasmids with the $pUC(GEM)_{rep}/256_{rep}$ replicon, which can indicate a higher expression level of HIVp24. Western blots of the strains also indicated a higher amount of HIVp24 being produced in strains expressing HIVp24 from plasmids containing the $SH71_{rep}$ replicon. In this respect, the results obtained from flow cytometry analysis were very surprising; the cells harbouring plasmids with the $SH71_{rep}$ replicon seemed to display less HIVp24 on the cell surface. These cells could be victims of the same fate as cells expressing lipoanchored HIVp24 from a plasmid containing the

pUC(GEM)$_{rep}$/256$_{rep}$ replicon, as described earlier. An increase in transcription could have led to an overload of the secretion system, thus clogging the translocase, aggregation of HIVp24 and exposure of the proteins to intracellular proteases. A change of replicon to increase the presence of surface located HIVp24 was thus unsuccessful, and is not recommended for further experiments in *L. plantarum* WCFS1. But the plasmids are functional and can most likely be used in other *Lactobacillus* strains.

The morphological changes observed during confocal microscopy of cells producing HIVp24cwa2 were further investigated using scanning electron microscopy (SEM). Wild type *L. plantarum* WCFS1 normally shows a characteristic rod-shaped, smooth-surface morphology (like the cells depicted in Figure 4.20a), while cells exposed to different types of stress show morphological changes such as filamentous shape and bulky structures on the cell surface (Bron et al. 2004; Ingham et al. 2008; Pieterse et al. 2005). The strains producing anchored HIVp24 showed some morphological changes resembling those described by Bron et al. (2004), who studied exposure to bile salts, Ingham et al. (2008), who studied exposure to acid stress, and Pieterse et al. (2005) who studied lactic acid stress. The micrographs of bacteria producing HIVp24, displayed in Figure 4.20, show no change in surface patterns for the strains harbouring pSIP-EV (Fig. 4.20b) and secreted HIVp24 (Fig. 4.20c) compared to the wild type (Fig. 4.20a). The strain producing cwa2-fused HIVp24 (Fig. 4.20d-e) showed a very bulky surface and many cells appeared flat and empty or lysed. The strains producing lipo- and LysM-fused HIVp24 (Fig. 4.20f-i) showed lower levels of bulkiness and no lysed cells were detected. The cells producing lipo- and LysM-fused HIVp24 appeared more in chains than the other strains, typically 4-6 bacteria per chain. As shown by Ingham et al. (2008), stress can induce both surface structure changes and changes in division- or growth patterns. Change in growth pattern for the cells producing lipo- and LysM-fused HIVp24 could thus be due to expression and anchoring of HIVp24. Another explanation could be that these cells appear at a different growth stadium at the time of harvest than the others strains and thus have a different appearance. The cells producing anchored HIVp24 all have a bulky surface, while the cells secreting HIVp24 show no sign of bulkiness. The stress is therefore most likely not only due to production of HIVp24, but must somehow relate to over-expression of anchor proteins and anchoring of HIVp24 to the cell surface. The anchors are all domains of cell envelope bound proteins; Lp_1261 and Lp_3014 are enzymes, while Lp_2578 is an adherence protein. If the active part of the anchor protein is intact, the enzyme

might still be able to exert its function, giving extra stress to the bacteria as the protein is overexpressed from the plasmid. The active site is still present in Lp_3014 while the active sites of Lp_1261 and Lp_2578 (cwa2) were removed during truncation of the anchor sequences (Lp_1261 only contains the first 75 residues of the N-terminal end of the protein, the protein was truncated upstream of the catalytic site predicted by Pfam (Fredriksen et al. 2012), and cwa2 was truncated downstream of the active sites predicted by Pfam and only contains the last 194 residues). As the strains of all three anchors show bulks, disruption of the cell envelope integrity is more due to over-expression of anchor-fused heterologous proteins in general than enzyme function.

5.2 Effects of the HIVp24 expressing strains on mice

An increase in IFN-γ producing cells indicates a protective immune response towards the antigen, mimicking the response observed when the innate and adaptive immune system are activated to battle viral or intracellular pathogens (Lea 2005). Results from the ELISPOT assay shown in Figure 4.23 show no significant increase in cells producing IFN-γ after immunisation with HIVp24 antigen displayed on the surface of *L. plantarum* WCFS1. A tendency towards an increased response could possibly be seen for groups 3 and 4, but the response was not antigen specific.

