Secretion and anchoring of invasin from Yersinia pseudotuberculosis in Lactobacillus plantarum

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Abstract

The work conducted in this thesis is a part of a project whose main goal is to develop oral vaccines for humans with *Lactobacillus* as a vaccine delivery vector. Lactic acid bacteria can potentially produce therapeutic heterologous proteins and deliver them to mucosal sites. *L. plantarum* is an interesting candidate for this purpose because it is resistant to bile and low pH and it is a versatile bacterium with GRAS-status (generally regarded as safe). One approach that could make the bacteria a better oral vaccine is to make them target specific receptors (like β_1-integrin) on the apical surface of M-cells by surface display of proteins such as invasin. This could promote transport of the bacterium by the M-cells, from the lumen of the intestine, across the epithelial barrier into organized lymphatic system below, which may promote an effective immune response. The work described in this thesis was aimed at secretion and anchoring of invasin from *Yersinia pseudotuberculosis* in *Lactobacillus plantarum*, using an inducible gene expression system formerly developed for efficient intracellular protein production.

Invasin is a membrane protein with an extracellular region that binds host cell integrin receptors and promotes uptake of the bacteria. This C-terminal extracellular region comprises five domains (referred to as Inv in this thesis), and it has been shown that the two domains (referred to as InvS) at the C-terminus comprises the shortest fragment of invasin that is capable of binding and inducing uptake. Both versions of invasin were expressed in *L. plantarum* WCFS1. To anchor these invasin proteins, lipo-anchors from the lipoproteins Lp_1261 and Lp_1452 from *L. plantarum* WCFS1 were selected. Five plasmids were constructed, pLp_1261InvS, pLp_1261Inv, pLp_1452InvS, pLp_1452Inv and pCytInv (invasin without anchor), that all were transformed into *L. plantarum* WCFS1. *L. plantarum* strains harbouring the pLp_1452InvS or pLp_1452Inv constructs showed strongly reduced growth upon induction of gene expression, whereas *L. plantarum* harbouring the Lp_1261-based constructs showed normal growth. All *L. plantarum* harbouring the different invasin constructs produced invasin, which could be detected intracellularly and for bacterial strains with an anchor, in the culture supernatant. Despite testing and optimization of various approaches to do so, it was difficult to convincingly demonstrate anchoring of invasin to the bacterial surface. However, several experiments did indicate anchoring. Experiments with Caco-2 cells did not convincingly show internalization of invasin-expressing bacteria, but clearly showed that several of the recombinant bacterial strains had increased affinity for the

Caco-2 cells. In conclusion, the work described in this thesis shows that *L. plantarum* is able to produce, secrete, and most likely anchor invasin to the surface of the bacteria, by using lipo-anchors. The results further indicate that there is a need to optimize the anchoring strategy to make invasin more available on the surface of *L. plantarum*. The latter is likely to improve the ability to detect the protein as well as its *in vitro* functionality.

Sammendrag

Arbeidet som ble utført i denne masteroppgaven er en del av et prosjekt som har som mål å utvikle orale vaksiner for mennesker med *Lactobacillus* som en leveringsvektor av vaksinen. Melkesyrebakterier kan potensielt produsere terapeutiske heterologe proteiner og levere dem til mukosa. *L. plantarum* er en interessant kandidat til dette formålet fordi den er motstandsdyktig mot galle og lav pH, og den er en allsidig bakterie med GRAS-status (generelt betraktet som trygg). En fremgangsmåte som kan gjøre bakterien til en bedre oral vaksine er å få bakterien til å binde spesifikke reseptorer (som β_1-integrin) på oversiden av M-celler ved at proteiner som invasin er lokalisert på overflaten av bakterien. Dette kan fremme transport av bakterien fra tarmlumen, gjennom epitelbarrieren, til det underliggende organiserte lymfesystemet som kan fremme en effektiv immunrespons. Arbeidet beskrevet i denne masteroppgaven hadde som hensikt å få til sekresjon og ankring av invasin fra *Yersinia pseudotuberculosis* i *Lactobacillus plantarum*, ved å utnytte et induserbart uttrykningssystem tidligere utviklet for effektiv intracellulær protein produksjon.

Membranproteinet, invasin, har en ekstracellulær region som binder til vertcellens integrin reseptorer og fremmer opptak av bakterien. Denne C-terminale ekstracellulære regionen består av fem domener (forkortet som Inv i denne oppgaven), og de to domene (forkortet som InvS) i den C-terminale enden er demonstrert til å være det korteste fragmentet av invasin som kan binde og indusere opptak. Begge versjonene av invasin er uttrykt i *L. plantarum* WCFS1. For å ankre disse invasin proteinene ble lipoankrer fra lipoproteinene Lp_1261 og Lp_1452 fra *L. plantarum* WCFS1 valgt ut. Det ble konstruert fem plasmider, pLp_1261InvS, pLp_1261Inv, pLp_1452InvS, pLp_1452Inv og pCytInv (invasin uten anker), som alle ble transformert inn i *L. plantarum* WCFS1. *L. plantarum* med pLp_1452InvS eller pLp_1452Inv plasmidet viste redusert vekst etter induksjonen av invasin produksjon, mens *L. plantarum* med Lp_1261-baserte konstrukter viste normal vekst. *L. plantarum* med de forskjellige invasin plasmidene produserte invasin, som kunne detekteres intracellulært og for bakteriestammer med et anker, i supernatanten til kulturen. Selv med testing og optimalisering av flere metoder var det vanskelig å bevise helt sikkert at invasin var ankret til den bakterielle overflaten, men flere eksperimenter indikerte ankring. Eksperimenter med Caco-2 celler viste ikke noen overbevisende resultater for internalisering av invasin uttrykkende bakterier, men viste at flere av de rekombinante bakteriestammene hadde økt affinitet for Caco-2 celler. Ut i fra arbeidet beskrevet i denne masteroppgaven ble det konkludert at *L. plantarum* kan

produsere, sekretere, og mest sannsynlig ankre invasin til overflaten av bakterien, ved bruk av lipoankrer. Resultatet indikerer også at det er nødvendig å optimalisere ankrestrategien for å gjøre invasin mer tilgjengelig på overflaten til *L. plantarum*. Dette vil mest sannsynlig forbedre evnen til å detektere proteinet på overflaten av bakterien og sannsynligvis forbedre bakteriens *in vivo* funksjoner.

Abbreviations

APC	antigen-presenting cell
ATP	adenosine triphosphate
BHI	Brain Heart Infusion
bp	base pair
CFSE	carboxyfluorescein succinimidyl ester
CFU	colony forming units
DCs	dendritic cells
FAE	follicle-associated epithelium
FITC	fluorescein isothiocyanate
dH_2O	sterile water (Milli-Q)
DNA	deoksyribonukleinsyre
dNTP	deoxynucleoside triphosphate
GI	gastrointestinal
GRAS	generally regarded as safe
HPK	histidine protein kinase
IgA	immunoglobulin A
IL	interleukin
IP	inducing peptide
LAB	lactic acid bacteria
MALT	mucosa-associated lymphoid tissue
MHC	major histocompatibility complex
MRS	de Man, Rogosa, Sharpe
OD	optical density
PCR	polymerase chain reaction
RR	response regulator
SDS-PAGE	sodium dodecyl sulphate polyacryl amide gel electrophoresis
SOE	splicing by overlapping extension
SPase	signal peptidase
SRP	signal recognition particle
v/v	volume/volume
w/v	weight/volume

CONTENTS

1. INTRODUCTION

Lactic acid bacteria (LAB) are commonly used in food products and several species are believed to have positive effects on human health. Many LAB species are a natural part of the gastrointestinal (GI) tract of humans and animals and they are generally regarded as safe (GRAS) organisms. Several systems for heterologous expression of genes have been developed for LAB and these systems may be exploited to develop LAB as *in situ* delivery vehicles for interesting therapeutic proteins and peptides. This thesis deals with the expression and anchoring of a heterologous protein, invasin, in *L. plantarum*, with the aim of improving this bacterium's potential as a vaccine delivery vector.

1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of Gram-positive, non-pathogenic, non-invasive, non-sporulating, usually non-motile, bacteria, which are defined by their ability to produce lactic acid as an end product from carbohydrate fermentation. *Lactobacillus, Lactococcus, Streptococcus, Enterococcus, Leuconostoc* and *Pediococcus* are examples on LAB (Tao et al. 2011; Willey et al. 2008). Generally, LAB are fastidious organisms with limited biosynthetic capabilities that needs to get vitamins, amino acids, purines and purimidines supplied. They lack cytochromes, so they generate energy by substrate-level phosphorylation (Willey et al. 2008). LAB are used in the food industry, mainly in food fermentation and preservation of milk, vegetables and meat. They have for a long time been generally regarded as safe (GRAS) for humans and some strains have also health promoting effects (Ahrne et al. 1998; Leroy & De Vuyst 2004). LAB occupy a range of ecological niches, including, in addition to food products, the oral cavity, gastrointestinal tract and vagina of vertebrates (Tao et al. 2011; Wells & Mercenier 2008).

1.2 *Lactobacillus*

The genus *Lactobacillus* contains non-sporing rods and sometimes coccobacilli, that lack catalase and cytochromes and are usually facultative anaerobic or microaerophilic. They prefer slightly acidic conditions and show optimal growth when the pH is between 4.5 and 6.4 (Willey et al. 2008). This is a genus with a considerable number of different species that display a relatively large degree of diversity (Kleerebezem et al. 2010). Some strains of lactobacilli are considered probiotics, providing health benefits through interactions within the

GI tract. The way lactobacilli interacts with their hosts and influence different factors encountered within the GI tract is dependent on their extracellular characteristics. For instance, lactobacilli produce lactic acid that lowers the pH and inhibits growth and adhesion of pathogenic microorganisms (Lebeer et al. 2008; Marco et al. 2006; Seegers 2002). Also as a part of their probiotic contribution to the host, some strains produce antimicrobial peptides (bacteriocins) that inhibits pathogens such as *Listeria* (Diep et al. 2009b; Eijsink et al. 2002). Other probiotic mechanisms are resistance to bile salt and acid (Marco et al. 2006; Seegers 2002), the capacity to attach to or colonize the intestinal tissue, at least temporarily, to prevent attachment of pathogens, and competition with pathogens for the same receptors (Styriak et al. 2003; Vaughan et al. 2002; Velez et al. 2007). Even though probiotic bacteria are considered to have health-promoting effects, the molecular mechanisms promoting these effects are largely unknown (Kleerebezem et al. 2010; Marco et al. 2006). Lactobacilli seem to contribute to maintaining the balance of the intestinal microflora, and it appears that they also modulate the intestinal immune system, detoxify colonic toxins, promote lactose tolerance, lower serum cholesterol levels, and produce metabolites that are essential to the function of intestinal epithelial cells (Liu et al. 2010).

Of the *Lactobacillus* species, *Lactobacillus plantarum* is one of the most studied and best understood, and *L. plantarum* WCFS1 was the first strain of *L. plantarum* to have the complete genome sequence determined (Klaenhammer et al. 2002; Kleerebezem et al. 2003). *L. plantarum* is versatile, which is reflected by its relatively large number of regulatory and transport functions and the fact that this bacterium has one of the largest genomes known among LAB (Daniel 1995; Kleerebezem et al. 2003). *L. plantarum* strains vary considerably in their ability to induce pro- and anti-inflammatory cytokines (Meijerink et al. 2010), in intestinal survival rates and in their ability to adhere to epithelial cells. One promising strain is *L. plantarum* NCIMB8826, which was originally isolated from human saliva, and which has high survival capacity in the intestine (Vesa et al. 2000). *L. plantarum* WCFS1 is a single colony isolated from the NCIMB8826 strain (Kleerebezem et al. 2003) and it has been shown that this bacterium has several genetic loci that influence the human immune system. The predicted exoproteome of this strain contain at least twelve proteins putatively involved in adherence to host components such as collagen and mucin (Kleerebezem et al. 2010; Meijerink et al. 2010). Generally, *L. plantarum* WCFS1 is a good candidate to utilize in oral vaccination due to its resistance to bile acid and its persistence in the GI tract.

1.3 LAB as delivery vectors for therapeutic proteins

LAB have several advantages for surface display applications, including a common mechanism for surface anchoring of proteins. Furthermore, several LAB surface proteins are known to be relatively permissive for the insertion of extended sequences of foreign proteins, giving interesting engineering opportunities. The thick cell wall that covers the bacteria makes it more resistant to rigorous manipulation conditions. Finally, since LAB are Gram-positive microorganisms with just a single cell membrane, theoretically protein secretion should be relatively easy to achieve (Motin & Torres 2009; Samuelson et al. 2002). Due to these features several adequate cloning system have been developed where the key quality for delivering of antigens involve promoter sequences that allow either constitutive or induced expression (Kleerebezem et al. 1997; Sørvig et al. 2003). Vectors for gene expression may also contain secretion and anchoring signals that allow targeting of proteins to different cell compartments (Reveneau et al. 2002). Plasmid based expression system are generally used because plasmids are easy to manipulate. Systems based on chromosomal integration can also be used, but are more complicated to develop and use. In every expression system, the promoters can have different activity levels in different *Lactobacillus* strains, and plasmid copy numbers (i.e. gene dosages) can also differ (McCracken & Timms 1999; Seegers 2002).

LAB are less exploited as vaccine delivery vectors than attenuated pathogens such as *Salmonella*, *Listeria*, and *Shigella* (Detmer & Glenting 2006; Guimaraes et al. 2005). LAB are safer than attenuated pathogenic bacteria, because there is a potential risk of reversion to the virulent wild-type for the latter (Tao et al. 2011). It is already known that LAB can generate antigen immune responses. LAB have successfully been used as delivery vector of several antigens (Cortes-Perez et al. 2007; Detmer & Glenting 2006). Initially, *L. lactis* was the most common LAB used as a delivery vehicle, because this LAB was the first one that had a genetic toolbox available and its genome sequence determined (Bolotin et al. 2001; Klaenhammer et al. 2002; Mierau & Kleerebezem 2005). *L. lactis* is a well studied organism and some landmark studies been developed from work on lactococci (Diep et al. 2009a). Lactococci have successfully been used to produced diverse molecules, such as tetanus antigen (Robinson et al. 1997), cancer antigen (Bermudez-Humaran et al. 2002; Cortes-Perez et al. 2003) and cytokines (Bermudez-Humaran et al. 2003; Steidler et al. 1995; Steidler et al. 2000).

One landmark study concerns an engineered *L. lactis* strain that expresses interleukin-10 (IL-10), to treat Crohn's disease. The first human trial with this method of treatment has been completed (Braat et al. 2006). Crohn's disease is a type of inflammatory bowel disease which is a chronic intestine inflammation (Bouma & Strober 2003). According to scientific literature, IL-10 is a good candidate for inflammatory bowel disease treatment, but injection of IL-10 induces side effects. Delivery of IL-10 *in situ* by using a genetically modified bacterial carrier was expected to give a better response, but this method raised concerns about the bacterium's survival and possible propagation in the environment (Steidler et al. 2003). Spreading of antibiotic selection markers and other genetic modifications from recombinant strains to other microorganisms in nature is not desirable. Steidler et al. (2003) replaced the *thyA* gene of *L. lactis* with the *hIL10* gene, resulting in bacteria that are dependent on extracellular thymidine or thymine and are unable to survive outside the human body. The result of the human trial with the *L. lactis* strain lacking the *thyA* gene, but secreting IL-10, indicated that this strategy is beneficial for the patient and that the bacteria can be biologically contained (Braat et al. 2006; Steidler et al. 2003; Wells & Mercenier 2008).

More recently, *L. plantarum* has become another important species for use as a delivery vector, mainly because many genetic tools and the genome sequence are now available (Kleerebezem et al. 2003; Seegers 2002; Sørvig et al. 2003). When used for *in situ* protein delivery *L. plantarum* can have an advantage compared to *L. lactis*, because *L. plantarum* has a high tolerance against bile acid and low pH. They will survive the passage of the gut, and they have an intrinsic immunogenicity (Seegers 2002). The increasing numbers of papers about expression of medicinal proteins in lactobacilli indicate that *L. plantarum* strains tend to give better immune responses than *L. lactis* strains when they present the same antigen. For instance, Cortes-Perez et al. (2007) showed that *L. plantarum* expressing E7 antigen was more immunogenic than *L. lactis* producing the same antigen. Grangette et al. (2002) compared *L. plantarum* and *L. lactis* strains producing equivalent amounts of the tetanus toxin fragment C (TTFC) in an oral immunization study, and found that *L. plantarum* gave a better immune response.

1.4 Inducible gene expression in *L. plantarum* using the SIP-system

LAB and many other bacteria produce antimicrobial peptides, often referred to as bacteriocins, to combat competing Gram-positive bacteria. Bacteriocins differ from antibiotics in that they are more strain specific and more powerful against target bacteria than antibiotics. In addition bacteriocins are ribosomally synthesized while antibiotics are made by multi-enzyme complexes (Diep et al. 2009b; Nes et al. 2007). Production of bacteriocins is often strictly regulated via quorum-sensing mechanisms mediated by a secreted peptide-pheromone, a membrane located pheromone sensor (histidine protein kinase) and a cytoplasmic response regulator (Eijsink et al. 2002; West & Stock 2001). The discovery of these regulatory systems in lactobacilli (Diep et al. 1995; Eijsink et al. 1996), and the discovery of an analogous "nisin"-system in lactococci (Kuipers et al. 1995; Mierau & Kleerebezem 2005), have been extremely important for development of gene expression systems in LAB. Use of these regulated promoters, which are very strong when induced, allows gene expression to be both highly efficient and strongly regulated, and has been exploited to make strictly regulated gene expression system in lactobacilli (Diep et al. 2009a).

The quorum-sensing mechanism is a method where the bacterium can monitor its own growth. It involves a secreted peptide pheromone (induction peptide) that functions as a sensor for cell density. During growth there is a low constitutive expression of genes required for production of the induction peptide (IP) and the IP slowly accumulates in the medium over time. At a certain cell density, the accumulated IP reaches a critical threshold concentration and will bind to and activate the histidine protein kinase (HPK), through a highly specific interaction (Figure 1.1). Interaction of IP with the HPK receptor leads to autophosphorylation of the HPK. Subsequently, the phosphate group is transferred from the HPK to an intracellular response regulator (RR), which then binds to specific promoter elements and activates transcription of all genes involved in bacteriocin production (Nes et al. 1996; Nes & Eijsink 1999).

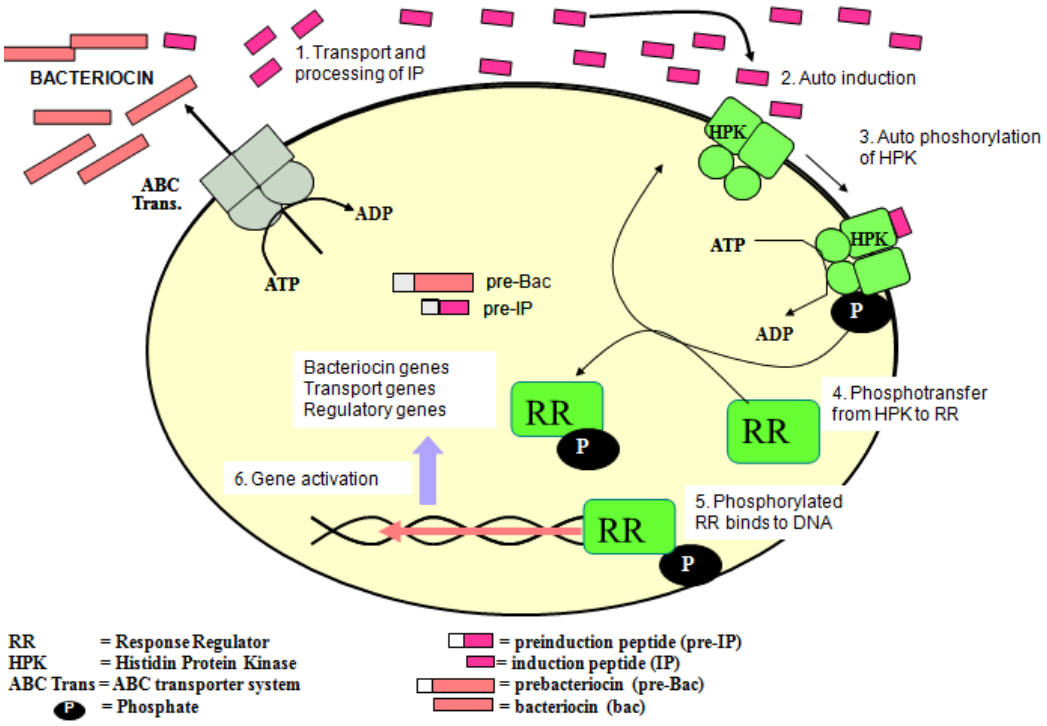


Figure 1.1: Schematic illustration of pheromone regulated bacteriocin production. (1) Low amounts of the constitutively produced induction peptide (IP) are produced as the cell grows, and transported out of the cells by an ABC transporter. The IP concentration increases as the cell density increases. (2) At a certain threshold, the concentration of IP will reach a level that makes the IP bind to the receptor histidine protein kinase (HPK). (3) This results in autophosporylation of a conserved histidine residue in the HPK. (4) The phosphoryl-group is transferred to the response regulator (RR) through interaction with the HPK. (5) The phosphorylated RR binds to DNA and (6) activates transcription of all genes involved in bacteriocin production (Mathiesen 2004; Nes & Eijsink 1999). The genes involved in bacteriocin production are often spread over several operons, each of which is preceded by a promoter that is activated by binding of the activated RR (Brurberg et al. 1997; Risøen et al. 2000). The figure is modified from Mathiesen (2004)

Sørvig et al. (2003) constructed vectors for an inducible expression system in lactobacilli. A schematic overview of their basic pSIP vector is shown in Figure 1.2 These pSIP expression vectors are based on promoters and regulatory genes involved in the production of the class II bacteriocins sakacin A (*sap* gene cluster) or sakacin P (*spp* gene cluster). Three genes are responsible for the regulation of this system, as explained above. One gene codes for a peptide whose primary function is to act as a pheromone. One gene codes for a membrane

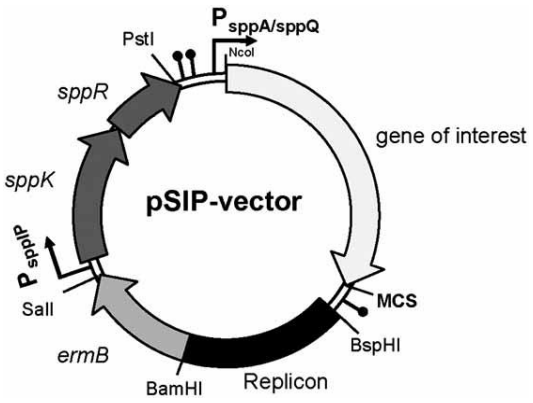


Figure 1.2: The pSIP vector expression system, illustrated by a schematic picture of the pSIP400 vector series. The sppK and sppR genes encode for the HPK and RR from the spp regulon, respectively. SppA/sppQ are regulated promoters that drive expression of the gene of interest. ermB is erythromycin resistance marker. The vector has unique restriction sites for easy exchange of different parts (Diep et al. 2009a). The figure is taken from Diep et al. (2009a).

located HPK that senses the pheromone, and one gene encode a cognate RR protein. pSIP-vectors have the genes for HPK and RR, but the gene for the IP is deleted in the plasmid. When IP is added to the medium it will interact with HPK and induce the expression system, as described above (Sørvig et al. 2003; Sørvig et al. 2005).

1.5 Protein secretion in bacteria

All proteins that have a task outside the bacterium need to be transported out of the cell to their final destination. In Gram-positive bacteria seven main protein secretion pathways have been characterized (Kleerebezem et al. 2010):

- The secretion pathway (Sec)
- Fimbrilin-protein exporter (FPE)
- Peptide efflux ABC
- Holin (pore-forming)
- Twin-arginine translocation (Tat)
- Flagella export apparatus (FEA)
- WXG100 secretion system (Wss)

Published *Lactobacillus* genomes indicate that lactobacilli contain genes for the Sec, FPE, peptide-efflux ABC, and holin secretion systems (Kleerebezem et al. 2010). Of these pathways, the secretion (Sec) pathway, is the most commonly explored in genetic engineering. The Sec pathway is also the naturally most commonly used system for protein transport across and into the cytoplasmic membrane. This secretion pathway is steered by the Sec translocase, as shown in Figure 1.3. The Sec translocase consists of a protein-conducting channel, the SecYEG (SecY, SecE and SecG) complex, which is membrane-embedded. It also consists of an ATPase motor protein (SecA), which is peripherally associated and deliver the energy to the process. In addition the proteins SecDF(yaiC) (the SecD, SecF and YajC proteins) and YidC are normally associated with Sec translocase. SecDF(yaiC) stimulates preprotein translocation and YidC facilitates the insertion of a some membrane proteins into the cytoplasmic membrane, in cooperation with the Sec-system or on its own (Driessen & Nouwen 2008; du Plessis et al. 2011; Kleerebezem et al. 2010).

Proteins that are going to be secreted or anchored as lipoproteins need to be recognized and targeted to the Sec translocase. Therefore, all secretory proteins and lipoproteins are synthesized with a characteristic N-terminal extension, called signal sequence or leader peptide. The signal peptide is removed by a signal peptidase after translocation (Driessen & Nouwen 2008). Signal sequences usually have three distinct domains, the N-terminal region (1-5 residues) with positively charged amino acids, the H region (7-15 residues) which is a central region with hydrophobic residues, and the C-terminal region (3-7 residues) with a more polar character (von Heijne 1990). Even though signal peptides show little sequence conservation, their presence can be predicted on the basis of protein sequences with computer algorithms such as SignalP (Bendtsen et al. 2004).

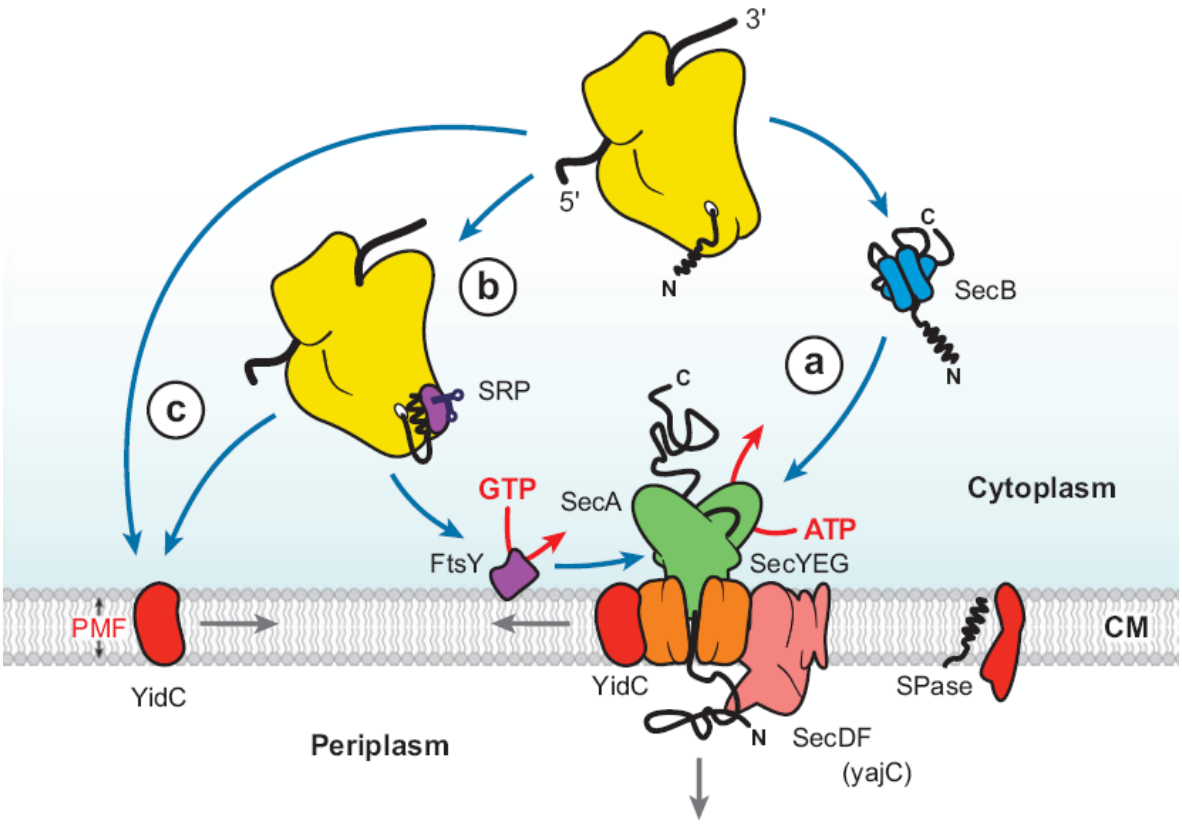


Figure 1.3: Protein targeting to the Sec translocase. The bacterial Sec translocase spans the cytoplasmic membrane (CM) and consists of the protein conducting channel SecYEG (SecY, SecE and SecG) (here in orange) and SecA (green) which acts as the peripheral motor protein. Other accessory proteins interacting with the translocase include SecDF (pink) and YidC (red). On the periplasmic side the signal sequence is cleaved by the membrane-bound Signal peptidase (SPase). There are three options: (a) After the protein is translated by the ribosome (yellow) they bind to the Sec translocase by their signal sequence, which is recognized directly by SecA or the molecular chaperone SecB (blue). (b) The signal sequence of the nascent protein chain binds to the signal recognition particle (SRP) and then to the SRP receptor FtsY (purple). Afterwards, the whole ribosome-FtsY complex binds to the Sec translocase. (c) Some membrane proteins insert into the CM via YidC (Driessen & Nouwen 2008). The figure is taken from Driessen & Nouwen (2008).

The preprotein with the N-terminal signal sequence is targeted to the Sec translocase by the molecular chaperone SecB or together with the ribosome by the signal recognition particle (SRP), as shown in Figure 1.3. If the signal sequence displays a high level of hydrophobicity and helicity the SRP will bind tightly to the ribosome nascent chain (Figure 1.3, b). This complex will bind to the membrane-associated signal-particle receptor, FtsY. The GTPase activity is activated and the ribosome nascent chain is transferred to the translocon pore, where the ribosomal exit tunnel makes close contact with the pore. Then the elongation of the polypeptide chain provides the energy for the insertion of the protein into the SecYEG complex. If the signal sequence does not display a high level of hydrophobicity, the polypeptide will be translated to its full length by the ribosome and released in cytosol. The still unfolded preprotein is recognized directly by SecA or by the molecular chaperone SecB (Figure 1.3, a). If the protein binds to SecB, this complex will target to the translocon where it binds SecA, leading to transfer of the preprotein and release of SecB (Driessen & Nouwen 2008; du Plessis et al. 2011).

After contact between the preprotein and the Sec system has been established, protein translocation starts with the binding of ATP to SecA. This provides energy that allows the insertion of the signal sequence, which adopts a hairpin-like loop structure, into the translocation pore. ATP hydrolysis results in release of the bound preprotein from SecA. Subsequently, SecA can either rebind to the preprotein located in the SecYEG pore or it can dissociate from SecYEG. The stepwise translocation of the preprotein is driven by multiple rounds of ATP binding and hydrolysis by SecA (Driessen & Nouwen 2008; du Plessis et al. 2011).

After the N-terminal signal peptide has initiated translocation of the protein across the cytoplasmic membrane, signal peptidases (SPases) remove the signal peptide. SPase is a membrane-bound enzyme, and different SPases recognize unique cleavage sites (Driessen & Nouwen 2008; Kleerebezem et al. 2010). The lipobox cleavage site, L-x-x-C, is recognized by Type-II SPase and its cleavage is linked to coupling of a lipo-anchor (for more details, see below) (Sutcliffe & Harrington 2002). The AxA-like cleavage site typical for regular Sec-driven secretion is recognized by Type-I SPase (van Roosmalen et al. 2004).

1.5.1 Heterologous protein secretion

Studies with Gram-positive bacteria have shown that the secretion efficiencies of heterologous proteins depend on the signal peptide, the secreted protein, and the host organism. It is difficult to predict which combination of these factors will lead to efficient secretion (Brockmeier et al. 2006; Mathiesen et al. 2008; Perez-Martinez et al. 1992). The genome of *L. plantarum* WCFS1 is predicted to encode many proteins with signal peptides that can direct secretion of a heterologous target protein (Kleerebezem et al. 2003). There are over 200 genes that are predicted to encode proteins with an N-terminal signal peptide, and approximately 100 of these are likely to contain a signal peptidase I cleavage site (Boekhorst et al. 2006; Kleerebezem et al. 2003). Currently, signal peptides derived from the lactococcal Usp45 protein (Cortes-Perez et al. 2005; Dieye et al. 2001; Slos et al. 1998), the M6 protein from *Streptococcus pyogenes* (Hols et al. 1997; Reveneau et al. 2002; Slos et al. 1998) and the S-layer protein from *Lactobacillus brevis* (Oh et al. 2007; Savijoki et al. 1997) are the most exploited for heterologous protein secretion in *Lactobacillus/Lactococcus*. In a recent study, Mathiesen et al. (2009), studied 78 signal peptides from *L. plantarum* WCFS1 for their efficiency in secretion of heterologous protein and identified several promising candidates.

1.6 Protein anchoring in Gram-positive bacteria

After translocation some secreted proteins are attached to the bacterial cell surface through covalent or non-covalent binding to the cell wall or membrane (Boekhorst et al. 2006). These surface-exposed proteins are considered to play an important role in the interaction between the bacterium and the environment, including processes such signal transduction, recognition, binding and degradation of complex nutrients, nutrient uptake, cell-cell recognition, colonization and surface adherence (Boekhorst et al. 2006; Samuelson et al. 2002). Proteins that are usually coupled to the cell surface can be divided into four major types (Desvaux et al. 2006):

- Transmembrane proteins (single or multiple)
- Lipoproteins
- Cell wall binding proteins
- LPXTG-like proteins

Transmembrane proteins are proteins anchored to the cytoplasmic membrane by one or several hydrophobic transmembrane helixes, often located N- or C- terminally. Lipoproteins

are covalently attached to a lipid in the cell membrane through a conserved cysteine in the lipobox sequence. Cell wall binding proteins have specific domains that recognize some cell wall components. They can bind non-covalently using domains/motifs called LysM, SLH or WXL. LPXTG-like proteins have a Sec-dependent N-terminal signal peptide, a Type-I SPase cleavage site, and a LPXTG-like motif in the C-terminal end drives covalently attachment of the protein to peptidoglycan by a enzyme sortase (Desvaux et al. 2006; Kleerebezem et al. 2010).

The most commonly applied anchor for cell surface display in biotechnology is the LPXTG motif (Leenhouts et al. 1999). However, there are problems with using LPXTG motif for cell surface display. Firstly, differences in sortase activity between strains can cause problems and result in insufficient display of the target protein (Kim et al. 2008). Secondly, the LPXTG motif is in the C-terminal region (Boekhorst et al. 2005), meaning that proteins can only be attached in one orientation (with their N-terminal end protruding); this can be a problem when the to-be-displayed proteins have their functional sites close to the C-terminal domain or need to be oriented with a protruding C-terminus (such as in the case of invasin, described below).

1.6.1 Lipoproteins

Lipoproteins are an important class of membrane bound proteins with many different functions. They typically represent ca. 2% of the bacterial proteome and can be involved in adhesion, sensory processes, nutrient uptake, signal transduction, conjugation, sporulation, antibiotic resistance, and cell-envelope homeostasis, as well as in protein secretion, folding and translocation, especially extracytoplasmic protein folding (Kleerebezem et al. 2010; Kovacs-Simon et al. 2011; Rahman et al. 2008). In Gram-positive bacteria about 40% of the putative lipoproteins are ABC transporters (Hutchings et al. 2009).

Lipoproteins have a signal peptide with a lipobox sequence (Figure 1.4) that directs them to the Sec machinery. All lipoproteins contain a cysteine directly downstream of the signal peptidase cleavage site, which is part of a well conserved lipobox. A typical lipobox motif is [LVI][ASTVI][GAS]C (Kovacs-Simon et al. 2011). After translocation of the pre-prolipoprotein through the Sec pathway, a prolipoprotein diacylglyceryl transferase (Lgt) will add the diacyl glyceryl group from a glycerophospholipid to the SH-group of the cysteine residue of the lipobox, resulting in a prolipoprotein. This prevents release of the protein

because the diacylglyceryl group is now inserted into the lipid bilayer of the cytoplasmic membrane (Desvaux et al. 2006). This is followed by an N-terminal cleavage of the signal peptide by lipoprotein signal peptidase (Lsp or SPase II), leaving the lipid-modified cysteine at the N-terminus of the mature lipoprotein. Thus the protein is anchored to the membrane via a thioether linkage (Hutchings et al. 2009; Tjalsma et al. 2000) (Figure 1.4). In Gram-negative bacteria and some Gram-positive bacteria, the prolipoprotein is amino-acylated at the N-terminal cysteine residue, by lipoprotein N-acyl transferase, adding an amide-linked fatty acid at the N-terminal cysteine residue. In those cases, the lipoprotein is anchored to the membrane by both the diacylglyceryl group and the amino-terminal acyl group (Kovacs-Simon et al. 2011).

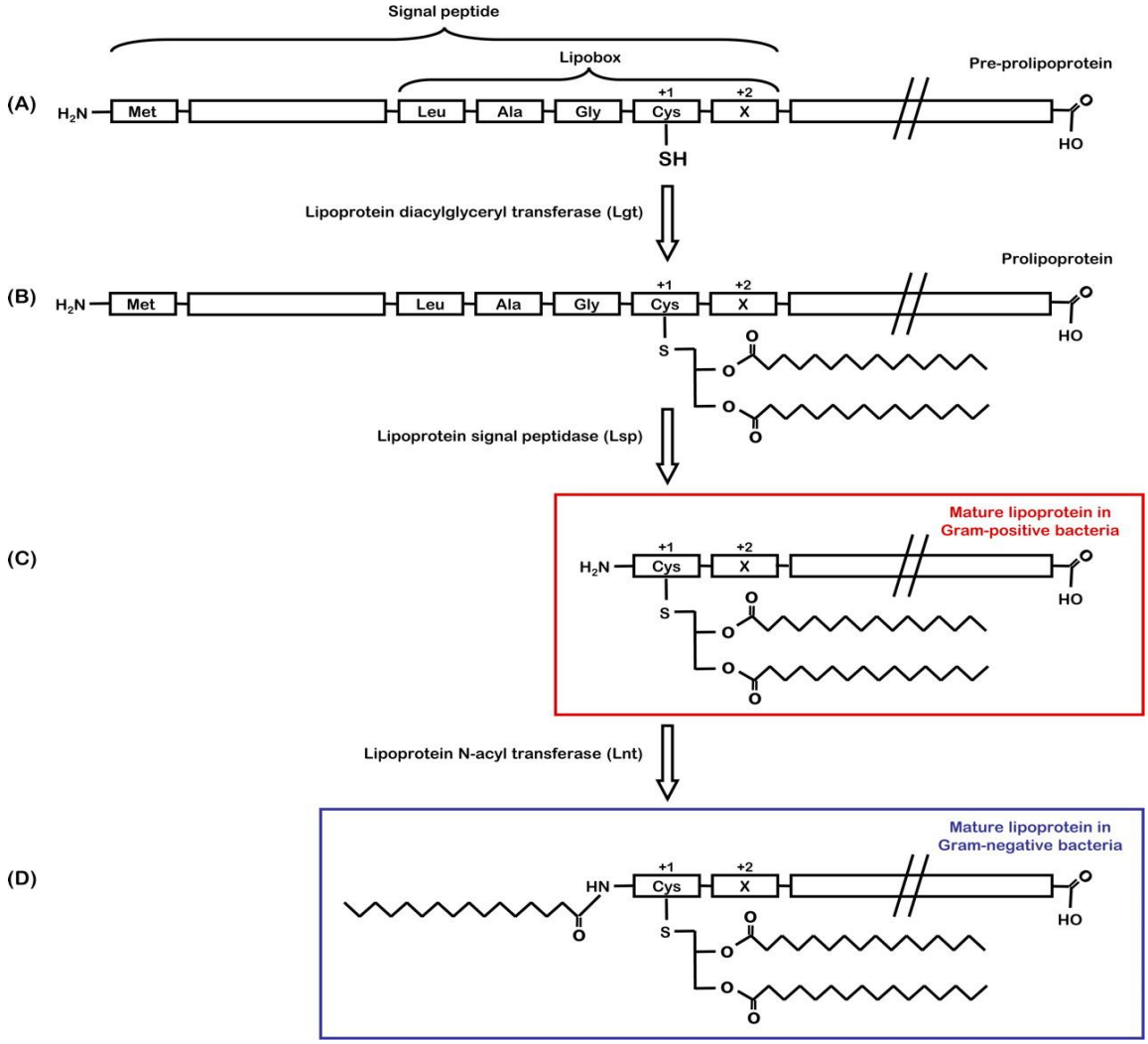


Figure 1.4: Biosynthesis of bacterial lipoproteins. (A) The pre-prolipoprotein has an N-terminal signal peptide with a characteristic consensus lipobox sequence. (B) The thiol group of the indispensable cysteine in the lipobox is modified by a diacylglyceryl moiety by lipoprotein diacylglyceryl transferase (Lgt). (C) The signal peptide is cleaved of by lipoprotein signal peptidase (Lsp) or Spase II, and the cysteine is left as the new amino-terminal residue forming the mature lipoprotein in Gram-positive bacteria. (D) In Gram-negative and some Gram-positive bacteria an additional amide-linked fatty acid is added to the mature lipoprotein at the N-terminal cysteine residue by lipoprotein N-acyl transferase (Lnt) (Kovacs-Simon et al. 2011). The figure is taken from Kovacs-Simon et al. (2011)

After the lipoprotein in Gram-positive bacteria is translocated across the cytoplasmic membrane and modified, the lipoprotein is anchored to the outer leaflet of the plasma membrane. In some pathogenic bacteria lipoproteins function as virulence factors in the host-pathogen interaction (Kovacs-Simon et al. 2011). Lipoproteins have attracted attention as vaccine candidates and many lipoproteins from different pathogenic bacteria have been evaluated (Ayalew et al. 2009; Erdile et al. 1993; Luo et al. 2009; Pimenta et al. 2006; Sardinas et al. 2009).

1.7 Bacteria and the immune system in the gut

The intestinal microflora in humans consists of approximately 10^{13}-10^{14} organisms and it is suggested that more than 1000 commensal species habitat the gastrointestinal tract (Velez et al. 2007). The immune system structure and function development are affected by the intestinal microflora (O'Hara & Shanahan 2006; Winkler et al. 2007). The mucosal immune system forms the largest part of the entire immune system, with about three-quarters of all lymphocytes, and it is the main site for host-microbe interactions (Didierlaurent et al. 2002; Shanahan 2002). There is a bi-directional adapted exchange between host and bacteria in the intestine, and the immune system has to discriminate between pathogenic and commensal microorganisms (Didierlaurent et al. 2002; Grainger et al. 2010; O'Hara & Shanahan 2006).

Intestinal epithelial cells form a barrier that functions as the first sensory line of defense and separates the bacterial community from the internal milieu (Figure 1.5) (Niedergang et al. 2004; O'Hara & Shanahan 2006). This constantly exposed barrier is covered by a protective layer of mucus (see Figure 1.5), which contains various protective and antimicrobial substances that are secreted by epithelial cells and have a broad spectrum of activities (Velez et al. 2007). In addition to protecting the host against bacterial invasion, the mucus layer digests and absorbs nutrients, and comprises a habitat for symbiotic bacteria (Deplancke & Gaskins 2001).

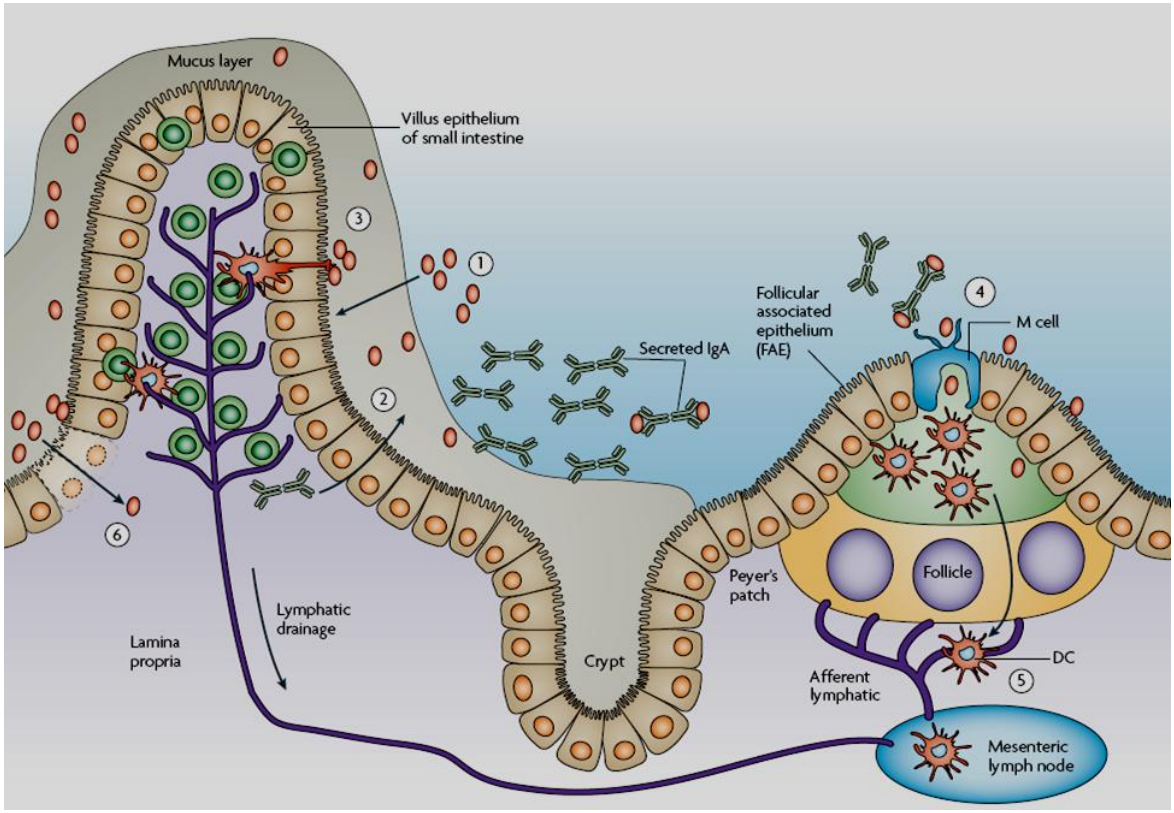


Figure 1.5: An overview of interactions between bacteria and the immune system in the intestinal tract. After their introduction into the intestinal tract, bacteria end up in the lumen or trapped in the mucus layer. (1) Bacteria and their secreted proteins or proteins from lysed cells will come into contact with the mucosal epithelium. (2) Immunoglobulin A (IgA) is secreted into the gut lumen. (3) Dendritic cells (DCs) can sample bacteria that are in contact with the apical surface, because DCs can extend between surface enterocytes without destroying the tight junctions. (4) M-cells are responsible for transporting luminal bacteria and antigens across the epithelium to cells of the immune system below. (5) Peyer's patches are sites where bacteria and different molecules can gain increased access to the epithelium that is located above the mucosal lymphoid follicles. Peyer's patches contain many DCs which can phagocytose bacteria and may move to mesenteric lymph nodes, where they can present antigens that are derived from the bacteria and then directly prime T-cell responses (Wells & Mercenier 2008). The picture is taken from Wells & Mercenier (2008).