The protective immune response modulated by the vaccine strongly depends on the nature of the antigen, the delivery system and the location of the antigen (Norton et al. 1996; Wells et al. 1996). Oral immunisation is able to modulate substantial immune responses in the small intestine, which is possible to detect through analysis of lymphocytes from Peyer's patches, mesenteric lymph nodes and the spleen (Holmgren & Czerkinsky 2005). Thus, a feasible immune response should be possible to detect through the ELISPOT analysis carried out in this study. Interestingly, the values for the NaCl group are about twice as high as normally observed for mice in the control group given NaCl (Pers. com. Dr. I. Øynebråten). The reason for these high values and how they influence the results for the rest of the groups are not known.

Previous research indicates that surface anchored antigens are most effective in terms of the strength of the immune response caused by engineered LAB (Bermudez-Humaran et al. 2011; Reveneau et al. 2002). Such antigens are more prone to degradation during passage through

the GI tract, but *L. plantarum* should be able to protect antigen against acid and bile and to preserve a sufficient amount of antigen to reach the intestine and trigger an immune response.

As the antigen most likely is present on the bacterial surface, the most plausible reason for a possible absence of an immune response is induction of oral tolerance. The type of immune response generated by the vaccine strongly depends on the entrance strategy and the administration regime; oral and intravenous immunisations have a higher chance of inducing tolerance than other immunisation strategies (Lea 2005), and repeated administration of very large doses of antigen, or doses too small to stimulate an immune response, is very prone to induce tolerance, or lack of reactivity (Lea 2005; Tomasi 1980). A lack of immune response after oral administration is found in several other studies resembling the experiment performed in this thesis (Dieye et al. 2003; Lee et al. 2001). Lee et al. (2001) tested several different immunisation regimes, all yielding different results, which strongly indicates that the antigen needs to be present at a certain concentration range and be able to interact with the immune system over a certain period of time to gain a maximum response. van Baarlen et al. (2009) also discovered that the immune response generated by a vaccine-producing recombinant *L. plantarum* WCFS1 strongly depended on whether the bacteria were harvested during the logarithmic or stationary growth face. The antigen dosage thus needs to be tailored to the specific antigen, and depends on the nature of the antigen and its delivery vector (Lea 2005; Lee et al. 2001).

The CCL17 cytokine fused to the HIV antigen can also have influenced the immune response. A strong T cell response is required for developing memory cells for long term protection. The intention of using a chemokine as part of the fusion protein was to influence the local cytokine production and thereby skewing the immunoprofile towards a stronger T-cell response by recruiting immune cells containing the CCR4 receptor. After the experiments were conducted, literature was found which indicates that CCL17 can induce T_H2 and T_{reg} responses (Pers. com. Dr. I. Øynebråten), responses which are strongly associated with development of oral tolerance (Holmgren & Czerkinsky 2005). If the cytokine has been able to exert its function, it might have resulted in a cytokine profile which lowers the amount of IFN-γ producing cells. The use of CCL17 was thus not optimal for our setting, although no final conclusions can be made before the results of the multiplex cytokine analysis are ready.

5.3 Concluding remarks and future perspectives

This thesis describes the expression and anchoring of a HIV antigen fused to a cytokine to the cell surface of *L. plantarum* WCFS1, using the pSIP expression system. The fusion protein was successfully expressed from all plasmids and detected by fluorescent staining on the cell surface of all strains, but to a varying degree. The replicon change was no success in terms of an increase in surface-located HIVp24, but it is absolutely necessary if one is to explore the antigen in other strains of the *Lactobacillus* genus. Regarding the results from ELISPOT, the vaccine seems unsuccessful in inducing immunity, although a proper conclusion cannot be drawn from only one assay and one cytokine. The multiplex analysis will give a better indication regarding if and what type of immune response was induced by the recombinant bacteria. The multiplex analysis can also yield some information regarding the effects of the vaginal immunisations.