There are three main types of immunesensory cells in the intestine: surface enterocytes, Microfold (M)-cells and intestinal dendritic cells (DCs). These cells are responsible for recognizing and active sampling of bacteria and antigens present in the gut (O'Hara & Shanahan 2006). Surface enterocytes are cells that secrete chemokines and cytokines that lead and alert innate and adaptive immune responses to the infected site (O'Hara & Shanahan 2006; Shanahan 2005). Polymeric immunoglobulin A (IgA) is secreted to the lumen and can potentially play a role in controlling bacterial persistence and uptake (Cerutti & Rescigno 2008; Wells & Mercenier 2008). M-cells are one type of cells that are responsible for transport of proteins and microbes across the epithelium cells layer to subadjacent DCs and other antigen-presenting cells (APC) (Figure 1.5). M-cells are found in the follicle-associated epithelium (FAE), above mucosal lymphoid tissue (Corr et al. 2008). Specialized

accumulations of lymphoid cells in one place are called lymphoid follicles where B-cells, T-cells and DCs are located. Lymphoid follicles are spread through the human intestine; part of these follicles are grouped in larger aggregates, referred to as Peyer's patches (Keita et al. 2006; Niedergang et al. 2004) (Figure 1.5). DCs do not only occur in Peyer's patches but also at other locations in the intestine. Several DCs have direct sensory roles, as illustrated in the left part of Figure 1.5. DCs are important cells because they can sample antigens directly from the lumen (O'Hara & Shanahan 2006) (Figure 1.5), and they are responsible for appropriate immune responses to commensal and pathogenic bacteria. DCs can stimulate any kind of response by phagocytosis of the bacteria, move to mesenteric lymph nodes where they present antigens that are derived from the bacteria to T-cells, and directly prime T-cell responses (Niedergang et al. 2004; Pasetti et al. 2011; Wells & Mercenier 2008).

Lymphoid microcompartments such as the Peyer's patches, the mesenteric lymph nodes, the appendix and isolated lymphoid follicles in the intestine constitute the mucosa-associated lymphoid tissue (MALT). The MALT consists of phenotypically and functionally distinct B-cells, T-cells and accessory cell subpopulations. The immune response in mucosal tissue is dependent on the nature of the antigen, the type of professional antigen-presenting cells (APC) involved and the local microenvironment. APCs include DCs, B lymphocytes and macrophages, and they present the antigen via their cell membrane-anchored major histocompatibility complex (MHC) proteins to conventional $CD4^+$ and $CD8^+$ T-cells which generate different responses (Holmgren & Czerkinsky 2005).

The MHC proteins play an important role in the immune system. The MHC proteins act as an "bulletin board" that serves to alert the immune system if foreign material is present inside a cell. They achieve this by displaying fragmented pieces or antigens on the host cell's surface (Lea 2006). The MHC class I molecules are found on almost every nucleated cell of the body and usually present peptides derived from endogenous proteins (proteins from cytosol in the cell). MHC class II molecules are found only on APC and usually present exogenous proteins (proteins from endocytose; from the environment around the cell). Cells of the epithelial mucosa mainly express MHC class I molecules, and only low amounts of MHC class II molecules. Antigens presented on MHC class I can activate $CD8^+$ T cytotoxic cells, which subsequently kill the host cells infected with intracellular microorganisms. Antigens presented on MCH class II can activate $CD4^+$ T_H-cells. T_H cells can be directed into T_H1 or T_H2

depending on several factors including the dose of the antigen. Inflammatory reactions are normally associated with the T_H1 response, whereas T_H2 responses are normally associated with allergic responses and parasite clearance. Another class of cells, T_{reg} cells, prevents overreactions by downregulating the immune response (Lea 2006; Ryan et al. 2001; Winkler et al. 2007).

1.8 Mucosal delivery of therapeutic and prophylactic molecules

A mucosal route of vaccination is in theory a very promising strategy because most infections start at mucosal surfaces (Wells & Mercenier 2008). With mucosal immunization the result is not always just a local immune response, since additional effects include production of mucosal-IgA antibodies at distant mucosal effectors sites. Furthermore, mucosal immunization stimulates systemic immune responses and T-cells activities to defeat infections (Cortes-Perez et al. 2007). One drawback of mucosal delivery is that the mucus layer probably limits the antigen uptake that is important for induction of adequate immune responses.

The immune responses of vaccines delivered through mucosal tissue are influenced by several different parameters, including the choice of bacterial host and the final subcellular location of the expressed foreign antigen (cytoplasmic, secreted or anchored to the cell wall or membrane). Active bacteria with *de novo* synthesis of the antigen can influence the immune response. It is not clear if a non-replicating vaccine would be as effective as a live bacterium and give the same immune responses (Wells & Mercenier 2008). This means that productions of antigens from active bacteria could be a good vaccine choice. The host does not necessarily have to be a bacterium, plant-based oral vaccines have also been considered (Tacket et al. 1998; Walmsley & Arntzen 2000). Other possible oral vaccine delivery vehicles are liposomes (Amin et al. 2009), dendrimers, multiple emulsions, immune stimulating complexes and biodegradable polymers (Azizi et al. 2010). The best mucosal route for delivering of the therapeutic molecules is also an issue. Delivering through genitals or rectum has demonstrated to be unpractical in human trials (Kozlowski et al. 1997). Oral and nasal administrations seem to be better alternatives when it comes to mucosal vaccines for humans (Azizi et al. 2010).

Oral delivery is considered as the preferred route of administration for a vaccine. This delivery has several advantages including easy administration (needle free), reduced risk of infection and possible contamination by the medical personnel, easy mass production, and potentially, low costs (Kim et al. 2010; Rieux et al. 2005). The drawbacks are related to difficulties in the delivery of active molecules to the mucosa because of the harsh digestion conditions in the GI tract (Critchley-Thorne et al. 2006; Kim et al. 2010). Several efforts have been made to handle this, such as the use of live organisms or viral carriers, and coating of the antigen (Palumbo & Wang 2006).

1.9 Delivery of vaccines to the immune system via M-cells

M-cells are considered a promising target for oral vaccination because they transport antigens, particles, viruses and bacteria from the lumen of the intestine, across the epithelial barrier into organized lymphoid structures below, where T cells, B cells and macrophages are ready to process any antigen present (Figure 1.6) (Corr et al. 2008; Gullberg et al. 2006). M-cells are located throughout the GI tract. They are found in the FAE of intestinal Peyer's patches, in isolated lymphoid follicles, in the appendix, as well as in mucosal-associated lymphoid tissue (MALT) sites outside the GI tract (Clark et al. 2001; Corr et al. 2008).

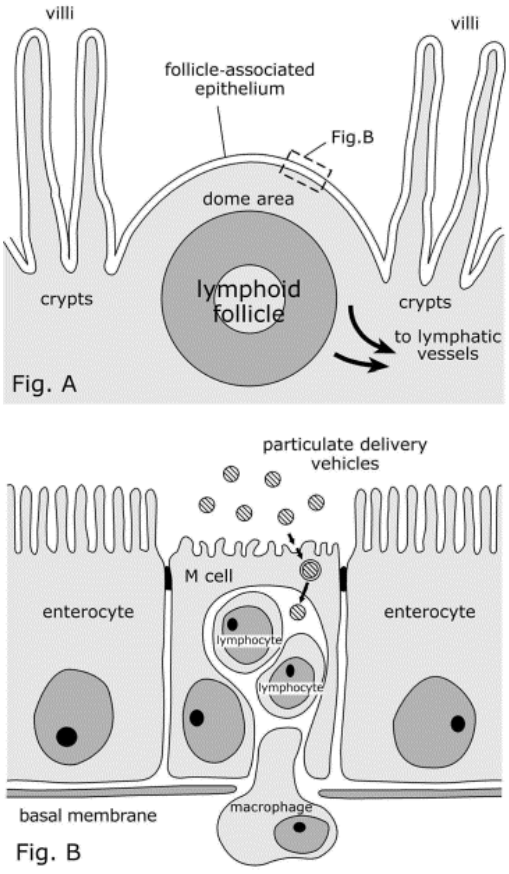


Figure 1.6: Overview of M-cell location found in the follicle-associated epithelium of Peyer's patches. The picture is taken from Clark et al. (2001)

Many pathogens exploit M-cells in invasion of the host, even though M-cells are specialized on antigen sampling. The invasion strategies used by pathogens have been examined in several studies, one reason being that their invasive abilities perhaps could be exploited for delivery of vaccines (Clark et al. 2001; Kraehenbuhl & Neutra 2000; Sansonetti & Phalipon 1999). However, the mechanisms involved in the uptake and transport of microorganisms by M-cells are poorly understood, primarily because M-cells are difficult to work with (Tyrer et al. 2007). Specific markers for M-cells are not completely known and the

result from *in vivo* studies using M-cells are not always relevant because there are a high variability in proportion and phenotype of M-cells among different species (Brayden & Baird 2001). Since M-cells are difficult to work with *in vivo*, *in vitro* models of M-cell/FAE are developed, which maintain the phenotypic and physiological features of the FAE and M-cells (Gullberg et al. 2000).

Translocation of antigens, particles, viruses and bacteria by M-cells is a very efficient and rapid process. The mechanisms involved when M-cells take up microorganisms and molecules are different and vary according to the nature of the material. Several factors of the material influence the transport mechanism, including size, local surface pH, surface charge, hydrophobicity, concentration, temperature and the presence or absence of an M-cell specific receptor (Corr et al. 2008; Ragnarsson et al. 2008; Rieux et al. 2005). It is assumed that M-cells contain many different surface receptors but only a limited number of receptors and their ligands have been identified. Most of the identified receptors are not only found on M-cells but in neighboring enterocytes as well (Azizi et al. 2010). β_1-integrin is a receptor found on the apical surface of M-cells, but not on the apical surface of enterocytes (Gullberg et al. 2006). Interesting, a protein from *Yersinia pseudotuberculosis* called invasin interacts with β_1-integrin with a higher affinity than the natural ligands (fibronectin, collagen, laminin and vitronectin) and can be used to target M-cells (Palumbo & Wang 2006; Ragnarsson et al. 2008).

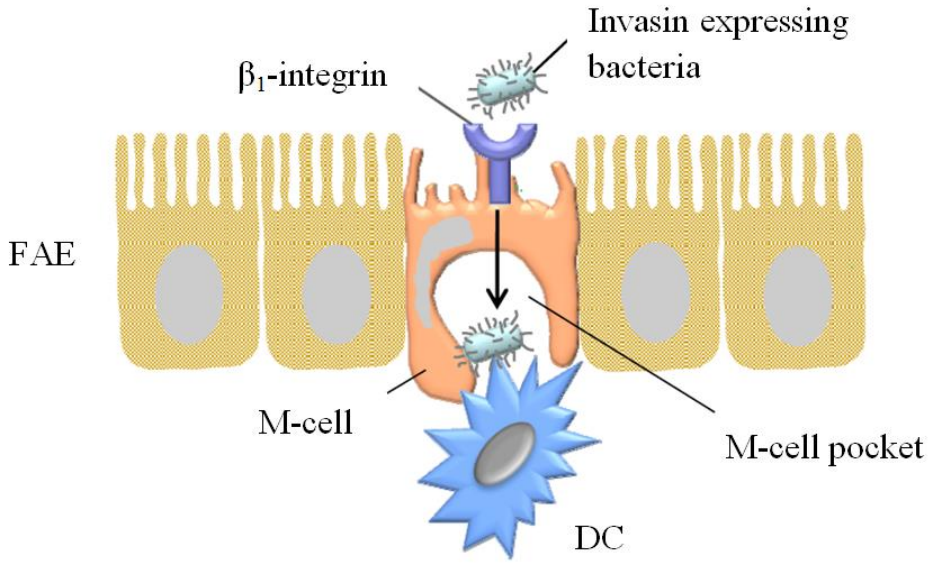


Figure 1.7: β_1-integrin dependent antigen transcytosis of invasin expressing bacteria. β1-integrin expressed on the apical surface of M-cells functions as transcytotic receptor for invasin expressing bacteria. The bacterium is transcytosed across FAE to the APCs underneath, such as DCs (Azizi et al. 2010). The figure is modified from Hase et al. (2009).

Y. pseudotuberculosis is a Gram-negative enteropathogenic bacterium that causes gastroenteritis in humans. The bacterium crosses the intestinal epithelium by translocation across M-cells to enter Peyer's patches (Hamburger et al. 1999). Invasin is the protein that promotes bacterial entry by binding to host cell β_1-integrin receptors (Leo & Skurnik 2011; Niemann et al. 2004; Palumbo & Wang 2006). The invasin gene, *inv*, of *Y. pseudotuberculosis* encodes a 986-residue protein (Grassl et al. 2003). About 500 amino acids in the N-terminal part are thought to anchor the protein in the outer membrane and this part is believed to form a β-barrel (Niemann et al. 2004). The C-terminal part of invasin comprises 497 amino acids that make up the extracellular region that binds to host cell β-integrin receptors and promotes uptake of the bacteria (Grassl et al. 2003; Hamburger et al. 1999). The crystal structure of this fragment shows five tandem domains with an elongated, rod-like structure (Figure 1.8) (Niemann et al. 2004). A fragment comprising the last 192 residues of this C-terminal fragment is the shortest fragment of invasin that is capable of binding integrins and inducing bacterial uptake by mammalian cells (Grassl et al. 2003; Hamburger et al. 1999). This C-terminal integrin binding fragment consists of domain D4 and D5 (Figure 1.8). The remaining domains, D1, D2 and D3 strengthen the binding further and enhance the efficiency of cell uptake (Palumbo & Wang 2006). The first four domains (D1, D2, D3 and D4) consist almost exclusively of β-strands, whereas the fifth domain (D5) has both α helices and -strands. The D4 and D5 domains that are essential for binding have an interface that is predominantly hydrophobic, but several hydrogen bonds are also present (Hamburger et al. 1999).

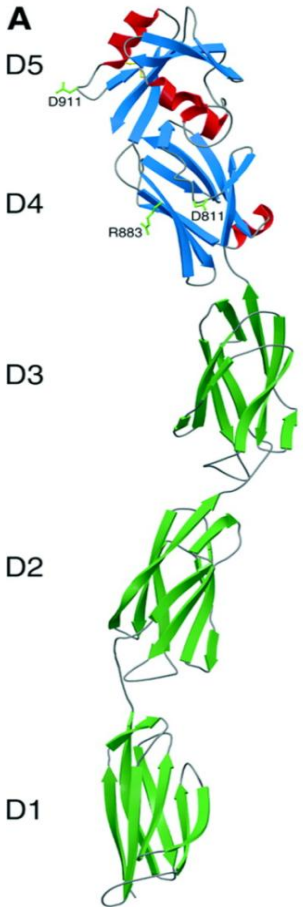


Figure 1.8: The 497 residues in the C-terminal end of invasin from *Y. pseudotuberculosis* shown as a ribbon diagram (Hamburger et al. 1999). The figure is taken from Hamburger et al. (1999).

The invasin protein is very attractive to utilize in oral delivery of molecules because of its efficiency and specificity (Palumbo & Wang 2006). In addition, it is now believed that invasin has more effect than just integrin binding and induction of uptake. It is believed that binding of invasin results in the activation and production of pro-inflammartory cytokines (Grassl et al. 2003; Leo & Skurnik 2011; Palumbo & Wang 2006). Invasin is also able to

activate B- and T-lymphocytes directly, because non-activated leukocytes express β_1-integrin, and invasin binds efficiently to these cells because of its high affinity for this receptor (Grassl et al. 2003). Bacteria coated with invasin have been used to deliver either proteins or genes to mammalian cells in several experiments (Acheson et al. 1997; Critchley-Thorne et al. 2006; Harms et al. 2009; Suzuki et al. 2006).

1.10 Goals of this study

This study is part of a project where the long term goal is to develop oral vaccines based on *L. plantarum*, for example vaccines against cancer or tuberculosis The aim of the present study was to achieve production, secretion and cell membrane anchoring of the M-cell binding protein invasin from *Y. pseudotuberculosis* in *L. plantarum* WCFS1, by using an inducible gene expression system previously developed for efficient intracellular protein production (Sørvig et al. 2003). M-cells are considered a promising target for oral vaccination because they transport antigens, particles, viruses and bacteria from the lumen of the intestine, across the epithelial barrier into the organized lymphoid structures called Peyer's patch (Corr et al. 2008; Gullberg et al. 2006). It is also of great basic interest to investigate the immunological changes induced when a probiotic organism such as *L. plantarum* is translocated to the Peyer's patches in the gut, even when there is no additional vaccine antigen present. *L. plantarum* WCFS1 is an interesting candidate to utilize as an oral vaccine because it is resistant to bile and has shown high survival capacity in the intestinal tract. In addition, it is a versatile bacterium with GRAS-status and genetic tools for strain engineering are available. As to these tools, an additional goal of the present study was to evaluate the possibilities for expressing and secreting proteins as complex as the invasin in lactic acid bacteria and to study the use of lipo-anchors for protein anchoring.

In the present study, lipoprotein anchors with an N-terminal signal peptide and a lipobox motif (Kovacs-Simon et al. 2011) were tested to anchor invasin to the cell membrane of *L. plantarum.* It was necessary with an anchor in the N-terminal end of invasin since the active binding-domain is located at the C-terminal end (Hamburger et al. 1999). Since it was uncertain which length of the anchor would optimally expose the invasin protein for binding to β_1-integin receptors, two different lipo-anchors with variable lengths were tested. Likewise, it was not known which form of invasin would give the best result. Two versions of the invasin consisting of five (Inv, comprising D1-D5) or two domains (InvS, comprising D4-D5)

was therefore studied. Thus at the start of the project, four different types of anchoring constructs were envisaged containing different combination of two lipo-anchors and two invasin forms (for details see Result section).

In short the experimental work of this study consisted of the following five parts:

- Construction of invasin expression vectors, including controls.
- Transformation of expression vectors into *L. plantarum* WCFS1.
- Analysis of invasin production in the different recombinant *L. plantarum* strains.
- Analysis of secretion and anchoring of invasin in *L. plantarum* (localization studies).
- Analysis of internalization of *L. plantarum* strains harbouring different invasin constructs by Caco-2 cells (i.e. a human intestinal cell line).

2. MATERIALS

2.1 Laboratory equipment

Laboratory equipment	Supplier
2 ml tubes	Axygen
13 ml tubes, PP	Sarstedt
15 ml cellstar tubes, PP	Greiner bio-one
50 ml cellstar tubes, PP	Greiner bio-one
Automatic pipettes	ThermoLabsystems
Cuvettes	
Disposable cuvettes, 1.5ml	Brand
Electroporation cuvettes	Bio-rad
Eppendorf tubes	Axygen
FastPrep ® Tubes	MP
Mikro tube 2 ml, PP	Sarstedt
NuPAGE SDS-gels and buffers	Invitrogen
Qubit assay tubes	Invitrogen
Sterile filters, 0.22 µm pore size	Millex GP
Vortex-machine	Ika
Various glass equipments	Labsystems
Waterbaths	Julabo
Western blot equipment	
Immun-Blot PVDF Membrane	Bio-Rad
Trans-blot Transfer Medium, Nitrocellulose membrane	BioRad
iBlot Gel Transfer Stacks Nitrocellulose	Invitrogen
Film cassette	
Film: CL-XPosure™ 18x24 cm	Kodak

Instruments	Supplier
ABI®PRISM 3100 DNA Sequencer	AME Bioscience
BioPhotometer	Eppendorf
Cell density meter	Swab
Centrifuges	
Table centrifuge	VWR/Biofuge
Cooling centrifuge	Eppendorf
Vacuum centrifuge	Savant
Centrifuge 5430R	Eppendorf
CertoClav CV-EL	One-Med
CP124S weight	Sartorius
Electrophoresis equipment	
Agarose gel: Power Pac 300 and Basic gel driver	Bio-rad
SDS-PAGE: Xcell Surelock™ Mini-Cell	Invitrogen
FastPrep-24 tissue and cell homogenizer	MP

Gel electrophoresis	Bio-rad
iBlot machine	Invitrogen
LC 621P weight	Sartorius
LEICA DMIL microscope	Leica
MACSQuant® Analyzer & MACSQuantify™ Software	MACS Miltenyl Biotec
Multi RS-60, Programmable rotator mixer	BIOSAN
Multitron eco incubator	Infors
PCR-machine	
Mastercycler gradient	Eppendorf
VWR	VWR
RCT classic stirrer	IKA
pH-meter, 827 pH lab	Metrohm
Qubit fluorometer	Invitrogen
Rotamax 120 rotate	Heidolph
SNAP i.d. Protein Detection System	Millipore
Steri-Cycle CO_2 Incubator, HEPA CLASS 100	Thermo Scientific
Telstar AV-100 sterile bench	Telestar
Universal Hood II, gel image	Bio-rad

Software	Source
ExPASy Proteomics Server	http://au.expasy.org/
LipoP 1.0	http://www.cbs.dtu.dk/services/LipoP/
NCBI	http://www.ncbi.nlm.nih.gov/guide/
pDRAW32	http://www.acaclone.com/
SignalP 3.0	http://cbs.dtu.dk/services/SignalP/

2.2 Chemicals

Chemicals	Supplier
1,10-phenanthroline, $C_{12}H_8N_2$	Sigma-Aldrich
Acetic acid, $C_2H_4O_2$	Merck
Acetone, CH_3COCH_3	Prolabo
Agar	Merck
Agarose Nusieve GTG	Cambrex
Agarose Seakem LE agarose	Promega
Ampicillin	Sigma
Bromphenol blue, $C_{19}H_{10}Br_4O_5S$	Kodak
Calcium chloride, $CaCl_2$	sds
EDTA, $C_{10}H_{16}N_2O_8$	Sigma
Erythromycin, $C_{37}H_{67}NO_{13}$	Sigma-Aldrich
Ethanol, C_2H_5OH	Arcus
Ethidium Bromide, EtBr	Sigma
Gentamicin	Sigma
Glass Beads, acid-washed	Sigma
Glycerol, $C_3H_8O_2$	Merck
Glycine, $C_2H_5NO_2$	Merck

Kalium chloride, KCl	Merck
Kanamycin	Sigma-Aldrich
Magnesium chloride, $MgCl_2$	Merck
Metanol, CH_3OH	Merck
Monopotassium phosphate, KH_2PO_4	Merck
Pepstatin A	Sigma
Polyethylen glycol, PEG_{1450}	Sigma
Sodium acetate, $NaC_2H_3O_2x3H_2O$	Novagen
Sodium chloride, NaCl	Merck
Sodiumdihydrogenphosphate monohydrate, Na_2HPO_4	Merck
Sodium hydroxide, NaOH	Merck
Sucrose, $C_{12}H_{22}O_{11}$	VWR Prolabo
Tris-base, $C_4H_{11}NO_3$	Sigma
Trichloroacetic acid (TCA), $C_2HCl_3O_2$	Sigma
Tris-HCl	Sigma
Tween-20	Sigma-Aldrich

2.3 Proteins and enzymes

Protein/enzyme	Supplier
Antibodies	
pAb invasin PAS Bleed #2 and Bleed #3 (Animal (Rabbit) ID 13619, 13620) Recognizes the C-terminal epitopes: YSSDWQSGEYWVKK and NGQNFATDKGFPKT	ProSci incorporated
HRP-Goat Anti-Rabbit IgG (H+L)	Invitrogen
Anti-Rabbit IgG (whole molecule)- FITC	Sigma
Bovine Serum Albumine (BSA)	Sigma-Aldrich
Lysozyme	Sigma
Mutanolysin from *Streptomyces globisporus*	Sigma
Phusion High Fidelity DNA polymerase (with 5x Phusion HF buffer)	Finnzymes
Proteinase K	Sigma-Aldrich
Quick T4 DNA ligase (with 2x Quick ligation reaction buffer)	Biolabs
Ribonuclease A (RNAse A)	Sigma
Restriction enzymes (with restriction buffers)	NewEnglandBiolabs
EcoRI	
EcoRIHF	
NdeI	
SalI	
T4 DNA ligase (with 10x ligase reaction buffer)	Biolabs
Taq DNA polymerase (with 10x reaction buffer)	Invitrogen

2.4 DNA & nucleotides

DNA	Supplier
dNTP-mix, F-560S	Finnzymes
DNA standards	
GeneRuler™ 1 kb DNA ladder	Fermentas
100 bp DNA ladder	NEB

2.5 Primers

Primers for traditional cloning and In-Fusion cloning are included in this study. Because traditional strategies were not successful to construct invasin expressing vectors, focus was shifted towards using In-fusion cloning technologies. Therefore, only primers for traditional cloning of a plasmid with the intracellular version of invasin (pCytInv) and a plasmid containing the Lp_1261 lipo-anchor (pLp_1261) were made before In-Fusion cloning were used instead.

Table 2.1: Primers by name and sequence

Name	Sequence	Restriction site in sequence
	Primers for use in traditional cloning	
CytInvF	*CATATG*AGCGTCACCGTTCAGCAGC	NdeI
InvR	*GAATTC*TTATATTGACAGCGCACAGAGC	EcoRI
Lp_1261F	*CATATG*AATTTCAAAACAGCTGCAAAAGT	NdeI
Lp_1261R	*GTCGAC***CGCCGCGATAGTACC**CCCGTTCTTACCGAGACGGTATAAC	SalI
	Primers for use in In-Fusion cloning	
HR1261F	GGAGTATGATT*CATATG*AATTTCAAAACAGCTGCAA	NdeI
1261R	*GTCGAC*CGCCGCAATCGTGCCCCCGTTCTTACCGAGACGGT	SalI
HR1452F	GGAGTATGATT*CATATG*AAGAAATGGCTCATTGCC	NdeI
1452R	*GTCGAC*CGCCGCAATCGTGCCTTGAACCGTGACTTTAGGTTCGT	SalI
SOE1261InvSF	CGGG**GGCACGATTGCGGCG***GTCGA*CACGCTGACCGGTATTCTGGT	SalI
SOE1261InvF	CGGG**GGCACGATTGCGGCG***GTCGA*CAGCGTCACCGTTCAGCAGC	SalI
SOE1452InvSF	TCAA**GGCACGATTGCGGCG***GTCGA*CACGCTGACCGGTATTCTGGT	SalI
SOE1452InvF	TCAA**GGCACGATTGCGGCG***GTCGA*CAGCGTCACCGTTCAGCAGC	SalI
HRCytInvF	GGAGTATGATT*CATATG*AGCGTCACCGTTCAGC	NdeI
HRInvR	CCGGGGTACC*GAATTC*TTATATTGACAGCGCACAGAGC	EcoRI
	Primers for use in sequencing	
SeqInvF	CTTGGCTGATGGCACGATGAGT	
SeqInvR	TCGCCGTCACAGCCACTT	
psecF	GGCTTTTATAATATGAGATAATGCCGAC	
secInvF	GTCGACAGCGTCACCGTTCAGCAGCCT	
psecAcc65IR	TGGCTATCAATCAAAGCAACACGT	
pSipSecR	CCGCCCTTATGGGATTTATCT	

Restriction sites in sequence are indicated in italic and the linker sequences (see section 4.3) are indicated in bold.

Table 2.2: Primers by name and description

Name	Description
	Primers for use in traditional cloning
CytInvF	Forward primer for the long version of invasin (the D1-D5 domains)
InvR	Reverse primer for the invasin sequence
Lp_1261F	Forward primer for the lipo-anchor sequence from *Lp_1261* in *L. plantarum* WCFS1
Lp_1261R	Reverse primer with **linker** (without silent mutations) for the lipo-anchor sequence from *Lp_1261* in *L. plantarum* WCFS1.
	Primers for use in In-Fusion cloning
HR1261F	Forward primer for the lipo-anchor sequence from *Lp_1261* in *L. plantarum* WCFS1, including an overlapping sequence with the vector.
1261R	Reverse primer for the lipo-anchor sequence from *Lp_1261* in *L. plantarum* WCFS1.
HR1452F	Forward primer for the lipo-anchor sequence from *Lp_1452* in *L. plantarum* WCFS1, including an overlapping sequence with the vector.
1452R	Reverse primer for the lipo-anchor sequence from *Lp_1452* in *L. plantarum* WCFS1.
SOE1261InvSF	Forward primer for the short version of invasin (the D4 and D5 domains) including a **linker** sequence (with silent mutations) and an overlap sequence with the Lp_1261 lipo-anchor.
SOE1261InvF	Forward primer for the long version of invasin (the D1-D5 domains) including a **linker** sequence (with silent mutations) and an overlap sequence with the Lp_1261 lipo-anchor.
SOE1452InvSF	Forward primer for the short version of invasin (the D4 and D5 domains) including a **linker** sequence (with silent mutations) and an overlap sequence with the Lp_1452 lipo-anchor.
SOE1452InvF	Forward primer for the long version of invasin (the D1-D5 domains) including a **linker** sequence (with silent mutations) and an overlap sequence with the Lp_1452 lipo-anchor.
HRCytInvF	Forward primer for the long version of invasin (the D1-D5 domains) including an overlapping sequence with the vector.
HRInvR	Reverse primer for invasin (both short and long version) including an overlapping sequence with the vector.
	Primers for use in sequencing
SeqInvF	Forward primer for the sequencing of the invasin
secInvF	Forward primer for the sequencing of the invasin
SeqInvR	Reverse primer for the sequencing of the invasin
psecF	Forward primer for the sequencing of inserts in the p2588sAmy-based vectors
psecAcc65IR	Reverse primer for the sequencing of inserts in the p2588sAmy-based vectors
pSipSecR	Reverse primer for the sequencing of inserts in the p2588sAmy-based vectors

2.6 Bacterial strains and plasmids

Table 2.3: Bacterial strains

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

Table 2.4: Plasmids

Plasmid	Description	Source or reference
pCR-BluntII-TOPO	Vector for cloning of PCR fragments; Kan^r.	Invitrogen
pLp_0373sNucA	pSIP401-derivative for secretion of NucA using the Lp_0373 signal peptide and the sakacin P promoter (P_{sppA}) for *nucA* expression.	(Mathiesen et al. 2008)
p2588sAmy	pSIP401-derivative for secretion of Amy using the 2588 signal peptide and the sakacin P promoter (P_{sppA}) for Amy expression.	(Mathiesen et al. 2008)
pTinvasin	Vector containing the invasin gene.	L. Fredriksen, unpublished
pEV	pSIP401-derivative lacking any target gene	L. Fredriksen, unpublished
pLp_1261	Vector containing anchor sequence from Lp_1261 in *L. plantarum*	This work
pCytInv	p2588sAmy-derivative with invasin (Inv) instead of Amy.	This work
pLp_1261InvS	p2588sAmy-derivative with a short sequence of Lp_1261 as anchor sequence and the D4 and D5 domains from invasin (InvS) instead of Amy.	This work
pLp_1261Inv	p2588sAmy-derivative with a short sequence of Lp_1261 as anchor sequence and invasin (Inv) instead of Amy.	This work
pLp_1452InvS	p2588sAmy-derivative with a short sequence of Lp_1452 as anchor sequence and D4 and D5 domain from invasin (InvS) instead of Amy.	This work
pLp_1452Inv	p2588sAmy-derivative with a short sequence of Lp_1452 as anchor sequence and invasin (Inv) instead of Amy.	This work

2.7 Kits

Kit	Supplier
Big Dye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
Big Dye® Terminator v3.1 Ready Reaction Premix	
Big Dye® Terminator v3.1 Sequencing Buffer (5x)	
Erase-It™ Background eliminator Kit	Pierce
Erase-It reagens A	
Erase-It reagens B	
E.Z.N.A.™ bacterial DNA kit	Omega
DNA Wash Buffer	
Buffer BTL	
Buffer BDL	
Buffer HB	
Elution buffer	
Equilibration Buffer	

In-Fusion™ Advantage PCR Cloning Kit	Clontech
In-Fusion enzyme	
5x In-Fusion reaction buffer	
pUC19 control vector, linearized (50ng/µl)	
2kb control insert (40ng/µl)	
JET Star, The Novel Plasmid Purification System	GENOMED
Resuspension solution E1	
Lysis solution E2	
Neutralization solution E3	
Equilibration solution E4	
Wash solution E5	
Elution solution E6	
Nucleic Acid and Protein Purification, NucleoSpin Extract II	Macherey-Nagel
Binding Buffer NT	
Wash buffer NT3	
Elution Buffer NE	
Nucleic Acid and Protein Purification, NucleoSpin Plasmid	Macherey-Nagel
Resuspention Buffer A1	
Lysis Buffer A2	
Neutralization buffer A3	
Wash Buffer AW	
Wash Buffer A4	
Eluation buffer AE	
Qubit™ dsDNA BR Assay Kits	Invitrogen
QubitTM dsDNA BR reagent	
QubitTM dsDNA BR buffer	
QubitTM dsDNA BR standard #1	
QubitTM dsDNA BR standard #2	

2.8 Agars and media

Medium		Supplier
BHI (Brain-Heart-Infusion)		Oxoid
Medium:	37 g BHI	
	dH_2O to 1 litre	
	Sterilized in a certoclave for 15 minutes at 115ºC	
Agar:	BHI medium with 1.5% (w/v) agar	
	Appropriate antibiotics were added after cooling to ~60ºC	

MRS (de Man, Rogosa, Sharpe) Oxoid

Medium:	52 g MRS
	dH_2O to 1 litre
	Sterilized in a certoclave for 15 minutes at 115ºC
Agar:	MRS medium with 1.5% (w/v) agar
	Appropriate antibiotics were added after cooling to ~60ºC

MRSSM medium (1l)

MRS		52 g
Sucrose	(0.5 M)	171 g
$MgCl_2x6H_2O$	(0.1 M)	2.0 g

dH_2O to 1 litre, filter sterilized (0.22 μm pore size)

RPMI 1640 medium with L-glutamine and 10% fetal calf serum (FCS) Invitrogen

2xTY medium:	15 g tryptone
	10 g yeast extract
	5 g 10 mM NaCl
	Sterilized in a certoclave for 15 minutes at 115ºC

3. METHODS

3.1 Growing of bacterial strains

Diverse bacterial strains have different nutritional needs and require distinct growth conditions. Optimal growth can be obtained by ensuring that each bacterial strain receives what it requires. When the bacterium is used as a host for a plasmid construct harbouring an antibiotic resistance gene, antibiotics must be added to the growth medium to ensure stable maintenance of the plasmid.

Escherichia coli cells were grown in BHI-medium (Brain-Heart-Infusion) either on solid agar plates or in liquid medium, and incubated overnight in a 37ºC heating cabinet (for plates) or in a shaker incubator (for liquid cultures). Appropriate antibiotics were added to the medium when growing *E. coli* strains harbouring plasmids, at the following concentrations:

- Erythromycin 200 μg/ml in both agar plates and liquid medium
- Kanamycin 50 μg/ml in both agar plates and liquid medium

L. plantarum was grown either in MRS-medium (Man-Rogosa-Sharpe) or on solid MRS-agar plates. *L. plantarum* cultures were incubated overnight in a 30ºC or 37ºC heating cabinet without shaking. *L. plantarum* cells containing plasmids harbouring an antibiotic resistance gene were grown in medium containing the appropriate antibiotic at the following concentrations:

- Erythromycin 10 μg/ml in agar plates, 5-10 μg/ml in liquid medium

3.2 Long-term storage of bacteria

Glycerol enables bacterial cultures to be frozen for an indefinitely long time without harming the cells. Cultures of different bacterial strains of both *E. coli* and *L. plantarum*, harbouring different plasmids, were preserved by glycerol by making the following mixes:

- 1000 μl bacterial culture
- 300 μl glycerol (87% v/v, sterile)
- After mixing, the glycerol stocks were kept at -80ºC.

Bacterial cultures were grown from glycerol stocks by scraping small amounts of the frozen culture with sterile toothpicks and transferring the toothpicks to tubes containing appropriate growth medium.

3.3 Plasmid isolation from *Escherichia coli*

In order to isolate plasmids from *E. coli* the NucleoSpin Plasmid isolation kit was used. The procedure was performed according to the user manual provided by the manufacturer for "isolation of high-copy plasmid DNA from *E. coli*".

Materials:

NucleoSpin Plasmid kit
Resuspension Buffer A1
Lysis Buffer A2
Neutralization buffer A3
Wash Buffer AW
Wash Buffer A4
Elution buffer AE

Procedure:

1. 1.5 ml overnight culture of *E. coli* containing the desired plasmid was centrifuged at 11 000 x g for 1 minute in a microcentrifuge to pellet and harvest bacterial cells. The medium was poured off and as much of the liquid as possible was removed.
2. 250 µl Buffer A1 was added and the pellet was resuspended by pipetting up and down. It was important to make sure that no cell clumps remained in the tube before addition of Buffer A2
3. To lyse the cells, 250 µl Buffer A2 was added and the tube was gently inverted 6-8 times. The lysis reaction was not allowed to proceed for more than 5 minutes and was carried out in room temperature.
4. The lysed cell suspension was neutralized by adding 300 µl of Buffer A3, which stops the lysis reaction. The tube was gently inverted 6-8 times and precipitation of cell debris occurred.
5. The tube was centrifuged at 16 100 x g for 10 minutes at room temperature, in order to separate the lysate from the cell remnants.

6. The resulting supernatant was transferred to a column to bind DNA, while the pellet was not disturbed. The column was centrifuged for 1 minute at 11 000 x g and the flow-through was discarded.
7. To wash the silica membrane in the column, 500 µl of the wash Buffer AW, preheated to 50ºC, was added. The column was centrifuged for 1 minute at 11 000 x g and the flow-through was discarded. Then the membrane was washed with 600 µl Buffer A4. The column was centrifuged for 1 minute at 11 000 x g and the flow-through was discarded.
8. The column was centrifuged for another 2 minutes at 11 000 x g to dry the silica membrane and remove residual ethanol, a critical step for further applications because residual ethanolic wash buffer might inhibit enzymatic reactions.
9. After placing the column in a clean eppendorf tube 50 µl Buffer AE was added followed by incubation on the bench for 1 minute. To elute the plasmid, the column was centrifuged for 1 minute at 11 000 x g.
10. The eppendorf tube with plasmids were stored at -20ºC

The midi kit from JET Star was used for plasmid isolation on a larger scale. The procedure was carried out as suggested by the supplier.

Materials:

Ethanol, 70%

JET Star, Midi kit

- Resuspension solution E1
- Lysis solution E2
- Neutralization solution E3
- Equilibration solution E4
- Wash solution E5
- Elution solution E6

Procedure:

1. The column was equilibrated before the cleared lysate was prepared, by adding 10 ml solution E4 to the column and allowing the column to empty by gravity flow.
2. *E. coli* cells from 100 ml culture were pelleted by centrifugation at 5000 rpm for 5 minutes. Afterwards, traces of medium were carefully removed.

3. 4 ml of solution E1 was added to the pellet and the cells were resuspended by using the pipette until the suspension was homogeneous.
4. 4 ml of solution E2 was added to the sample and the sample was mixed gently by inverting the tube until the lysate appeared to be homogeneous. The lysis reaction was incubated at room temperature for 5 minutes.
5. The lysed cell suspension was neutralized by adding 4 ml of solution E3, which stops the lysis reaction. The suspension was mixed immediately by multiple inversions of the tube until a homogeneous suspension was obtained and no remainders of the viscous matter that appeared after cell lysis were left. The entire suspension was transferred to several 2 ml tubes, and centrifuged at 16 100 x g at room temperature for 10 minutes.
6. The supernatant from step 5 was applied to the equilibrated column (from step 1), and the lysate was allowed to pass through the column by gravity flow.
7. To wash the column, 10 ml of solution E5 was added twice and each time the wash solution was allowed to pass through the column by gravity flow.
8. After placing the column in a clean tube, 5 ml of solution E6 was added to the column to elute the plasmid using gravity flow.
9. After removing the column 3.5 ml isopropanol was added to the solution. The entire solution was transferred to several 2 ml tubes, and centrifuged at 16 100 x g at 4ºC for 30 minutes. The supernatant was removed and 100 µl of 70% ethanol was added to each tube and the tubes were recentrifuged. The sample pellet was vacuum dried for 5 minutes to remove residual ethanol and the pellet was redissolved in 10 µl of dH_2O and stored at -20ºC.

3.4 Isolation of genomic DNA from *Lactobacillus plantarum* WCFS1

Isolation of genomic DNA from *L. plantarum* was performed according to the bacterial DNA Spin protocol from the E.Z.N.A bacterial DNA kit (Omega).

Materials:

Ethanol, 96%

Glass Beads

Lysozyme (50 mg/ml)

Proteinase K (25 mg/ml)

RNase A (10 mg/ml)

E.Z.N.A bacterial DNA kit

- DNA Wash Buffer
- Buffer BTL
- Buffer BDL
- Buffer HB
- Elution buffer
- Equilibration Buffer

Procedure:

1. 1.5 ml overnight culture was centrifuged at 4000 x g for 10 minutes at room temperature. Afterwards, the medium was poured off and as much of the liquid as possible was removed.
2. The pellet was resuspended in 180 µl sterile dH_2O. 18 µl lysozyme was added and the suspension was incubated in a 30ºC water bath for 10 minutes.
3. The suspension was centrifuged at 5000 x g for 5 minutes at room temperature. Afterwards, the supernatant was aspirated and about 10 µl of residual liquid was left in the tube. The cell pellet was resuspended by vortexing.
4. 200 µl Buffer BTL was added to the suspension. The suspension was transferred to a tube with ~25 mg glass beads. The tube was placed in the FastPrep-24 tissue and cell homogenizer operated with the settings 6.0 m/s and 45 seconds for crushing the cells mechanically. The crushed cells were centrifuged at 13 000 x g for 1 minute and the supernatant was transferred to a new eppendorf tube.
5. 20 µl Proteinase K was added to the supernatant, to break peptide bonds in proteins in the sample, and the sample was vortexed to mix it thoroughly.
6. The sample was incubated in a 55ºC water bath for 1 hour and briefly vortexed every 20 minutes during the incubation.
7. 5 µl RNase A was added to the sample and the tube was inverted several times followed by incubation on the bench for 5 minutes.
8. The sample was centrifuged at 10 000 x g for 5 minutes to pellet insoluble debris. Afterwards the supernatant was carefully aspirated and transferred to a new eppendorf tube, leaving behind any insoluble pellet.

9. 220 µl Buffer BDL was added to the sample, the tube was briefly vortexed followed by incubation at 65ºC for 10 minutes.
10. 220 µl 96% ethanol was added to the sample and the sample was mixed thoroughly by vortexing the tube at max speed for 20 seconds.
11. A column for DNA binding was prepared by adding 100 µl Equilibration Buffer to it, followed by incubation for 4 minutes at room temperature in a collection tube. The column was then centrifuged at 13 000 x g for 1 minute.
12. The entire sample from step 10 was transferred to the column, including any precipitate that might have formed, and the column was centrifuged at 10 000 x g for 1 minute. The collection tube and the filtrate were discarded.
13. The column was placed into a second collection tube and washed with 500 µl Buffer HB. The column was centrifuged at 10 000 x g for 1 minute and the flow-through was discarded.
14. 700 µl DNA Wash Buffer diluted with ethanol was added to the column. The column was centrifuged at 10 000 x g for 1 minute and the flow-through was discarded.
15. The wash step was repeated with a second 700 µl DNA Wash Buffer and the column was centrifuged as described in step 14.
16. The column was centrifuged at 10 000 x g for 2 minutes to remove ethanol.
17. After placing the column in a clean eppendorf tube 50 µl Elution Buffer, preheated to 65ºC, was added followed by incubation for 5 minutes at room temperature.
18. The column with the eppendorf tube was centrifuged at 10 000 x g for 1 minute to elute genomic DNA. Afterwards, the eppendorf tube was stored at -20ºC.

3.5 Nucleic acid precipitation with Pellet Paint

This method is based on using Pellet Paint Co-Precipitant which is a brightly colored (pink), dye-labeled carrier, designed specifically for use in alcohol precipitation of nucleic acids. Both RNA and DNA can be precipitated from solutions by alcohols and such a precipitation step can be used for cleanup or/and concentration of DNA, for example after a PCR reaction.

Materials:

- Pellet Paint Co-Precipitant
- 3M sodium acetate pH 5.2
- Ethanol, 96% and 70%

Procedure:

1. The tubes with Pellet Paint Co-Precipitant and 3M sodium acetate were put at room temperature. The Pellet Paint stock solution tube was inverted carefully several times, until a uniform suspension was achieved.
2. 2 µl of Pellet Paint was added to the sample (independent of sample volume).
3. 0.1 volumes of 3 M sodium acetate were added to the sample and the sample was mixed briefly.
4. 2 volumes of 96% ethanol were added to the sample and the sample was mixed by vortexing the tube briefly for 5-10 seconds.
5. The sample was incubated at room temperature for 2 minutes.
6. The sample was centrifuged at 16 100 x g for 5 minutes, leading to a pink pellet containing the DNA becoming visible at the bottom of the tube. The supernatant was carefully aspirated.
7. The pellet was rinsed with 500 µl 70% ethanol and the sample was briefly vortexed. The sample was recentrifuged, and the supernatant was carefully aspirated.
8. The pellet was rinsed with 100 µl 96% ethanol. The sample was recentrifuged, and the supernatant was carefully aspirated.
9. The sample pellet was vacuum dried for 5 minutes to remove residual ethanol.
10. The dried pellet was solved in a desired volume of dH_2O.

3.6 Determination of DNA concentration with Qubit

The Qubit method is a method for DNA quantification. Qubit™ dsDNA BR Assay Kit is selective for double-stranded DNA and consists of a fluorescence-based dye that binds specifically to DNA. The fluorescent dye emits signals when it is bound to the specific target molecules. The standards included in the kit are used to make the standard curve which is used to determine the concentration of DNA in the samples. Common contaminants, such as salts, free nucleotides, solvents, detergents, or protein are tolerated in the assay.

Materials:

Qubit™ assay tubes

Qubit™ dsDNA BR Assay Kits

- Qubit™ dsDNA BR reagent
- Qubit™ dsDNA BR buffer

- Qubit™ dsDNA BR standard #1
- Qubit™ dsDNA BR standard #2

Procedure:

1. All reagents were at room temperature before use.
2. The working solution was made by diluting the Qubit™ dsDNA BR reagent 1:200 in Qubit™ dsDNA BR buffer. The final volume in each assay tube was 200 µl. Each standard tube required 190 µl of the working solution, whereas each sample tube required from 180 µl to 199 µl (most often199 µl). A sufficient amount of working solution to accommodate all standards and samples were prepared.
3. Standards were prepared by mixing 190 µl of the working solution with 10 µl of each Qubit™ standard. The solutions were vortexed carefully, for 2-3 seconds, while avoiding formation of air bubbles.
4. Samples were prepared by mixing 180-199 µl (most often 199 µl) working solution with 1-20 µl (most often 1 µl) DNA sample. The solutions were vortexed carefully for 2-3 seconds, while avoiding formation of air bubbles.
5. All tubes were incubated at room temperature for 2 minutes.
6. The tubes were read in Qubit™ fluorometer by selecting dsDNA BR assay type (the staining signal was stable for 3 hours).

3.7 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a method for amplifying particular fragments of DNA *in vitro*. To amplify the desired DNA sequence, specific oligonucleotides (primers), a DNA polymerase and deoxynucleotides (dNTPs) are mixed. The DNA is first denatured at a high temperature, and then each strand of the target DNA will serve as a template for DNA synthesis. When lowering the temperature the primers will anneal to their target sequence in the template DNA. The DNA polymerase extends the two primers with available dNTPs. This reaction generates double-stranded DNA over the region of interest on both stands of DNA. Additional repeated cycles of denaturation and primer-directed DNA synthesis lead to exponential amplification of the region between the two primers. Several DNA polymerases are used, depending of the purpose of the PCR reaction.

3.7.1 *Taq* DNA Polymerase

Taq DNA polymerase is purified from *E. coli* expressing a cloned *Thermus aquaticus* DNA polymerase gene. This enzyme has a 5'→ 3' DNA polymerase and a 5'→ 3' exonuclease activity but lacks a 3'→5' exonuclease activity. *Taq* DNA polymerase was used to check if a plasmid contained the desired gene after transformation.