L. plantarum WCFS1 is still regarded as a promising vaccine delivery vector, but several aspects of the current approach should be reconsidered, both regarding vaccine design and regarding the interactions between the bacteria and the host immune system. Reconsiderations regarding vaccine design include (i) changes in the combination of the homologous anchor and the signal peptide to find a more optimal anchoring mechanism and production level for HIVp24; (ii) reconsideration regarding the nature of the fusion protein, especially the question whether one should use a fusion protein, which may cause difficulties in protein folding. One could change the cytokine, or use no cytokine at all. The last suggestion is to (iii) investigate the possibility of down-regulating both intra- and extracellular housekeeping proteases to avoid degradation of the heterologous protein, as suggested by Dieye et al. (2003).

Improvements regarding the interactions between the bacteria and the host immune system include: (i) Reconsider the immunisation route and the immunisation regime to avoid oral tolerance, e.g. try nasal immunisation and change the time period of immunisation, days between immunisation and succeeding days of immunisation. Lee et al. (2001) saw differences in immune response depending on immunisation regime when investigating *Lc. lactis* as a live delivery vector of a *Helicobacter pylori* antigen. (ii) As mentioned in the introduction, section 1.6, several research groups have seen positive results when immunising mice with recombinant LAB. Other researchers who were unsuccessful the first time tried a different approach involving the co-expression of another peptide with the antigen. An alternative to co-expressing a cytokine with the HIV antigen, as done in the present thesis, is thus to co-express a Toll like receptor ligand derived from a pathogen with the antigen, as

successfully done by Kajikawa et al. (2012), or fuse the antigen sequence to a DC binding sequence. Fusion to a DC-binding peptide has proven very successful in improving the immune response to *L. plantarum* displaying a tuberculosis antigen (Pers. com. Dr. G. Mathiesen) and to other antigens displayed in other LAB strains (Mohamadzadeh et al. 2009). (iii) Although *L. plantarum* WCFS1 should be able to protect the surface-displayed antigen from degradation during passage through the GI tract, this might not be entirely successful. It can thus be clever to administer a "protective solvent", such as bicarbonate buffer or soybean trypsin inhibitor, in the administration buffer to avoid degradation of surface located antigen from proteolytic enzymes during passage through the GI tract (Kajikawa et al. 2011).

The fact that it at all is possible to anchor a protein as complex as HIVp24 to the cell surface of LAB is a major achievement. Clearly further improvements are needed in order to produce a successful vaccine. Probably, a combination of the improvements mentioned above will be necessary to make *L. plantarum* WCFS1 a serious candidate for live-vaccine delivery of an HIV antigen.

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APPENDIX

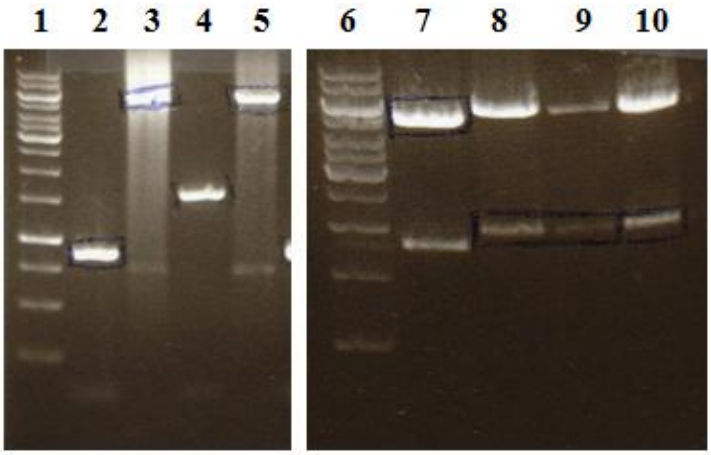

Figure A.1: Images of gel electrophoreses of digested plasmids. The images show the result of restriction enzyme digestions of plasmid from *Lc. lactis* MG1363 (left image, lanes 3 and 5) and *E. coli* TG1 (right image, lanes 7-10).

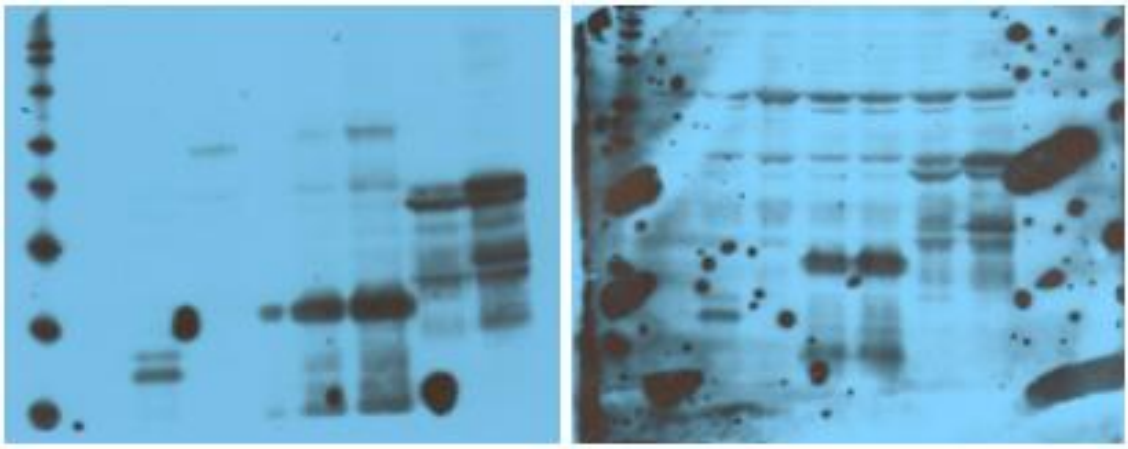

Figure A.2: Optimisation of Western blotting. The figure shows the result of two different Western blotting techniques performed on SDS-PAGE gels of the same samples. The left blot was obtained using the Tank transfer system Mini Trans-Blot Cell, and the right blot was obtained using the iBLOT^{TM} Dry Bloting System. Blotting through the Mini Trans-Blot Cell system clearly yielded the best blot.

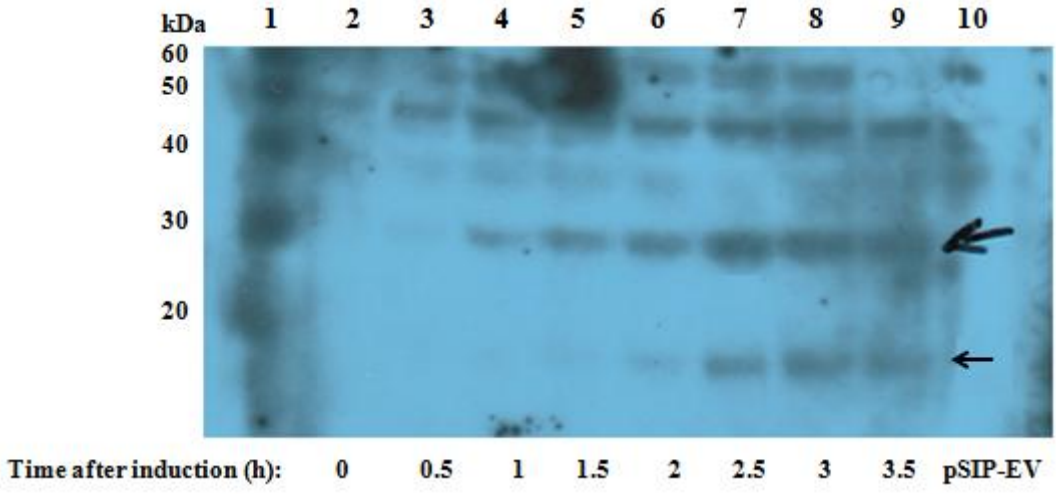

Figure A.3: Western blot analysis of production of membrane anchored HIVp24 over time. The blot shows crude total cell extract from induced *L. plantarum* strain harbouring pLp_1261HIVp24. The lane labelling is as follows: 1, MagicMark protein ladder; 2.-9., crude total cell extract from induced *L. plantarum* strain harbouring pLp_1261HIVp24 harvested at 0-3.5 hours after induction (time intervals are given below the lanes). 10, pSIP-EV. The volume loaded on the gel from each sample was calculated using OD_{600} measured at the time of harvest; this allows approximate comparison of amount of protein produced at the different time intervals. The large arrow marks expressed Lp_1261HIVp24 and the small arrow marks possible degradation products.