Materials:

- Taq DNA Polymerase
- 10X PCR Buffer, Minus Mg^{++}
- 50 mM Magnesium Chloride
- dNTP-mix
- Primers (see Materials, section 2.5)

Procedure:

1. PCR reactions were carried out as suggested by the *Taq* DNA polymerase suppliers (Invitrogen). The components listed in Table 3.1 (for making of master mix) or in Table 3.2 (for use of the ready-to-use master mix) were mixed in a sterile 0.5 ml microcentrifuge tube placed on ice. When a colony was examined directly 1 µl of template DNA was replaced with bacteria from the colony. A toothpick was used to pick a colony and transfer it to the bottom of the PCR tube. The PCR tube was placed in a microwave oven for 2 minutes at maximum intensity to lyse the bacterial cells. Subsequently, reactants were added.

Table 3.1: PCR reagents in a *Taq* DNA polymerase reaction

Components	Volume	Final Concentration
Autoclaved distilled water (dH_2O)	To 100 µl	
10X PCR buffer minus Mg^{++}	10 µl	1X
10mM dNTP mixture	2 µl	0.2 mM each
50mM $MgCl_2$	3 µl	1.5 mM
Primer mix (10µM each)	5 µl	0.5 µM each
Template DNA	1 µl	
Taq DNA polymerase (5U/µl)	0.2-0.5 µl	1.0-2.5 unites

Table 3.2: PCR reagents in a Red *Taq* DNA polymerase Master Mix reaction

Components	Volume	Final Concentration
Autoclaved distilled water (dH_2O)	To 50 µl	
Taq Master Mix RED	25 µl	1X
Primer mix (10µM each)	3.5 µl	0.7µM each
Template DNA	1 µl	

2. The reaction mixtures were placed in a thermal cycler and the settings shown in Table 3.3 were applied.

Table 3.3: PCR settings in a *Taq* DNA polymerase reaction

Temperature	Action	Time	Number of cycles
94ºC	Initial denaturation	3 minutes	1
94ºC	Denaturation	45 seconds	
55ºC*	Annealing	30 seconds	35
72ºC	Extension	30 seconds	
72ºC	Final extension	10 minutes	1
4ºC	Storage	Until use	

* The temperature was varied in order to be approximately 5ºC below the average melting point of the primers used.

3.7.2 Phusion High-Fidelity DNA Polymerase

Phusion DNA polymerase is a *Pyrococcus*-like enzyme with a processivity-enhancing domain. This enzyme has a 5'→ 3' DNA polymerase activity and a 3'→5' exonuclease activity. The Phusion DNA polymerase was used for PCR-amplification of the to-be-cloned DNA fragments because Phusion DNA polymerase has approximately a 50-fold lower error rate than *Taq* DNA polymerase.

Materials:

- Phusion DNA Polymerase
- 5x Phusion HF Buffer
- dNTP-mix
- Primers (see Materials, section 2.5)

Procedure:

1. PCR reactions were carried out as suggested by the Phusion DNA polymerase suppliers (Finnzymes). The components listed in Table 3.4 were added to a sterile 0.5 ml microcentrifuge tube placed on ice.

Table 4.4: PCR reagents in a Phusion DNA polymerase reaction

Components	Volume	Final Concentration
Autoclaved distilled water (dH_2O)	To 50 µl	
5X Phusion HF Buffer	10 µl	1X
10 mM dNTP mixture	1 µl	0.2 mM each
Primer mix (10 µM each)	2.5 µl	0.5 µM each
Template DNA	1 µl	
Phusion DNA polymerase	0.5 µl	0.02 U/µl

2. The reaction mixtures were placed in a thermal cycler and the settings shown in Table 3.5 were applied.

Table 3.5: PCR settings in a Phusion DNA polymerase reaction

Temperature	Action	Time	Number of cycles
98ºC	Initial denaturation	30 seconds	1
98ºC	Denaturation	10 seconds	
65ºC*	Annealing	10 seconds	25
72ºC	Extension	15-30 seconds×	
72ºC	Final extension	10 minutes	1
4ºC	Storage	Until use	

* The Tm calculator and instructions on Finnzymes website was used to determine the optimal annealing temperature.
× For low complexity DNA (e.g. plasmid) was an extension time of 15 seconds per 1kb used.

3.8 Agarose gel electrophoresis

Agarose gel electrophoresis is a much used separation method to separate DNA fragments according to their size, i.e. the number of base pairs (bp), as well as for purifying DNA fragments. The fragments are separated by an electric current, since DNA is negatively charged and migrates to the positive pole at different speed, depending on size. It is possible to visualize these fragments on the gel due to the presence of the fluorescent dye ethidium bromide (EtBr), which binds to DNA. In the present study, fragments over 1000 bp were separated on 1.2% (w/v) agarose gels and DNA-fragments shorter than 1000 bp were separated on 2-3% (w/v) agarose gels. A special type of agarose (Nusieve GTG), specifically developed for separating smaller fragments, was used for analysis of fragments below 1000 bp.

3.8.1 Agarose gel electrophoresis

Materials:

Agarose Seakem LE agarose

Agarose Nusieve GTG

TAE-buffer (Tris-acetate), 50x:	242 g Tris-base
	57.1 ml acetic acid
	100 ml 0.5 M EDTA, pH 8.0
	dH_2O up to 1 l
Ethidium bromide	10 mg/ml
Loading dye (10x)	40% (w/v) sucrose
	0.25% bromphenol blue

	dH_2O to 10 ml
DNA ladders	See Materials, section 2.4

Procedure:

1. In order to make the 1.2% gels, 0.6 gram agarose was mixed with 50 ml 1xTAE buffer and heated in a microwave until the agarose was completely dissolved.
   For higher percentage gels, two different methods were applied:
   - The agarose was mixed with 1xTAE buffer with a magnet stirrer for 10 minutes before heating.
   - The 1xTAE buffer was chilled before use. The agarose powder was slowly sprinkled into the buffer while the solution was rapidly stirred. The solution was left on the bench for 15 minutes and then heated in the microwave oven on medium power for 2 minutes. The solution was gently swirled and then reheated on high power until the solution came to a boil. The solution was kept at boiling point for 1 minute or until all of the particles were dissolved.
2. The agarose solution was cooled to below 60ºC and 0.5 µg/ml ethidium bromide (EtBr) was added. The solution was poured into a moulding tray and a well-comb was placed in the chamber, to make wells in the gel. The solution was then allowed to cool in the tray until the solution was a solid gel.
3. The solid gel was transferred to an electrophoresis chamber and the chamber was filled with 1xTAE buffer.
4. 0.1 volume of 10x loading dye was added to the DNA containing samples. After mixing, the samples were applied to the wells in the gel.
5. The gels were normally run at 90V until the fragments were sufficiently separated, but for higher percentage gels, 50V was usually applied.
6. DNA-bands were visualized by UV-light using a GelDoc machine from BioRad.

3.8.2 DNA extraction from agarose gels

Fragments from PCR reactions and restriction cutting (see sections 3.7 and 3.9.2, respectively) were run on agarose gels, and agarose pieces containing the bands with correct size were excised from the gel with a scalpel and transferred to an eppendorf tube. The gel slices were weighed and DNA was isolated using the NucleoSpin Extract II kit, according to the protocol provided by the manufacturer (Macherey-Nagel).

Materials:

NucleoSpin Extract II, Purification of nucleic acids

- Binding Buffer NT
- Wash buffer NT3
- Elution Buffer NE

Procedure:

1. For each 100 mg of agarose gel 200 µl of Buffer NT was added to the gel piece. For a gel containing more than 2% agarose, the volume of Buffer NT was doubled. The maximum amount of gel slice per NucleoSpin Extract II column was 400 mg for normal gels and 200 mg of high percentage gels (more than 2%).
2. The sample was incubated for 5-10 minutes at 50ºC and the tube was vortexed every 2-3 minutes, until the gel slice was completely dissolved.
3. The sample was added to a NucleoSpin Extract II column placed in a collection tube. The tube was centrifuged for 1 min at 11 000 x g, in order to bind DNA to the column. The flow-through after centrifugation was discarded.
4. To wash the silica membrane in the column, 700 µl Buffer NT3 was added and the sample was centrifuged for 1 min at 11 000 x g; the flow-through was discarded.
5. The empty column was centrifuged for 2 minutes at 11 000 x g to remove Buffer NT3 quantitatively. The column was removed from the collection tube without any contact with the flow-through.
6. The column was placed into a clean eppendorf tube and 15-50µl Buffer NE was added. After incubation at room temperature for 1 minute the sample was centrifuged for 1 min at 11 000 x g to elute the DNA.

3.9 Construction of novel plasmids

In this study two main strategies were employed for the construction of new plasmids:

- Conventional Topo-cloning of to-be-cloned fragments (see section 3.9.1), followed by restriction enzyme digestions and ligations of appropriate fragments and vectors.
- In-Fusion cloning, PCR fragments with 15 bp ends overlapping with vector sequences were constructed for direct insertion into the desired vector.

3.9.1 TOPO-cloning and transformation

PCR products purified according to section 3.8.2 were cloned using the Zero Blunt® TOPO® PCR Cloning Kit from Invitrogen. TOPO-cloning uses topoisomerase instead of DNA ligase to insert the PCR fragment into the vector. TOPO-cloning was used because it is easier to get successful restriction digests when the PCR product is in a vector, compared to performing digests directly on the PCR products. Furthermore, the TOPO-vector can be used as storage vehicle for the PCR product.

Materials:

SOC medium

BHI agar plates and liquid with kanamycin (50 µg/ml)

Zero Blunt® TOPO® PCR Cloning Kit

PCR®-Blunt II-TOPO®

Salt solution

OneShot® Chemically Competent TOP10 *E. coli* cells

Procedure:

1. The TOPO-cloning was set up as described in Table 3.6 and the reagents were added in the order shown.

Table 3.6: Reagents in TOPO-reaction

Reagent	Volume
Fresh purified PCR product	2 µl
Salt solution	1 µl
Sterile water (dH_2O)	2 µl
pCR®-Blunt II-TOPO®	0.5 µl

2. The reaction mixture was briefly vortexed and centrifuged, and then incubated for 5 minutes at room temperature.
3. 50 µl TOP10 *E. coli* cells were transferred to Falcon tubes. After adding 2 µl of the TOPO-reaction mixture the cells were placed on ice for 30 minutes.
4. The Falcon tubes were transferred directly from ice to a 42ºC water bath for 30 seconds, to heat shock the *E. coli* cells.
5. 250 µl of room temperature SOC medium was added to the reaction.
6. The samples were incubated at 37ºC with shaking for minimum 1 hour.
7. 100 µl of the solution was spread on pre-warmed agar plates containing BHI with kanamycin (50 µg/ml) and the plates were incubated at 37ºC overnight.

8. The next day, colonies were picked with sterile toothpicks and grown in liquid BHI containing kanamycin in a shaking incubator overnight. The same colonies were checked for the desired gene with a PCR reaction with Taq DNA Polymerase and appropriate primers, described in section3.7.1. The PCR product was examined with agarose gel electrophoresis (see section 3.8).
9. The next day, the plasmids were isolated from the *E. coli* cells, but only if the PCR reaction in step 8 gave products with the correct size. The plasmids were stored at -20ºC for further processing.

3.9.2 Restriction endonuclease cutting

Restriction endonucleases are enzymes that cleave double stranded DNA molecules at sequence specific locations. Each enzyme recognizes a specific (short) sequence that directs DNA binding and cutting. If the cuts are at the same position in the two strands the resulting new chain ends are referred to as blunt ends. If the enzyme cuts the two DNA strands at different positions, usually two or four nucleotides apart, sticky (cohesive) ends will be the result. A successful reaction with a restriction enzyme depends on several factors. The different enzymes have varying optimal working-temperatures, the reaction has to be carried out in a buffer that is well-suited for the restriction enzyme, and some enzymes require BSA as an adjuvant to function in the best possible manner. When two restriction enzymes are used simultaneously, it is often required to make compromises to ensure that both enzymes have satisfactory activity in the reaction. Alternatively, such digestions may be carried out in two separate steps.

For a normal restriction enzyme cutting the following is needed:

Materials:

Restriction enzyme	Listed in Materials, section 2.3
10x enzyme buffer	Listed in Materials, section 2.3
BSA stock solution (Bovine serum albumin)	For some enzymes

Procedure:

1. An appropriate amount of DNA (depending on use) was mixed with dH_2O and the appropriate enzyme buffer (5 µl); BSA was added if it was recommended by the supplier.

2. 2.5 µl of each of the two restriction enzymes were added to the reaction and after brief mixing, the reaction was incubated at the temperature recommended by the enzyme supplier (usually 37ºC) for 3 hours.
3. After incubation, reaction products were separated and visualized on agarose gels as described in 3.8.1 and desired bands with the correct fragment size were cut out and purified as described in 3.8.2.

3.9.3 DNA ligation

DNA ligases are enzymes that catalyze the reaction which joins breaks in the sugar-phosphate backbone in double-stranded DNA. T4 DNA ligase is obtained from *E. coli* cells infected with T4 bacteriophage and it catalyzes the ATP-dependent formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA. Ligation of DNA strands was done either by setting up reactions with T4 DNA ligase (NEB) or by using the Quick Ligation™ Kit (NEB).

Materials:

10x T4 DNA ligase reaction buffer

T4 DNA ligase

Quick Ligation™ Kit (NEB)

2x Quick ligation reaction buffer

Quick T4 DNA ligase

Procedure:

For a typical ligation with T4 DNA ligase, the reagents were set up as described in Table 3.7

Table 3.7: Reagents and their amounts for ligation with T4 DNA ligase

Reagents	Amount
DNA	50ng vector + 3-fold molar excess of insert
dH_2O	to 20 µl
10x T4 DNA ligase reaction buffer	2 µl
T4 DNA ligase	1 µl

The reaction was mixed briefly and incubated at room temperature for 2 hours or at 16 º C for 2 hours or overnight. The samples were stored on ice prior to transformation

A typical ligation reaction with Quick T4 DNA ligase was set up as described in Table 3.8.

Table 3.8: Reagents and their amounts for ligation with Quick T4 DNA ligase

Reagents	Amount
DNA	50ng vector + 3-fold molar excess of insert
dH_2O	to 20 µl
2x Quick ligation reaction buffer	10 µl
Quick T4 DNA ligase	1 µl

The reaction mixture was mixed and centrifuged briefly followed by incubation at 25ºC for 5 minutes. After the incubation, the samples were stored on ice prior to transformation.

3.9.4 In-Fusion Cloning

In-Fusion Cloning is based on an In-Fusion™ enzyme which joins any two pieces of DNA that have 15 bp of identity at their ends, as shown in Figure 3.1. A typical use for this technology would be to clone PCR products into vectors, without the use of restriction enzymes, ligase or phosphatase. First in the In-Fusion method PCR primers are designed that share 15 bases of sequence overlap with the sequence at the ends of the linearized cloning vector. These primers are then used to PCR amplify the insert DNA. The resulting PCR product and the linearized vector are joined together in the In-Fusion reaction and then transformed into *E. coli*.

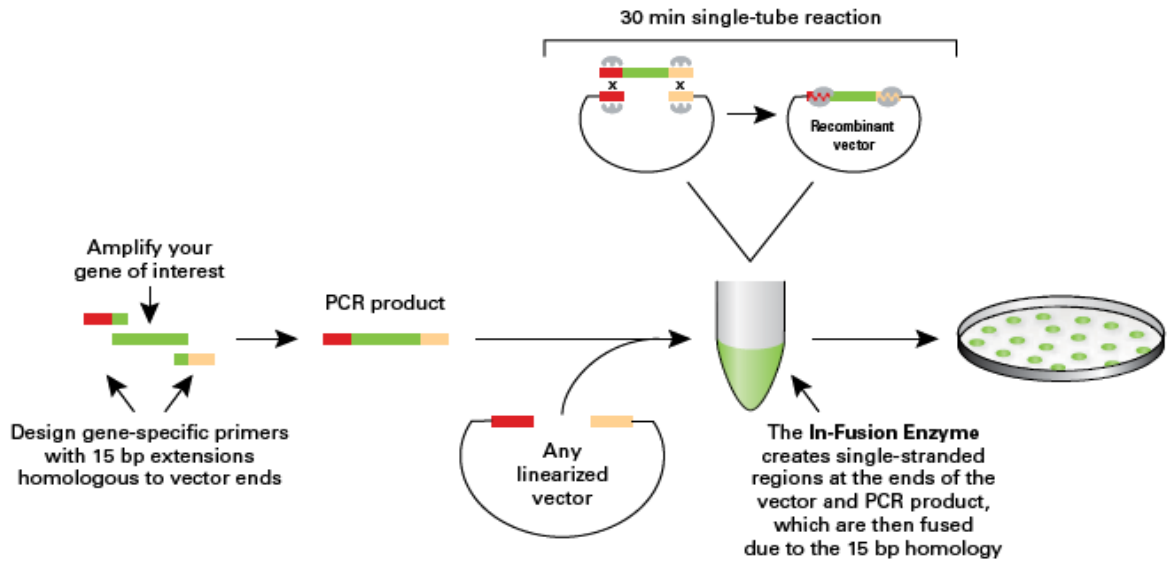


Figure 3.1: The In-Fusion cloning method. First, PCR primers are designed to share 15 bp sequence overlap with the vector, and these are used to amplify the gene of interest. The PCR product and the linearized vector are joined together in the In-Fusion reaction, and the plasmid is transformed into *E. coli*. The figure is taken from In-Fusion™ Advantage PCR Cloning Kit User Manual by Clontech.

Splicing by Overlapping Extension PCR (SOE-PCR) is a technique where two DNA fragments are fused together by the use of special primers. The primer designed to bind at the end (that later are going to be linked to the other fragment), is constructed to have an overlap of 25 bases with the end of the other fragment. Two separately PCR reactions are performed (Figure 3.2) where each of the DNA fragments is extended by a new sequence, complementary to the other fragment. Once both DNA fragments are extended in such a manner, they are mixed and a PCR is carried out using only the primers for the far ends. The overlapping complementary sequences introduced will serve as primers, fusing the two sequences.

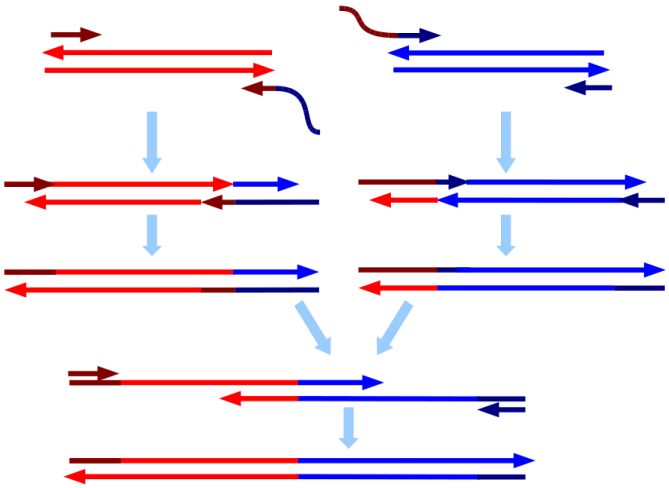


Figure 3.2: Splicing by Overlapping Extension PCR (SOE-PCR). First, two separately PCR reactions are performed where each of the DNA fragments is extended by a new sequence that is complementary to the other fragment. The two DNA fragments are mixed and a PCR is carried out using only the primers for the far ends. The overlapping complementary sequences introduced will serve as primers, fusing the two sequences.

Materials:

TE Buffer (pH 8.0): 10 mM Tris-HCl
1 mM EDTA

LB agar plate with ampicillin (100 µg/ml)

BHI agar plates and liquid with erythromycin (200 µg/ml)

Chemically Competent TOP10 *E. coli* cells (purchased or made as described in section 3.10)

NucleoSpin Extract II

In-Fusion™ Advantage PCR Cloning Kit

- In-Fusion enzyme
- 5x In-Fusion reaction buffer
- pUC19 control vector, linearized (50 ng/µl)

2 kb control insert (40 ng/µl)

Procedure:

1. A linearized vector was generated by using restriction enzymes as described in section 3.9.2
2. In-Fusion primers were designed to generate PCR products containing ends that are identical to the ends of the linearized vector. The 5' end of the primer contained 15 bases that correspond to 15 bases at one end of the vector and the 3' end of the primer contained sequence that was specific to the target gene. The 3' portion of each primer was between 18-25 bases in length and had a GC-content between 40-60% and a melting temperature (T_m) between 58-65ºC. The last five nucleotides did not contain more than two guanines (G) or cytosines (C).
3. The insert was made by PCR amplification with Phusion High-Fidelity DNA Polymerase as described in section 3.7.2. The PCR product was examined with agarose gel electrophoresis (see section 3.8.1) and it was purified by following the NucleoSpin Extract II protocol (see section 3.8.2)
4. Step 3 was repeated with an appropriate sets of primers (the primers had 25 bp of homology between the anchor and the invasin protein), to join together the two inserts (anchor and protein) in a SOE-PCR reaction.
5. The Qubit method, described in section 3.6, was used to determine the DNA concentration of the insert and vector solutions. In general, maximum cloning efficiency is achieved when a 2:1 molar ratio of insert:vector is used. Typically, 100 ng of a 4-5 kb linearized vector plus 50 ng of a 1 kb PCR product is found to work well in In-Fusion reactions. The In-Fusion® Molar Ratio Calculator (available at: http://bioinfo.clontech.com/infusion/molarRatio.do) and the Qubit results were used to calculate the amount of insert and vector for the In-Fusion reaction.
6. The In-Fusion cloning reaction was set up as described in Table 3.9

Table 3.9: Reagents and amounts in an In-Fusion reaction

Reagents	Amount
dH_2O	To 10 µl
5x In-Fusion reaction buffer	2 µl
Vector	x µl *
Purified PCR insert	x µl *
In-Fusion enzyme	1 µl

***** For reactions with larger volumes of vector and insert (>7 µl of vector + insert), the amounts of reaction buffer and enzyme were doubled and the total volume was adjusted to 20 µl.

7. The reaction was incubated for 15 minutes at 37ºC, followed by 15 minutes at 50ºC, and then the reaction was placed on ice.
8. After bringing the reaction volume up to 50 µl with TE buffer the solution were mixed thoroughly.
9. 50 µl of competent TOP10 *E. coli* cells (purchased or made as described in section 3.10) were transferred to Falcon tubes. After adding 5 µl of the diluted In-Fusion reaction to the *E. coli* cells, the mixture was placed on ice for 30 minutes.
10. The Falcon tubes were transferred directly from ice to a 42ºC water bath for 30 seconds, to heat shock the *E. coli* cells.
11. 250 µl of room temperature SOC medium was added to the reaction.
12. The samples were incubated at 37ºC with shaking for minimum 1 hour.
13. 100 µl of the solution was spread on pre-warmed agar plates containing BHI with erythromycin. 100 µl of the solution with the positive control included in the In-Fusion™ Advantage PCR Cloning kit was spread on pre-warmed agar plates containing LB with ampicillin. The plates were incubated at 37ºC overnight.
14. The next day, colonies were picked with sterile toothpicks and grown in liquid BHI containing erythromycin in a shaking incubator overnight. The same colonies were checked for the desired gene with a PCR reaction with Taq DNA Polymerase and appropriate primers, described in section 3.7.1. The PCR product was examined with agarose gel electrophoresis (see section 3.8).
15. The next day, the plasmids were isolated from the *E. coli* cells, but only if the PCR reaction in step 14 gave products with the correct size. The plasmids were stored at -20ºC for further processing.

3.10 Preparation of chemically competent *E. coli* TOP10 cells

Preparation of chemically competent *E. coli* TOP10 cells is a method to make the *E. coli* cells "ultra-competent".

Materials:

2xTY medium: 15 g tryptone
10 g yeast extract
5 g 10 mM NaCl

TOP10 *E. coli* cells

0.05 M $CaCl_2$

0.05 M $CaCl_2$ with 15% glycerol

Procedure:

1. A small amount of cells were scraped of the frozen TOP10 culture with a sterile toothpick and transferred to a tube containing 5 ml 2xTY medium. The culture was incubated at 37ºC overnight.
2. The overnight culture was poured in a flask with 200 ml 2xTY medium and the bacteria were grown at 37ºC until the OD_{600} was 0.5.
3. The culture was transferred to 50 ml centrifuge tubes.
4. The tubes were incubated on ice for 10 minutes and then centrifuged at 4000 rpm at 4ºC for 10 minutes.
5. After discarding the supernatant, the pellet was resuspended in 10 ml cold 0.05 M $CaCl_2$. The tubes were incubated on ice for 15-30 minutes followed by centrifugation at 4000 rpm at 4ºC for 10 minutes.
6. After discarding the supernatant, the pellet was resuspended in 10 ml cold 0.05 M $CaCl_2$ with 15% glycerol. The tubes were incubated on ice for 5-10 minutes.
7. 200 µl aliquots of the competent cells were distributed into 1.5 ml eppendorf tubes; the cells were then stored at -80ºC (for maximum 6 months).

3.11 Preparation of electro-competent *L. plantarum*

Preparation of electro-competent *L. plantarum* cells were required before transforming plasmids into them, and was done according to the protocol of Josson et al. (1989). *L. plantarum* cells were grown in media containing high amounts of glycine, to increase transformability. The presence of high amounts of glycine in the medium leads to the replacement of L-alanine in the cell wall with glycine, which increases cell wall permeability (Holo & Nes 1989). Cells grown in this way were washed with PEG because this has been demonstrated to increase the transformation efficiency of the electro-competent cells.

Materials:

MRS medium

20% glycine (w/v)

PEG_{1450} (polyethylen glycol)

Procedure:

1. *L. plantarum* cells were transferred to a tube with 10 ml MRS and the culture was incubated at 37ºC overnight.
2. A series of 10-fold dilutions were made in tubes with 10 ml of MRS containing 1% glycine and the dilutions were incubated at 37ºC overnight.
3. The next day, the culture with an OD_{600}= 2.5 ± 0.5 was diluted 1:20 in a tube with MRS containing 1% glycine. The culture was incubated at 37ºC until it reached the logarithmic phase and the OD_{600} was 0.7 ± 0.07. Then the culture was placed on ice for 10 minutes.
4. The culture was centrifuged at 4500 rpm for 10 minutes at 4ºC and the supernatant was discarded. The cells were resuspended in 5 ml ice-cold 30% PEG_{1450} and the suspension was transferred to a chilled corex-tube. After adding 10 ml ice-cold 30% PEG_{1450} to the suspension the tube was kept on ice for 10 minutes followed by recentrifugation and discarding of the supernatant.
5. The pellet was resuspended in 400 µl ice-cold 30% PEG_{1450}.
6. The cells were kept on ice and 40 µl portions of the suspension were transferred into sterile eppendorf tubes and the tubes were transferred immediately to dry ice. The tubes with *L. plantarum* cells were stored at -80ºC until use.

3.12 Transformation of *E. coli* and *L. plantarum*

3.12.1 Transformation of *E. coli*

E. coli cells used for transformation were either purchased competent TOP10 cells or *E. coli* TOP10 cells were made competent as described in section 3.10. Transformation of *E. coli* was performed as followed:

- 50 µl of *E. coli* cells were thawed on ice and transferred to Falcon tubes incubated on ice.
- 5 µl of the sample with the plasmid or ligation mixture was added to the *E. coli* cells in the Falcon tube, and the mixture was placed on ice for 30 minutes.
- The rest of the transformation was done according to the Invitrogen protocol described in section 3.9.1, step 4-9.

3.12.2 Transformation of *L. plantarum*

Transformation of *L. plantarum* was conducted according to the protocol by Aukrust et al. (1995).

Materials:

Electro-competent *L. plantarum* (from section 3.11)

MRSSM medium (see section 2.8)

MRS agar with erythromycin (10 μg/ml)

Procedure:

1. 40 μl electro-competent *L. plantarum* cells were thawed on ice and then 5 μl of plasmid DNA was added.
2. The mixture was immediately transferred to an ice-cold electroporation cuvette and the cuvette was gently tapped to mix and remove air bubbles from the mixture.
3. Electroporation was performed with a BLA electroporator with the following setting:
    - Tension 1.5 kV
    - Capacitance 25 μF
    - Resistance 400 Ω
4. The cuvette was placed in the electroporation handle and it was given the tension pulse.
5. After adding 950 μl ice-cold MRSSM directly to the cuvette the mixture was transferred to an eppendorf tube.
6. The eppendorf tube was incubated at 30ºC for at least 2 hours (normally 3-4 hours).
7. 100 μl of the cell suspension was spread on MRS agar plates with erythromycin and the plates were incubated at 30ºC or 37ºC overnight.

3.13 DNA sequencing

All novel plasmids that were constructed in this study were sequenced to ensure that the inserts were correct and no unwanted mutations had occurred.

Materials:

BigDye® Terminator v3.1 Cycle Sequencing Kit

125 mM EDTA

70% and 96% ethanol

Procedure:

1. The sequencing reaction was set up as shown in Table 3.10.

Table 3.10: Sequencing reaction reagents

Reagents	Amount
5x sequencing buffer	3 µl
Plasmid DNA	3 µl
Primer	3.2 pmol
Premix	2 µl
dH_2O	To 20 µl

2. The sequence reaction mixture was placed in a PCR machine and the settings shown in Table 3.11 were applied.

Table 3.11: PCR-settings in DNA sequencing

Temperature	Time	Number of cycles
96ºC	1 minute	1
95ºC 50ºC 60ºC	10 seconds 5 seconds 4 minutes	25
4ºC	Until use	

3. The reaction mixture was transferred to a sequencing eppendorf tube. 2 µl of 125 mM EDTA and 62.5 µl of 96% ethanol were added to the sample and the reaction was mixed by inverting the tube 5 times.
4. The reaction mixture was incubated at room temperature for 15 minutes followed by centrifugation at 16 100 x g for 30 minutes at 4ºC.
5. The supernatant was immediately aspirated and 60 µl 70% ethanol was added to the sample, then the tube was centrifuged at 16 100 x g for 10 minutes at 4ºC.
6. The supernatant was immediately aspirated and the tube was centrifuged in a vacuum centrifuge for 5 minutes to dry the pellet.
7. The eppendorf tube was stored at -20ºC until sequencing.

3.14 Harvesting of *L. plantarum* cells for analysis of invasin production

L. plantarum harbouring the plasmid of interest was grown at 30ºC or 37ºC and gene expression was induced with an inducing peptide (SppIP). The subcellular localization of the produced protein was subsequently analyzed.

Materials:
MRS medium (See section 2.8)

Erythromycin (10 mg/ml)

Inducing peptide SppIP (100 µg/ml)

TEN-buffer: 10 mM Tris-HCl, pH 8
1 mM EDTA, pH 8
100 mM NaCl

10x PBS: Dissolved the following in 800 ml distilled H_2O.
80 g of NaCl
2.0 g of KCl
14.4g of Na_2HPO_4
2.4 g of KH_2PO_4
Adjusted pH to 7.4 and adjusted the volume to 1 l with dH_2O

Procedure:

1. The *L. plantarum* strain harbouring the desired plasmid was grown at 30ºC or 37ºC overnight in MRS medium containing 10 μg/ml erythromycin.
2. The overnight culture was diluted in fresh MRS with 5 μg/ml erythromycin to an absorbance of OD_{600}~0.1
3. The diluted culture was incubated at 30ºC or 37ºC until the culture reached an OD_{600} in the range 0.27-0.33. The cells were then induced by adding 25 ng/ml inducing pheromone (SppIP).
4. The culture were incubated at 30ºC or 37ºC and harvested at different time points.
5. 50 ml culture was harvested in a Centrifuge 5430R (eppendorf) by centrifugation at 3000 x g for 10 minutes at 4ºC; the supernatant was discarded or stored at -20ºC for further analysis (see section 3.14.2).
6. 10 ml TEN or 1xPBS buffer (PBS buffer was only used prior to staining of cells; see section 3.18.1, 3.18.2 and 3.21) was added and the tube was recentrifuged. Afterwards the liquid was discarded.
7. The cells were then further processed or stored at -20ºC until the next day.

3.14.1 Cell disruption to analyze intracellular *L. plantarum* proteins

Materials:

TEN buffer (see section 3.14)

Glass beads (106 microns and finer)

Procedure:

1. FastPrep tubes were filled with ~1.5 grams of glass beads and the tubes were placed on ice.
2. The harvested cells were resuspended in 1 ml cold TEN buffer and the suspension was transferred to the FastPrep tubes.
3. The cells were crushed mechanically by shaking them in a FastPrep-24 tissue and cell homogenizer at speed 6.5 for 45 seconds.
4. Cell-debris and glass beads were removed by centrifugation at 13 000 rpm for 5 minutes at 4ºC.
5. The cell-free protein extract was transferred to an eppendorf tube and the tube was recentrifuged.
6. The protein extract was transferred to new sterile eppendorf tube and kept on ice prior to SDS-PAGE (described in 3.15) or stored at -20ºC.

3.14.2 TCA precipitation of proteins in supernatant

For analysis of supernatants the proteins were precipitated with trichloroacetic acid (TCA) to concentrate and denature the proteins.

Materials:

50 mM PMSF (Phenylmethylsulphonyl fluoride) dissolved in isopropanol
NaOH (6 M)
Deoxycholate (20 mg/ml)
100% (w/v) Trichloroacetic acid (TCA)
Acetone
Urea/Tiurea (7 M / 2 M)
Amidosulfobetaine-14 (ASB-14) (20%)
NuPAGE® Reducing agent (10x)
NuPAGE® LDS Sample buffer (4x)

Procedure:

1. The procedure described in section 3.14 was followed and the supernatant from step 4 was sterile filtrated (0.22 µM pore size) into a new tube. 1 mM PMSF (protease inhibitor) was added to the supernatant and the supernatant was stored at -20ºC until further processing.

2. The supernatant was thawed on ice and 4 ml of the supernatant solution was transferred to a new tube.
3. The supernatant was pH adjusted with 6 M NaOH to a pH above 7 (~8).
4. Sodium deoxycholate was added to the supernatant to a final concentration of 0.2 mg/ml, and the sample was incubated on ice for 30 minutes, to increase precipitation.
5. TCA was added to the solution to a final concentration of 16% (v/v), and the tube was incubated on ice for minimum 20 minutes.
6. 2 ml of the solution was transferred to a 2 ml tube and the tube was centrifuged at 16 100 x g for 15 minutes at 4ºC; the supernatant was carefully removed. An additional 2 ml of the solution was transferred to the same tube, the tube was recentrifuged and the supernatant was carefully removed.
7. 200 µl ice-cold acetone was added to wash the pellet. The solution was centrifuged at 16 100 x g for 5 minutes at 4ºC; the supernatant was carefully removed. This step was repeated once.
8. The supernatant was carefully removed and the pellet was dried in vacuum centrifuge for 5 minutes.
9. The pellet was dissolved in 4 M Urea, 1.14 M Tiourea, 1% ASB-14, 1x reducing agent and 1x sample buffer. The sample was boiled at 95ºC for 10 minutes, and vortexed shortly every second minute until the pellet was dissolved. At the end of this ten minutes boiling step, the sample was centrifuged for 1 minute and stored at -20ºC or used for further analysis by SDS-PAGE as described in section 3.15, and western blotting as described in section 3.16.

3.14.3 Preparation of cell wall fraction

Gram-positive bacteria have a thick cell wall composed primarily of peptidoglycan, where the glycan chains comprise units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) linked through β-1,4 linkages. For preparation of the cell wall fraction mutanolysin, an enzyme that cleaves the β-1,4 linkage between MurNAc and GlcNAc, was utilized. By carefully and partially hydrolyzing the peptidoglycan cell wall, proteins associated to the cell wall may be solublizied. This procedure was based on protocols described by Fredriksen et al. (2010) and Mujahid et al. (2007).

Materials:

TES-buffer:	1 mM EDTA (pH 8.0)
	50 mM Tris-HCl (pH 7.5)
	25% Sucrose
2x osmotic digestion buffer:	40 mM Tris-HCl
	20 mM $MgCl_2$
	40% Sucrose
	Adjusted to pH 7.0

50 mM PMSF

1 mM Pepstatin A

200 mM 1,10-phenanthroline

Mutanolysin (500 U) (Lysozyme, 40 mg/ml)

Procedure:

1. The harvested cells were washed once with 1.5 ml ice-cold TES buffer. The culture was centrifuged at 3000 x g for 5 minutes at 4ºC; the supernatant was discarded.
2. The cell pellet was resuspended in 500 µl 2x osmotic digestion buffer. 1 mM (final concentration) PMSF, 1 µM (final concentration) Pepstatin A and 10 mM (final concentration) 1,10-phenanthroline (protease inhibitors) were added to the solution.
3. 100 µl (or 50 µl) 500 U mutanolysin (or 60 U/ml mutanolysin and 15 mg/ml lysozyme if the resulting cells in step 5 are going to be stained as described in section 3.18.1) was added to the reaction and the volume was adjusted to 1 ml with 2x osmotic digestion buffer.
4. The sample was incubated at 37ºC for 2 hours (different incubations times were tested, but none gave a better result).
5. The sample was centrifuged at 14 000 x g for 5 minutes at 4ºC; the supernatant was transferred to a new eppendorf tube and recentrifuged. Alternatively, the sample was centrifuged at 3000 x g for 30 minutes, the supernatant was transferred to a new tube and the tube was centrifuged at 16 100 x g for 15 minutes at 4ºC.
6. Proteins were precipitated using the procedure described in section 3.14.2 step 3-9. Prior to step 5 (addition of TCA), dH_2O was added to a total volume of 1.5 ml, to dilute the sucrose concentration.

3.15 Gel electrophoresis of proteins by SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a separation method routinely used to separate denatured proteins according to their molecular weight. Sample buffer and reducing agent are added to the protein sample prior to the electrophoresis. The sample buffer has a slightly alkaline pH (8.4) that provides the optimal conditions for reductions of protein disulfide bonds. The proteins are denatured by the anionic detergent Lithium dodecyl sulfate (LDS), which disrupts secondary and non-disulfide-associated tertiary structure (analogous to SDS). LDS ions coat the polypeptide chain, giving it a uniform negative charge. While the reducing agent, dithiothreitol (DTT), removes disulfide-associated tertiary structure. After this treatment, every protein in the mixture is supposed to have a total charge of the protein proportional with its length. The samples are applied onto the gel and after application of power the denatured proteins will start moving towards the anode. The electrophoretic mobility of the protein molecules is only dependent on their length and short molecules will move faster than long molecules. The sizes of the proteins can hence be determined using a protein standard. The protein bands are visualized by staining them with a protein staining solution or by western blotting.

Materials:

NuPAGE® Novex® Bis-Tris Mini Gels
NuPAGE® LDS Sample buffer (4x)
NuPAGE® Reducing agent (10x)
NuPAGE® MOPS SDS Running Buffer (20x)
NuPAGE® Novex® Pre-Cast Gel cassette

Procedure:

1. A desired amount of cell-free protein extract was added to a tube and sample buffer (final concentration on 1x), reducing agent (final concentration on 1x) and dH_2O to a total volume of 10-25 µl were added.
2. The samples were heated at 70ºC (in some cases at 95ºC) for 10 minutes.
3. The plastic case and well-comb were removed from the pre-cast NuPAGE® Novex® Bis-Tris Mini Gel and the tape from the bottom of the cassette was peeled off.
4. The equipment was put together and 1x NuPAGE® MOPS SDS Running Buffer was added.

5. The Magic mark ladder and the protein samples were carefully applied to the gel wells.
6. Electrophoresis was carried out by applying 200 volts for 50 minutes.
7. The gel was removed and the plastic plates surrounding the gel were removed with a gel knife.
8. After placing the gel in a rectangular Petri dish, the gel was washed with dH_2O for minimum 5 minutes. The procedure described in 0 was then followed.

3.16 Western blotting

Western blotting is a method that utilizes antibodies to detect proteins. Electrophoretically separated proteins are made available for antibody hybridizations by transferring them from a gel to a membrane, by using an electric current. iBlot® Dry Blotting System was used to transfer the proteins to the membrane. The top and bottom stacks contain the necessary buffers and the bottom stack includes an integrated nitrocellulose or PVDF membrane (Figure 3.3). The membrane has high affinity for proteins and needs to be blocked prior to antibody hybridization to prevent interactions between the membrane and the antibody. The membrane is then incubated in a solution of an antibody that specifically recognizes the protein of interest. Subsequently, a horseradish peroxidase (HRP)-linked secondary antibody is added which will bind specifically to the primary antibody. Subsequently, a chemiluminescent substrate is added that will be converted by HRP, yielding a detectable signal. The western blot detection principle is illustrated in Figure 3.4.

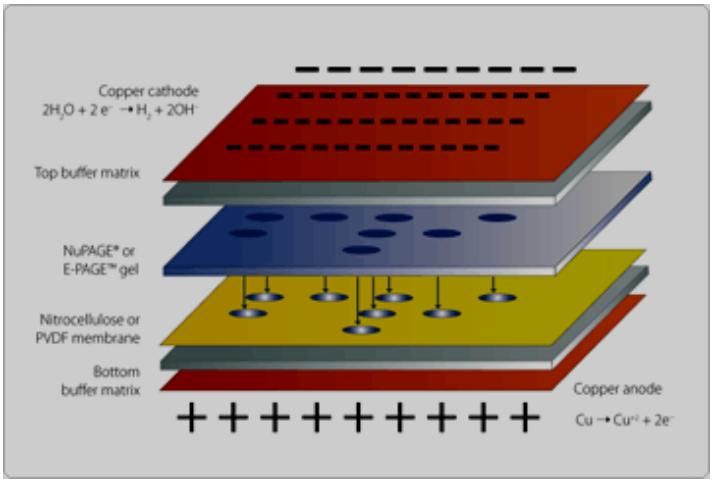


Figure 3.3: Western blotting. The primary antibody recognizes the protein of interest. The secondary antibody conjugated with HRP recognizes the primary antibody. Chemiluminescent substrat reacts with HRP and the emitted light is captured on X-ray film. The figure is taken from The Phototope®-HRP Western Blot Detection System protocol from Cell Signal Techology®.

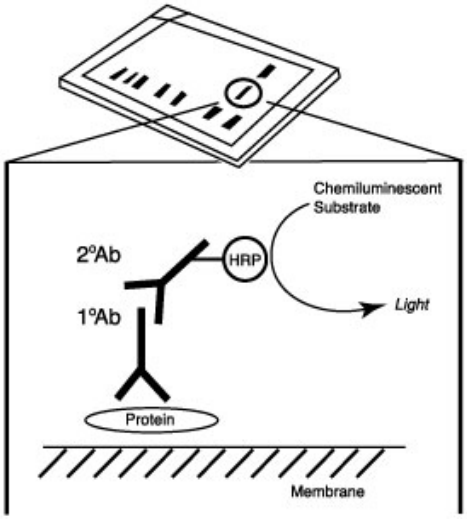


Figure 3.4: In iBlot® dry blotting. The proteins are transferred from the gel to the membrane by an electric current. The figure is taken from Invitrogen™.

Figure 3.5: The SNAP i.d.® Protein Detection System. The system is based on a vacuum-driven technology and a built-in flow distributor that actively drives reagents through the membrane. SNAP i.d. single well blot holder was used, but it exist three different sizes of blot holders. Two blot holders can be run in parallel in the system. The figure is taken from SNAP i.d.® Protein Detection System protocol by Millipore

Materials:

iBlot® Dry Blotting System

Gel Transfer Device

Cathode stack

Anode stack (with nitrocellulose membrane)

Filter paper

Disposable sponge

pAb invasin PAS Bleed #3, primary antibody (see section 2.3)

HRP-Goat Anti-Rabbit IgG (H+L), secondary antibody (see section 2.3)

Bovine Serum Albumine (BSA)

TBS buffer: 150 mM NaCl
20 mM Tris-HCl, pH 7.5

TTBS buffer: TBS buffer containing 0.1% (v/v) Tween-20

Super Signal® West Pico Chemiluminescent Substrat kit

Stable Peroxid Solution

Luminol/Enhancer Solution

Procedure:

1. The anode stack was placed in the transfer device and the SDS-PAGE gel from section 3.15 was placed on the membrane in the anode stack. A roller was used to remove air bubbles between the membrane and the gel.
2. One filter paper was soaked in dH_2O and placed on top of the gel, and a roller was used to remove air bubbles.
3. The cathode stack was aligned on top of the filter paper and a gel roller was used to ensure proper contact between all sandwich components (Figure 3.3).
4. The disposable sponge was placed in the lid of the transfer device. The lid was closed and blotting was carried out by using program 2 (on the iBlot machine from Invitrogen) for 9 minutes.
5. The cathode stack, the filter paper and the gel were removed. After transferring the membrane to a rectangular Petri dish, the membrane was washed with dH_2O for minimum 5 minutes.
6. 0.2 g BSA was dissolved in 40 ml TTBS to make the TTBS/0.5% BSA solution. 3 ml of this solution was transferred into a new tube and 5 µl pAb invasin PAS Bleed #3 primary antibody was added. Additional 3 ml of the TTBS/0.5% BSA solution was transferred into a new tube and 0.2 µl HRP-Goat Anti-Rabbit IgG (H+L) secondary antibody was added. The blocking solution containing TTBS with 1% BSA were made by solving 0.15 g BSA in 30 ml of the remaining TTBS/0.5% BSA solution.
7. The inner white face of the SNAP i.d. single well blot holder was wetted with Milli-Q water until it turned gray, and any excess liquid was removed using the roller.
8. The pre-wet blot membrane from step 5 was placed in the center of the blot holder, protein side down. The roller was used to remove any air bubbles.

9. The spacer (wetting not necessary) was placed on top of the blot. The roller was used again to ensure complete contact of blot spacer with blot membrane.
10. The blot holder was placed in the SNAP i.d. ® protein detection system chamber (Figure 3.5) with the well side up, and the blot holder tabs were aligned with notches of the chamber.
11. 30 ml of blocking solution (TTBS with 1% BSA) was added to the blot holder. The vacuum was immediately turned on using the vacuum control knob(s) (Figure 3.5), and the blocking solution was driven through the membrane.
12. The vacuum was turned off after the blot holder had emptied completely (10–20 seconds).
13. 3 ml TTBS containing 0.5% BSA and primary antibody was evenly added to the blot holder and the blot holder was left for 10 minutes at room temperature, with the vacuum off.
14. With vacuum running continuously, the blot holder was washed with 3x 10 ml of TTBS where each wash took 10 to 20 seconds to complete. When the blot holder was empty, the vacuum was turned off.
15. 3 ml TTBS containing 0.5% BSA and the secondary antibody was evenly added to the blot holder and the blot holder was left for 10 minutes at room temperature, with the vacuum off.
16. Step 14 was repeated.
17. The blot holder was removed from the SNAP i.d. system. With a forceps, the spacer was removed and discarded. After transferring the blot to a Petri dish 5 ml Stable Peroxid Solution and 5 ml Luminol/Enhancer Solution were added to the membrane followed by incubation on the bench for 5 minutes.
18. The membrane was placed with the protein side down on a plastic foil piece and the foiled was folded around the membrane. The membrane was turned so the protein side was up and the membrane was placed in a film cassette. The rest of the procedure was carried out in a dark room.
19. Kodak film was placed on the membrane in the film cassette. The cassette was shut and the film was exposed for some seconds.
20. The film was transferred to a vessel containing developer solution and incubated until the bands were visible.

21. The film was transferred to a vessel containing fixation solution and incubated for 2 minutes.
22. Finally, the film was washed for 1 minute in a vessel containing water and subsequently air-dried.

3.17 Cell dot-blot

A cell dot-blot is an antibody-detection method similar to western blotting, and was used in order to detect surface exposed proteins. Intact cells are applied to a membrane and the membrane is incubated with primary and secondary antibodies, after which the protein of interest is identified using chemiluminescent detection.

Materials:

Immun-Blot™ PVDF or Trans-Blot Transfer Medium Pure nitrocellulose membrane (0.20 μm pore size)
TES buffer or Ringers solution
TBS buffer
TBS-T buffer: TBS buffer containing 0.05% (v/v) Tween-20
BSA/TBS-T: 0.1% BSA in TBS-T
BSA
pAb invasin PAS Bleed #3, primary antibody (see section 2.3)
HRP-Goat Anti-Rabbit IgG (H+L), secondary antibody (see section 2.3)

Procedure:

1. The bacterial cells were harvested (from 50 ml culture) as described in section 3.14 and the resulting pellet was suspended in 1 ml TES or Ringers solution.
2. Pellet harvested at OD_{600}=0.6 was resuspended in 50 μl of TES buffer or Ringers solution and pellets harvested at higher OD_{600}-values were resuspended correspondingly increased in solution volumes (e.g. sample harvested at OD_{600}=1.2 would be resuspended in 100 μl solution. This was necessary in order to compare protein amounts in the samples.
3. 5-fold and 25-fold dilutions of each sample were made.
4. The PVDF membrane was soaked in methanol and the nitrocellulose membrane was soaked in dH_2O. The membrane was dried by evaporation.