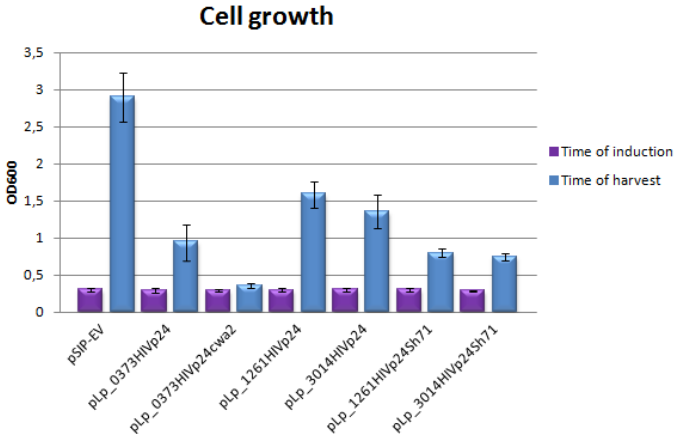

Figure A.4: Growth of strains harbouring plasmids for secretion and anchoring of HIVp24. Several cultivation and harvest experiments were conducted throughout this thesis. OD_{600} values measured at time of induction and time of harvest in all the experiments were collected and are presented in this figure. The figure shows the average OD_{600} values measured at time of induction (purple) and time of harvest (blue). The strains were all harvested at 3.5 hours post induction, except the strain harbouring pLp_0373HIVp24cwa2, which was harvested at 1 hour post induction.

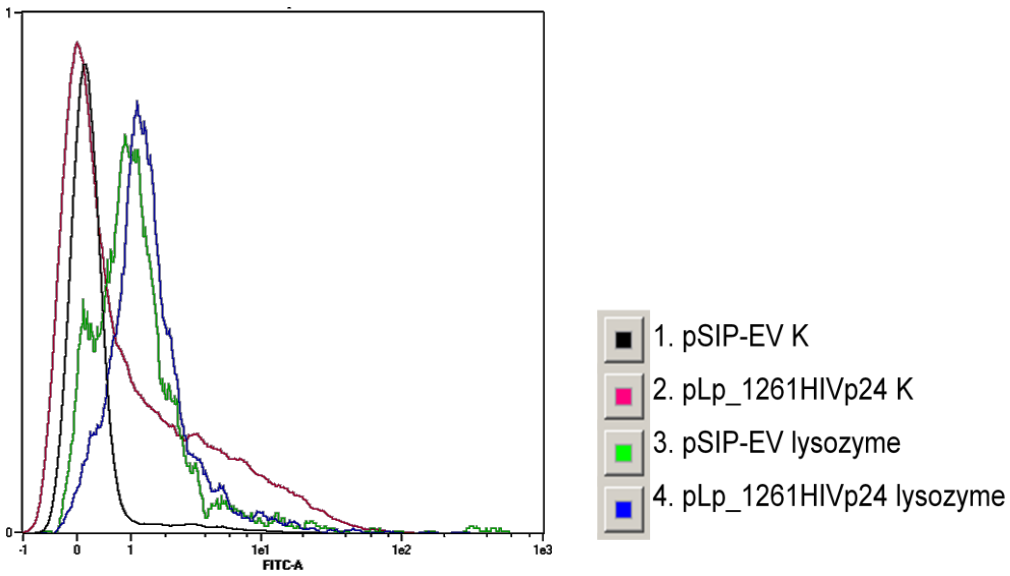

Figure A.5: Flow cytometry analysis of Alexa Fluor® 488-stained *L. plantarum* strains harbouring a plasmid for covalent membrane anchoring of HIVp24 (pLp_1261HIVp24). The results are shown as a single dimension histogram where relative fluorescence (x-axis) is plotted against the relative number of events (y-axis). The histogram depicts the graphs of strains harbouring pSIP-EV (negative control) and pLp_1261HIVp24, both untreated (K) and treated with lysozyme. The cells were immunostained using the HIVp24 specific mCCL17/TARC goat anti-mouse IgG primary antibody and Alexa Fluor® 488 donkey anti-goat secondary antibody after lysozyme treatment. The colour scheme is shown to the right of the histogram. The graphs of cells not treated with lysozyme (1 and 2, graphs to the left) remained like the standards presented in Figure 4.16. Graph 3 and 4 are both displaced to the right, showing an increase in fluorescent signal after treatment with lysozyme. 3 and 4 show the same displacement; hence one cannot conclude that HIVp24 becomes visible when the cell wall is degraded.