5. 2 µl of each cell suspension was pipetted onto the membrane. It was important to do this immediately after the methanol/dH_2O evaporated from the membrane in order to ensure proper cell binding.
6. After air drying of the membrane, the membrane was transferred to a vessel with a blocking solution, 50 ml TBS containing 3% BSA, for 50 minutes. After removal of the blocking solution 10 ml TBS containing 3% BSA and 10 µl primary antibody (pAb invasin PAS Bleed #3) was added and the membrane was incubated in the solution for 50 minutes (method 1). Alternatively, the membrane was soaked in 50 ml 5% BSA in TBS-T for 1 hour. After removal of the blocking solution 10 ml BSA/TBS-T containing 10 µl primary antibody (pAb invasin PAS Bleed #3) was added and the membrane was incubated in the solution for 30 minutes (method 2).
7. The membrane was washed with TTBS for 2 x 10 minutes and TBS for 10 minutes. Alternatively, the membrane was washed three times with TBS-T for 3 x 5 minutes. Alternatively, this washing step was omitted (this gave best results).
8. After removing of the washing solution 10 ml TBS containing 3% BSA and 0.5 µl secondary antibody (HRP-Goat Anti-Rabbit IgG (H+L)) was added and the membrane was incubated in the solution for 50 minutes (method 1). Alternatively, 10 ml BSA/TBS-T containing 0.5 µl secondary antibody (HRP-Goat Anti-Rabbit IgG (H+L)) was added and the membrane was incubated in the solution for 30 minutes (method 2).
9. After removing of the solution the membrane was washed in TTBS for 4 x 10 minutes (method 1). Alternatively, the membrane was washed three times with TBS-T, 1 x 15 minutes and 2 x 5 minutes, and then once with TBS for 5 minutes (method 2).
10. 5 ml Stable Peroxid Solution and 5 ml Luminol/Enhancer Solution were added to the membrane followed by incubation on the bench for 5 minutes. The procedure described in section 3.16, step 20-24 was followed.

3.18 Immunofluorescence techniques

In immunofluorescence techniques in general, antibodies are chemically conjugated to fluorescent dyes such as fluorescein isothiocyanate (FITC). These labeled antibodies will directly or indirectly bind to the antigen of interest. The fluorescence signal can then be quantified using a flow cytometer or visualized using fluorescence microscopy. In direct immunofluorescence, the fluorescent dye is conjugated to the primary antibody, which gives

the advantage of shorter sample staining time, but the disadvantage of a weaker signal. In indirect immunofluorescence, the fluorescent dye is conjugated to the secondary antibody, which gives the advantage of greater sensitivity, but increases the risk of background signals. An overview of these procedures is given in Figure 3.6.

Flow cytometry may be employed for identification of microorganisms. The flow cytometer forces a suspension of cells through a laser beam and measures the light they scatter or the fluorescence the stained cells emit as they pass through the beam. Each fluorescent cell should ideally be detected, counted and even separated from other cells in a suspension. The cytometer can also measure a cell's shape, size, and content of DNA and RNA.

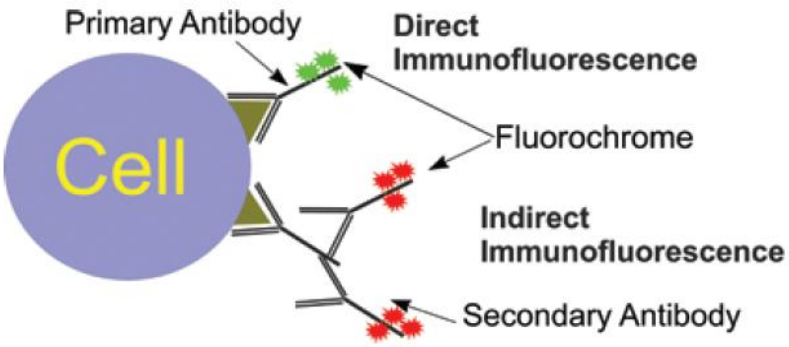


Figure 3.6: Overview over direct and indirect immunofluorescence techniques. In direct immunofluorescence the fluorescent dye is conjugated to the primary antibody, while in indirect immunofluorescence the fluorescent dye is conjugated to the secondary antibody. The picture is taken from Connection Molecular Pathology Protocol by Dako.

3.18.1 Primary antibody conjugated with FITC

The primary antibody that binds epitopes on invasin was conjugated with FITC. Before the conjugation with FITC it was necessary to transfer the primary antibody to PBS buffer by dialysis.

Materials:

PBS buffer (see section 3.14)

ShakeSkin Pleated Dialysis Tubing

pAb invasin PAS Bleed #3 (primary antibody) (see section 2.3)

Pierce® FITC Antibody Labeling Kit

- FITC (fluorescein isothiocyanate)
- 0.67 M Borate Buffer
- Purification resin

Amicon® Ultra-05 Centrifugal Filter Devices

Procedure:

1. A piece of about 10 cm of ShakeSkin Pleated Dialysis Tubing was soaked in 5 liter of cold PBS buffer. The superfluous buffer was pressed out and a clip was put on in one end. 10 ml of primary antibody was added and a clip was put on in the outer end.
2. The piece was attached to a magnet stirrer followed by stirring in the buffer in a cold room (4ºC).
3. The buffer was chanced with fresh PBS (5 liters) after 4 and 8 hours followed by overnight stirring.
4. The antibody solution from the ShakeSkin tube was transferred into a 15 ml tube and stored at -20ºC.
5. The concentration of primary antibody in PBS was measured as described in section 3.19
6. All the reagents in the Pierce® FITC Antibody Labeling Kit were brought to room temperature.
7. 40 µl of the 0.67 M Borate buffer was added to 0.5 ml of ~2 mg/ml primary antibody in PBS. If the protein concentration was >2 mg/ml, the concentration was adjusted to 2 mg/ml with PBS.
8. 0.5 ml of the protein solution from step 7 was added to a vial of FITC Reagent and the reagents were mixed by pipetting up and down 10 times until all the dye was dissolved. Brief vortexing steps were used if required.
9. The vial was briefly centrifuged to collect the sample in the bottom of the tube.
10. The reaction mixture was incubated for 60 minutes at room temperature, protected from light.
11. Two spin columns were placed in separate collection tubes.
12. The Purification Resin was mixed to ensure uniform suspension and 400 µl of the suspension was added into both spin columns. The samples were centrifuged for 30-45 seconds at ~1000 x g to remove the storage solution. The used collection tubes were discarded and the columns were placed in new collection tubes.
13. 250-270 µl of the labeling reaction was added to each spin column and the sample mixed by pipetting up and down or briefly vortexing.
14. The columns were centrifuged for 30-45 seconds at ~1000 x g to collect the purified proteins.

15. The labeled protein was stored protected from light at 4ºC for up to one month. Alternatively, labeled protein was stored in single-use aliquots at -20ºC (meaning that repeated freeze/thaw cycles were avoided).
16. In order to concentrate the primary antibody conjugated with FITC, a Amicon® Ultra-05 Centrifugal Filter Device was placed in a collection tube, and 100 µl of primary antibody conjugated with FITC was added to the column.
17. The sample was then centrifuged at 14 000 x g for 30 minutes.
18. The column was turned upside down in a new collection tube and centrifuged at 1000 x g for 2 minutes.
19. The concentrated primary antibody conjugated with FITC was stored at -20ºC.

3.18.2 Staining of cells with primary antibody conjugated with FITC

Materials:

pAb invasin PAS Bleed #3 (primary antibody) conjugated with FITC (see section 3.18.1)
PBS buffer (see section 3.14)

Procedure:

1. The cells were harvested (from 50 ml culture) as described in section 3.14.
2. The cell pellet (from 50 ml culture) was resuspended in 1 ml PBS
3. Different amounts of resuspended cells, PBS and primary antibody conjugated with FITC were tested.
4. The tubes were incubated protected from light at room temperature or at 37ºC for 30-60 minutes followed by centrifugation at 3000 x g for 5 minutes; the supernatant was discarded.
5. The cells were washed in 1 ml PBS and recentrifuged, and the supernatant was discarded. This step was repeated 1-3 times.
6. The pellet was resuspended in PBS and the samples were analyzed using a fluorescence microscope.

3.18.3 Staining of cells with secondary antibody conjugated with FITC

Materials:

pAb invasin PAS Bleed #3 (primary antibody) (see section 2.3)

Anti-Rabbit IgG (whole molecule)-FITC (secondary antibody) (see section 2.3)
PBS buffer (see section 3.14)
BSA

Procedure:

1. The cells were harvested (from 50 ml culture) as described in section 3.14.
2. The cell pellet (from 50 ml culture) was resuspended in 1 ml PBS. Different amounts of cell suspension were transferred to an eppendorf tube (different amounts of cells were tried stained)
3. The cells were washed with 1 ml PBS, the sample was centrifuged at 5000 x g for 2 minutes, and the supernatant was discarded.
4. 40 µl *L. plantarum* harbouring empty vector, 100 µl PBS with 2% BSA and 40 µl pAb invasin PAS Bleed #3 were mixed. The solution was incubated at 4ºC for 20 minutes followed by centrifugation at 16 100 x g for 5 minutes. The supernatant was used as the primary antibody solution.
5. The cells from step 3 were resuspended in 50 µl of PBS containing 2% BSA. 10 µl of the supernatant from step 4 was added and the reaction was incubated at 4ºC for 15-30 minutes.
6. The cells were washed with 1 ml PBS containing 2% BSA four times. The cells were collected by centrifugation at 5000 x g for 2 minutes between every wash.
7. The cells were resuspended in 50 µl of PBS containing 2% BSA. 0.2 µl of Anti-Rabbit IgG (whole molecule)- FITC was added and the reaction was incubated at 4ºC for 15-20 minutes.
8. Step 6 was repeated.
9. The cells were finally resuspended in 50 µl PBS containing 2% BSA. The cells were analyzed using a fluorescence microscope or flow cytometry.

3.19 Protein concentration measurement

Total protein concentration (mg/ml) in cell-free protein extracts can be measured using BioRad's protein assay reagent, which is based on Bradford's method. The method involves the addition of an acidic dye to the protein solution and then the sample is measured at 595nm in a spectrophotometer. By comparing the samples to a standard curve, relative measurements of protein concentrations can be made.

Materials:

BioRad Protein Assay, Dye Reagent Concentrate

Procedure:

1 Different dilutions of the sample were prepared, to find a dilution that was inside the standard curve (1.2 µg/ml - 15 µg/ml). The standard curve was made using different dilutions of BSA.
2 1 µl of cell-free protein was added to 800 µl PBS. Three parallels of each sample were made.
3 200 µl BioRad Protein Assay was added to the samples and the samples were vortexed; the samples were incubated at room temperature for 5 minutes. It was important to make sure that all of the samples stood in this solution for approximately the same time, because the signal is rather unstable and absorbance will increase over time.
4 1 ml PBS was added into a clean, dry test tube, and the absorbance at 595 nm was measured. This was repeated for the protein samples, where parallels had to be measured right after each other.

3.20 Relation between OD and CFU

Materials:

Ringers solution

MRS plates with erythromycin (10 µg/ml)

Procedure:

1. Cells were harvested (from 50 ml culture) as described in section 3.14.
2. A series of dilutions were made, starting by transferring 100 µl of the culture, to 10 ml of Ringers solution. The tube was vortexed for about 15 seconds and 1 ml from that tube was transferred to 9 ml of Ringers solution. Then this next tube was vortexed and 1 ml was transferred to yet another 9 ml tube, and this was repeated for as many dilutions as necessary.
3. 100 µl of the dilution was spread on a MRS plate, with minimum two replicates.
4. After incubation at 37ºC overnight, the colonies on the plates were counted and the CFU/ml was calculated.

3.21 Staining of bacteria with FITC or CFSE

To visualize bacterial cells after incubation with Caco-2 cells (to analyze internalization) in the microscope, the bacterial cells were strained with FITC or CFSE. FITC contains an isothiocyanate moiety and this moiety is very reactive with aliphatic amine groups. The result from this reaction is covalent attachment of FITC to cellular proteins (Parish 1999). Carboxyfluorescein diacetate, succinimidyl ester (CFDASE) is a highly membrane penetrating non-fluorescent molecule that is taken up by bacterial cells. Inside the cell esterases can remove the two acetate groups from CFDASE to yield fluorescent carboxyfluorescein succinimidyl ester (CFSE). CFSE is highly reactive with amino groups and can covalently couple carboxyfluorescein (CF) to intracellular molecules (Parish 1999). FITC will react with molecules on the surface (including invasin), but CFSE will only react with molecules inside the bacterial cell.

Materials:

FITC

CFSE

PBS buffer (see section 3.14)

Procedure:

1. The cells were harvested as described in section 3.14.
2. The cell pellet (from 50 ml of culture) was resuspended in 1 ml PBS. Different amounts of cell suspension were transferred to an eppendorf tube (different amounts of cells were stained).
3. The cells were centrifuged at 5000 x g for 1-2 minute; the supernatant was discarded.
4. The cells were resuspended in 400 µl of PBS containing 0.02 mg/ml FITC. Alternatively, the cells were resuspended in 400 µl of PBS containing 10 µM CFSE.
5. The sample was incubated in the dark for 40 minutes in room temperature for FITC staining and in the dark at 37ºC and constant shaking for 20 minutes for CFSE staining. After incubation, the sample was centrifuged at 5000 x g for 1-2 minute.
6. The cells were washed by adding 1 ml PBS followed by centrifugation at 5000 x g for 1-2 minute; the supernatant was discarded. This was repeated minimum 2 times.
7. The pellet was resuspended in a desired volume of PBS.

3.22 Incubation of bacteria with Caco-2 cells to visualized the cells with CLSM

Bacterial internalization was analyzed using CLSM (Confocal Laser Scanning Microscopy) and the procedure was based on protocols described in Agerer et al. (2004) and Innocentin et. al (2009).

Materials:

PBS buffer (see section 3.14)
RPMI 1640 medium with L-glutamine and 10% fetal calf serum (FCS)
CFSE (FITC)
250 mM Biotin
TRITC or Hoechst stain
MRS plates with erythromycin (10 μg/ml)

Procedure:

1. The cells were harvested as described in section 3.14.
2. The cell pellet (from 50 ml of culture) was resuspended in 1 ml PBS. Different amounts of cell suspension were transferred to an eppendorf tube (different amounts of bacteria were analyzed).
3. The bacteria were stained with CFSE (or with FITC) as described in 3.21.
4. An additional step was performed when the cells were TRITC stained; the cell pellet was resolved in 500 μl PBS with 7.5 mM Biotin and the reaction was incubated at room temperature for 30 minutes. The pellet was washed 5 times with 1 ml PBS, and between every wash the solution was centrifuged at 5000 x g for 2 minutes.
5. After staining the bacteria the bacterial pellet was resuspended in 1.1 ml RPMI. 500 μl of the cell suspension was added to each sample with Caco-2 cells, in duplo.
6. The bacteria suspension was incubated with Caco-2 cells for 1 or 1½ hour at 37ºC. Subsequently, 400 μg/ml gentamicin was added to the sample and the samples were incubated under the same conditions for an additional hour.
7. The reaction was washed and fixated on microscope slides (performed by Charlotte Kleiveland).
8. The Caco-2 cells were stained blue using Hoechst stain (done by Charlotte Kleiveland). This step was not done if the cells were TRITC stained
9. The Biotin-labeled bacteria were stained with TRITC (done by Charlotte Kleiveland).
10. The sample was analyzed with CLSM by Charlotte Kleiveland and Lene Olsen

3.23 The gentamicin survival assay

Bacterial invasiveness was also analyzed using the gentamicin survival assay. The procedure was based on the protocol described in Innocentin et al. (2009). In this assay the bacteria were incubated with Caco-2 cells, and then gentamicin was added. In theory only bacteria that have been internalized will survive the gentamicin treatment.

Materials:

PBS buffer (see section 3.14)

RPMI 1640 medium with L-glutamine and 10% fetal calf serum (FCS)

Gentamicin

Triton

MRS plates with erythromycin (10 μg/ml)

Procedure:

1. The cells were harvested as described in section 3.14.

11. The cell pellet (from 50 ml of culture) was resuspended in 1 ml PBS. Different amounts of cell suspension were transferred to an eppendorf tube (different amounts of bacteria were analyzed).

2. The sample was centrifuged at 5000 x g for 2 minutes and the supernatant was discarded.
3. The pellet was resuspended in 1.5 ml RPMI.
4. 1.4 ml of the bacterial suspension was added to each sample with Caco-2 cells. The last 100 μl of the bacterial suspension was used to spread out on agar-plates to find the amount of viable cells in the suspension just prior to incubation.
5. The cells were incubated at 37ºC for 3 hours.
6. The solution above the Caco-2 cells was removed and the cells were washed by adding 2 ml of PBS, three times.
7. 2 ml RPMI containing 400 μg/ml gentamicin was added, and the reaction was incubated overnight (ca. 15 hours)
8. The solution above the Caco-2 cells was removed, and the cells were washed by adding 2 ml of PBS, three times.
9. 300 μl PBS containing 0.1% triton was added to lyse the Caco-2 cells.

10. The presence of bacteria in the lysates from the Caco-2 cells were quantified by plating out all of the solution in every well on MRS plates, 100 µl per plate, 3 plates in total. The numbers of bacteria were expressed as colony forming units (CFU).

4. RESULTS

Construction of invasin expression vectors

The main goal in this study was to express, secrete and anchor the invasin protein from *Y. pseudotuberculosis* in *L. plantarum* WCFS1 using the SIP-system (see introduction) (Mathiesen et al. 2008; Sørvig et al. 2003). The length of invasin that would give the best result in *L. plantarum* was not known, therefore two versions of invasin consisting of all 5 C-terminal domains (Inv) (see Figure 1.8) or the 2 C-terminally domains (InvS), were selected. It was also uncertain which anchor length would expose the invasin protein in the best position for binding to β_1-integin receptors. Two different lipo-anchors with variable lengths were selected. Initially, much time was spent on the use of traditional cloning strategies for vector construction. Because these traditional strategies were not successful for construction of invasin expressing vectors, focus was shifted towards using In-fusion cloning technologies, which lead to successful construction of the various expression vectors.

Table 4.1 shows a list of the key expression vectors constructed in this study and their key properties. Further details about their construction are provided below, whereas technical details are provided in Table 2.4 and in the materials and method sections.

Table 4.1: The constructed expression vectors and their key properties

Plasmid	Properties
pLp_1261InvS	The anchor sequence from the lipoprotein 1261 (originally from *L. plantarum* WCFS1) and the D4 and D5 domains from invasin (originally from *Y. pseudotuberculosis*)
pLp_1261Inv	The anchor sequence from the lipoprotein 1261 (originally from *L. plantarum* WCFS1) and the D1-D5 domains from invasin (originally from *Y. pseudotuberculosis*)
pLp_1452InvS	The anchor sequence from the lipoprotein 1452 (originally from *L. plantarum* WCFS1) and the D4 and D5 domains from invasin (originally from *Y. pseudotuberculosis*)
pLp_1452Inv	The anchor sequence from the lipoprotein 1452 (originally from *L. plantarum* WCFS1) and the D1-D5 domains from invasin (originally from *Y. pseudotuberculosis*)
pCytInv	This plasmid express the longer version of invasin (all five domains; D1-D5) without any signals for secretion and anchoring

4.1 Construction of plasmid for intracellular invasin production

First, a plasmid for intracellular production of invasin (pCytInv; Table 2.4 and 4.1) was constructed. This construct was used as a control in various settings, e.g. to check for cell lysis and in functional studies (where *L. plantarum* harbouring an intracellular version of invasin was compared to *L. plantarum* harbouring the constructs encoding lipo-anchored invasin).

The *inv* gene was PCR amplified with the primers HRCytInvF and HRInvR (see Table 2.1 and 2.2) using pTinvasin (see Table 2.4) as template. The PCR amplified invasin gene was cloned into an NdeI and EcoRI digested p2588sAmy vector (Table 2.4) (Mathiesen et al. 2008) using the In-Fusion kit, yielding pCytInv (see section 3.9.4 for technical details). The pCytInv vector was transformed into *E. coli* cells prior to transformation into electro-competent *L. plantarum* WCFS1 cells, like all plasmids constructed in this thesis. In this pCytInv vector the longer version of the invasin (all five domains) is expressed by the inducible P_{sppA} promoter, and the protein is produced without any signals for secretion and anchoring.

4.2 Selection of *L. plantarum* WCFS1 lipoproteins to use as lipo-anchor

In the genome of *L. plantarum* WCFS1, 48 genes are predicted to encode lipoproteins (Boekhorst et al. 2006). Before selecting lipo-anchors, all of the predicted lipoproteins (Boekhorst 2006) were tested using LipoP 1.0 server prediction. LipoP 1.0 is a web-based program that predicts lipoproteins and their signal peptide cleavage sites (Juncker et al. 2003). Two different variants of *L. plantarum* lipo-anchors, with different lengths were selected to be studied further, namely the lipo-anchors from genes *Lp_1261* and *Lp_1452*. Lp_1261 is predicted to be an ABC transporter and Lp_1452 is predicted to be a peptidylprolyl isomerase. Both were predicted by LipoP 1.0 to be cleaved by signal peptidase II with a high score (~25). Both Lp_1261 and Lp_1452 lipoproteins have been found on the surface of *L. plantarum* using proteiomics tool based on surface "shaving" (Unpublished data, Lasse Fredriksen). Surface "shaving" is a method where the surface of intact bacteria is treated with trypsin and the released peptides are identified with tandem mass spectrometry (MS/MS).

Primers to PCR amplify the lipo-anchors were designed to amplify the N-terminal regions of the two lipoproteins, including the signal peptide, the lipobox and the N-terminal end of the

following protein up to the start of the predicted enzymatic domains. The enzymatic domains were found in the overview of predicted lipoproteins in *L. plantarum* WCFS1 (Boekhorst 2006). The enzymatic domain in Lp_1261 was predicted to begin at amino acid 76, therefore the 75 first amino acids from Lp_1261 were used as lipo-anchor (Figure 4.1). The enzymatic domain in Lp_1452 was predicted to begin at amino acid 143, therefore the 142 N-terminally amino acids were used as the anchor sequence (Figure 4.2). The length of the two lipo-anchors differed substantially; the Lp_1452 anchor was considerable longer than the Lp_1261 anchor.

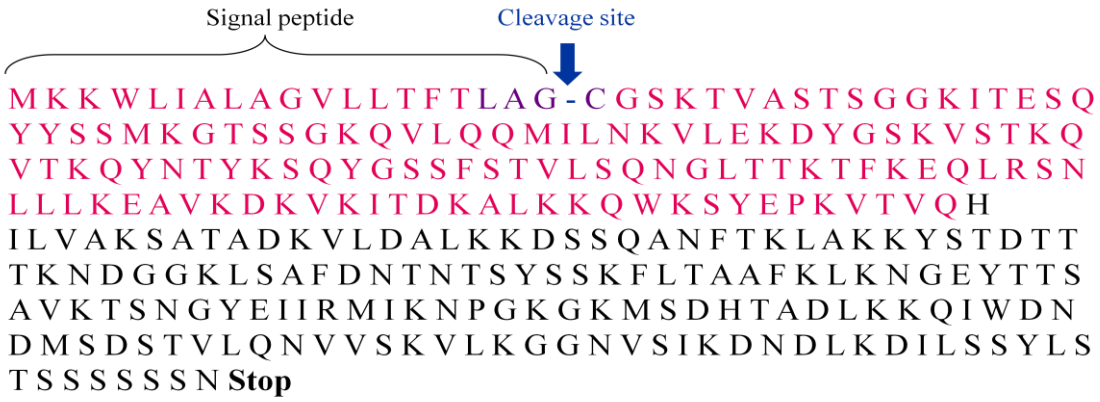


Figure 4.1: The amino acid sequence of Lp_1261. The enzymatic domain was predicted to start at residue no. 76; therefore the 75 N-terminally amino acids (marked in pink) were used as the lipo-anchor. The lipobox is marked in purple.

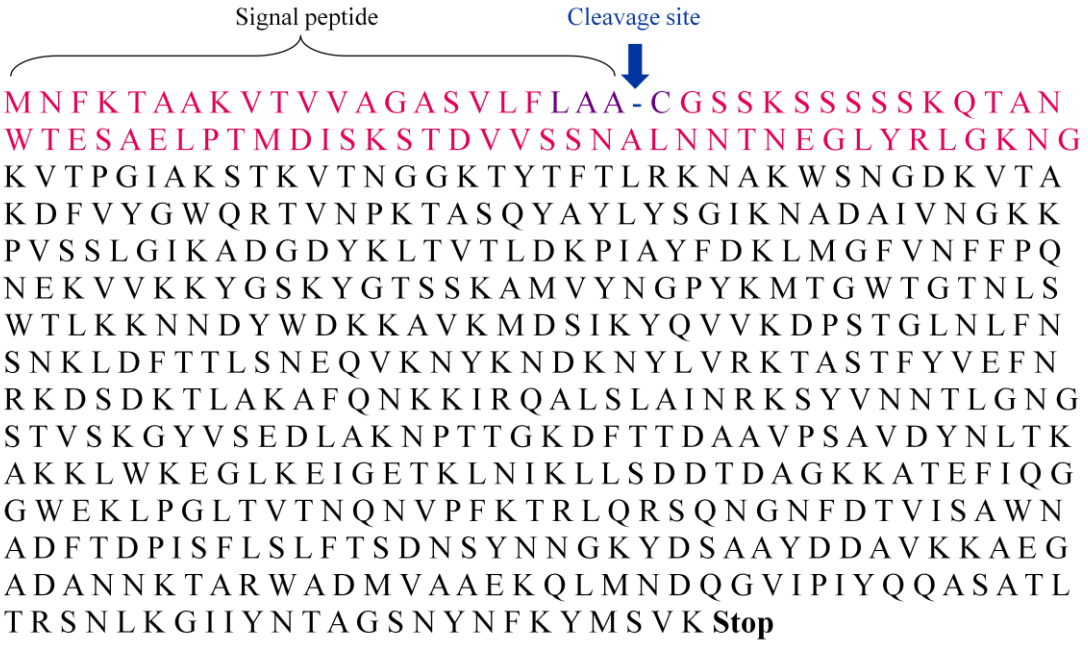


Figure 4.2: The amino acid sequence of Lp_1452. The enzymatic domain was predicted to begin at residue no. 143; therefore the 142 N-terminally amino acids (marked in pink) were used as the lipo-anchor. The lipobox is marked in purple.

4.3 Constructs for expressing invasin with lipo-anchors

In order to construct plasmids that express invasin with lipo-anchor, an expression cassette that includes the lipo-anchor and the invasin sequence was created (see Figure 4.3). A short linker sequence was inserted between the anchor and invasin in order to reduce the risk of invasin misfolding. The linker sequence selected was copied from the original invasin sequence, based on the fact that this sequence actually is a linker in the invasion protein structure (Hamburger et al. 1999). The linker with the DNA sequence: GGT ACT ATC GCG GCG, encodes the amino acid sequence GTIAA. In order to make sure that the primers with the included linker sequence did not bind to the natural linker sequence in invasin, it was made some silent mutations in the linker sequence. The DNA sequence used for GTIAA linker sequence with silent mutation was: GGC ACG ATT GCG GCG where 3 bases diverged from the natural linker sequence. This linker sequence was included in the forward primers used for amplification of the invasin gene (see Table 2.1 and 2.2). Furthermore, in between this linker and the invasion a SalI site was inserted (analogous to what was done in previous studies on protein secretion in *L. plantarum* (Mathiesen et al. 2008)), to permit easy exchange of fragments.

The invasin and the anchor were linked using a splicing by overlap extension (SOE) PCR (see section 3.9.4), meaning that the reverse primers used for anchor amplification and the forward primers used for invasin amplification had complementary parts. To permit In-Fusion cloning, the forward primers used for anchor amplification and the reverse primer used for invasin amplification had ends with the same sequence as the ends of vector (see section 3.9.4 for more details). The two lipo-anchors were amplified from the *L. plantarum* WCFS1 chromosome with the primers HR1261F and 1261R (see Table 2.1 and 2.2) for the Lp_1261 anchor and HR1452F and 1452R for the Lp_1452 anchor. Invasin with the 5 domains was PCR amplified from the pTinvasin using the primers HRInvR and SOE1261InvF primer for linking invasin to the Lp_1261 anchor, and HRInvR and SOE1452InvF for linking invasin to the Lp_1452 anchor. The last two domains in the C-terminus of invasin were amplified from the same plasmid but the forward primers were replaced with SOE1261InvSF and SOE1452InvSF, respectively. All primers used in this study are listed in Table 2.1 and 2.2.

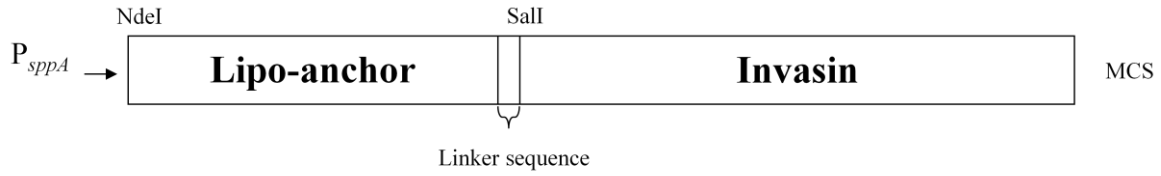


Figure 4.3: The modular lipo-anchor-linker-invasin cassette. This gene construct encodes a protein precursor consisting of a lipo-anchor fragment, the linker sequence, an additional Val-Asp linker corresponding to the SalI restriction site and the invasin protein. The length of the lipo-anchor domain determines how much of the target protein (In this case, invasin) is exposed to the environment outside the bacterial cell wall. MCS indicates a multiple cloning site (including EcoRI).

Initially four PCR products were made (two anchors and two invasin). Subsequently, overlap extension (SOE) PCR was performed creating various variants of the expression cassette shown in Figure 4.3. The Lp_1261 anchor was linked together with invasin (Inv) and the short version of invasin (InvS) with the outer primers HR1261F and HRInvR in the SOE-PCR, while the Lp_1452 anchor was linked together with Inv and InvS with the outer primers HR1452F and HRInvR. The pSIP vector p2588sAmy (Mathiesen et al. 2008) was used as a vector for all the constructs (Figure 4.4). The signal peptide-Amy insert (2588sAmy) was removed by restriction digestion with NdeI and EcoRI, and the four different PCR inserts from SOE-PCR were directly cloned into the linearized p2588sAmy vector with the In-Fusion kit (for more details see section 3.9.4), yielding pLp_1261InvS, pLp_1261Inv, pLp_1452InvS and pLp_1452Inv. As an example, the pLp_1261Inv vector is shown in Figure 4.4. All PCR amplified sequences were verified by DNA sequencing, see section 3.13 for details.

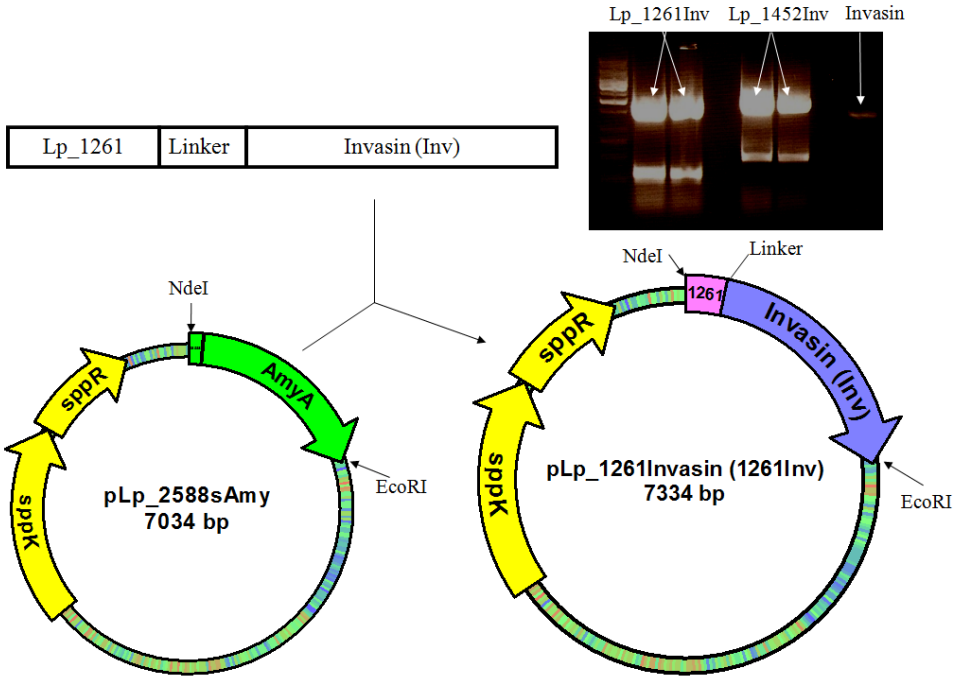


Figure 4.4: Overview of the construction of pLp_1261Inv. First, invasin (Inv) and lipo-anchor Lp_1261 were PCR-amplified with overlapping ends containing the linker sequence. Then, Lp_1261 anchor was fused with invasin in SOE-PCR. The fused PCR products are shown in the gel and the anchor-invasin PCR products was shown to be longer than invasin, indicating that the anchor is fused with invasin. Restriction enzymes (NdeI and EcoRI) were used to remove the signal peptide-Amy (2588sAmy) insert in the pLp_2588sAmy vector (basically leaving linearized pSIP401) and the lipo-anchor-linker-invasin insert was cloned into the vector using the In-Fusion technology. The other plasmids were constructed by the same principle, except pCytInv which does not have lipo-anchor and linker sequence and whose construction is described in section 4.1.

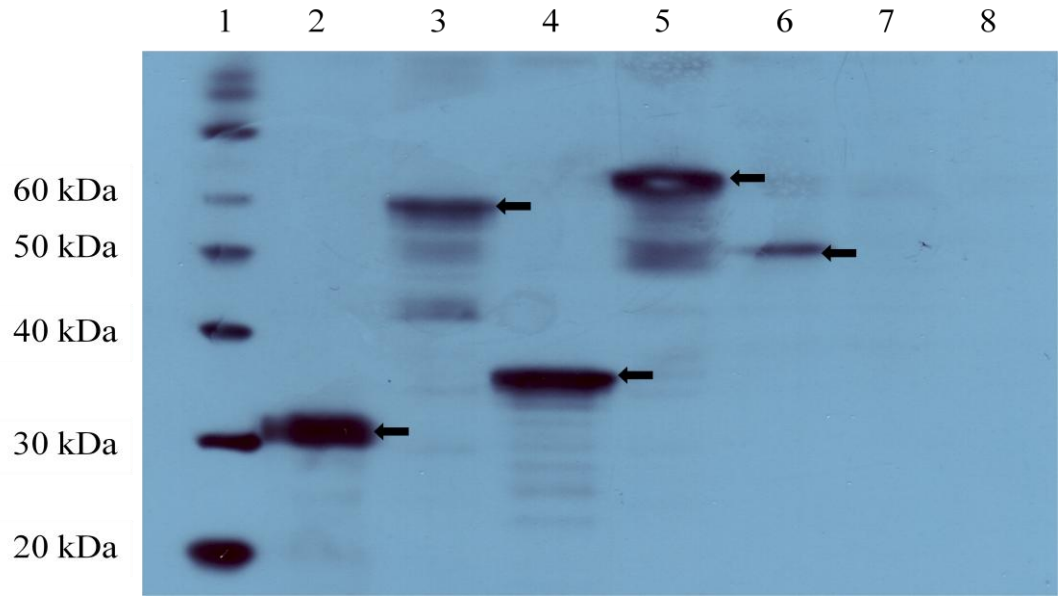
Intracellular invasin production in *L. plantarum*

4.4 Intracellular invasin production in *L. plantarum*

The vectors whose construction is described above were, without any problems, transformed to *L. plantarum* WCFS1 using the procedure described in section 3.12.2. In order to see if *L. plantarum* harbouring the different constructs actually produce invasin, intracellular proteins were analyzed by looking for the presence of invasin in cell free extracts. This is an easy and more rapid method for initial screening than detecting actual anchoring of invasin.

L. plantarum WCFS1 cells harbouring plasmids pLp_1261InvS, pLp_1261Inv, pLp_1452InvS, pLp_1452Inv, pCytInv or pEV (plasmid without any target sequence, i.e. empty vector) were grown under standard conditions (see Methods, section 3.1) and gene expression was induced by adding 25 ng/ml SppIP at OD_{600} ~0.3. The cells were grown at 30ºC and harvested four hours after induction (as described in Methods, section 3.14). The

initial harvesting time of four hours after protein induction was chosen because previous studies on protein expression with the pSIP-system in *L. plantarum* had shown good protein levels at this time point (Fredriksen et al. 2010). Cell-free protein extracts of the induced and harvested cultures of *L. plantarum* WCFS1 cells harbouring the plasmids were tested for invasin expression by SDS-PAGE and western blotting as described in section 3.15 and 3.16. Figure 4.5 shows that bands with invasin of expected size appear in all intracellular fractions from cells harbouring an invasin encoding vector. As expected no invasin was detected in extracts from cells harbouring the empty vector (pEV) nor in extracts from the wild-type bacterium (lacking plasmid). Interestingly, it seems that *L. plantarum* strains harbouring invasin with a lipo-anchor produce more intracellular invasin than *L. plantarum* harbouring the intracellular version of invasin (pCytInv).



4.4.1 Intracellular invasin production at 1 to 4 hours after induction

Figure 4.5: Intracellular production of invasin. The picture shows a western blot analysis of cell free protein extracts obtained from *L. plantarum* strains harbouring various expression vectors after growth at 30ºC for 4 hours after induction of protein production. The size marker is located in lane 1. The other lanes show extracts from *L. plantarum* strains harbouring the following plasmids (predicted molecular mass of the invasin protein in parenthesis):
2, pLp_1261InvS (~29kDa); 3, pLp_1261Inv (~60kDa); 4, pLp_1452InvS (~37kDa); 5, pLp_1452Inv (~68kDa); 6, pCytInv (~52kDa); 7, pEV (empty vector; no signal expected); 8, wild-type *L. plantarum* with no plasmid.
The arrows indicate invasin. The amount of cell-free protein extracts from each strain was adjusted according to the OD_{600}-value at the time of harvest to ensure that the cell-free protein extracts loaded onto the gel were from approximately the same amount of cells. The cell free protein extracts were added on the SDS-PAGE gel as described in 3.15 and the western blotting was performed as described in 3.16.

After the intracellular invasin production was detected 4 hours after induction, it was interesting to examine if invasin could be detected at an earlier time-point. The *L. plantarum* cells harbouring the plasmids of interest were grown at 30ºC, induced, and harvested 1 to 4 hours after induction, as described in section 3.14. Cell free protein extracts were obtained using glass beads (as described in section 3.14.1) and analyzed with SDS-PAGE and Western blot (see section 3.15 and 3.16 for details). The amount of cell-free protein extracts from each strain was adjusted according to the OD_{600}-value at the time of harvest to ensure that the cell-free protein extracts loaded onto the gel were from approximately the same amount of cells. Figure 4.6 show that invasin was detected in the intracellular fraction at all time points within 1 to 4 hours after pheromone induction for each stain carrying an invasion construct. Interestingly, Figure 4.6 demonstrates that intracellular invasin does not increase over time for the strains carrying constructs with an anchor, while the amount of invasin produced in the strain producing the intracellular version intracellular version (pCytInv) increases over time.

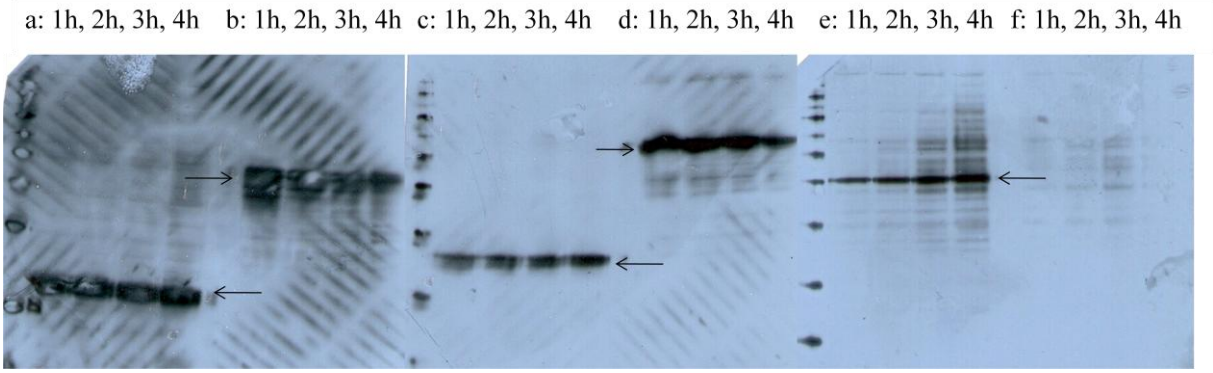


Figure 4.6: Intracellular production of invasin. The pictures shows western blot analysis of cell free protein extracts obtained from *L. plantarum* strains harbouring various expression vectors, after growth at 30ºC, 1 to 4 hours (h) after induction of protein production. Size markers are shown in the non-labeled lanes. The other lanes show extracts from *L. plantarum* strains harbouring the following plasmids: (a) pLp_1261InvS; (b) pLp_1261Inv; (c) pLp_1452InvS; (d) pLp_1452Inv; (e) pCytInv or (f) pEV (empty vector; no signal expected). The arrows indicate the invasin protein; in all cases, the size of the marked band corresponds to the expected size given in the legend of Figure 4.5. The amount of cell-free protein extracts from each strain was adjusted according to the OD_{600}-value at the time of harvest to ensure that the cell-free protein extracts loaded onto the SDS-PAGE gel (see section 3.15) were from approximately the same amount of cells. Western blot was performed as described in 3.16.

4.4.2 Intracellular invasin production at 37ºC

L. plantarum with pSIP vectors are normally grown at 30ºC (Mathiesen et al. 2008; Mathiesen et al. 2009; Sørvig et al. 2003; Sørvig et al. 2005), but because of the potential application of these constructs in a human vaccine, invasin production by the recombinant strains was evaluated at 37ºC as well. Therefore, an analogous experiment as described in

4.4.1 was performed at 37ºC to check the production level at this temperature. Figure 4.7 show that invasin production at 37ºC is a similar to the production expression at 30ºC. All *L. plantarum* constructs produced invasin at all times within 1 to 4 hours. Interestingly, also at 37ºC the amount of invasin increased over time in the intracellular version of invasin (pCytInv; Figure 4.7e). In addition, pLp_1261InvS shows some increasing of the invasin protein.

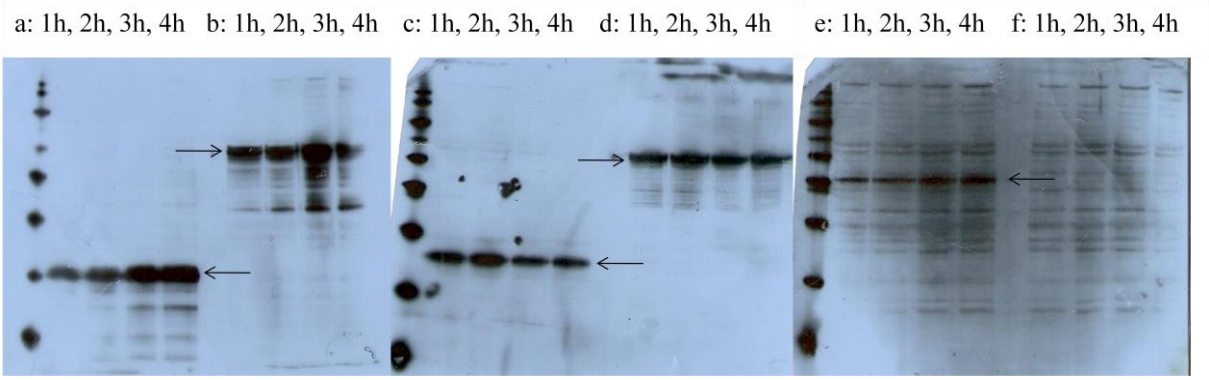


Figure 4.7: Intracellular production of invasin. The pictures shows western blot analysis of cell free protein extracts obtained from *L. plantarum* strains harbouring various expression vectors, after growth at 37ºC, 1 to 4 hours (h) after induction of protein production. Size markers are shown in the non-labeled lanes. The other lanes show extracts from *L. plantarum* strains harbouring the following plasmids: (a) pLp_1261InvS; (b) pLp_1261Inv; (c) pLp_1452InvS; (d) pLp_1452Inv; (e) pCytInv or (f) pEV (empty vector; no signal expected). The arrows indicate the invasin protein; in all cases, the size of the marked band corresponds to the expected size given in the legend of Figure 4.5. The amount of cell-free protein extracts from each strain was adjusted according to the OD_{600}-value at the time of harvest to ensure that the cell-free protein extracts loaded onto SDS-PAGE gel (see section 3.15) were from approximately the same amount of cells. Western blot was performed as described in 3.16.

4.5 Growth of *L. plantarum* harbouring different invasin constructs

In order to study the effect of invasin production on growth of the host stain, growth of induced strains harbouring various plasmids was monitored at 30ºC (Figure 4.8) and 37ºC (Figure 4.9).

L. plantarum harbouring the invasin encoding constructs grew slightly faster at 30ºC. The growth rates of the various recombinant strains showed major differences, which were similar at 30 and 37ºC. Interestingly, strains carrying the two invasin versions with the Lp_1452 anchor showed minimal growth after pheromone induction. Stains carrying the Lp_1261 anchor versions showed higher growth, especially for pLp_1261InvS. The strain carrying the intracellular version of invasin (pCytInv) grew almost at the same rate as the strain carrying

the empty vector (pEV). These differences in growth have been confirmed by repeated experiments (data not shown).

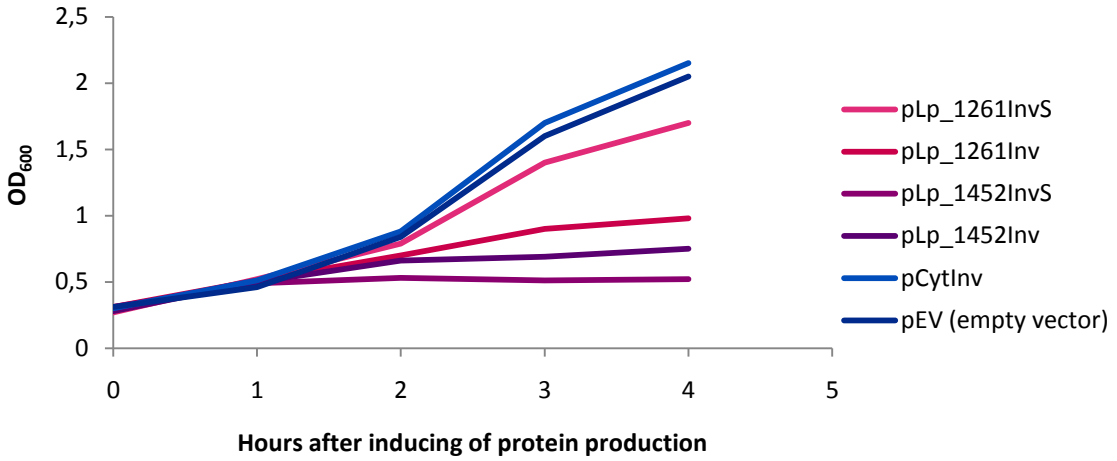


Figure 4.8: Growth of *L. plantarum* harbouring different constructs at 30°C. The cell density of the bacteria was measured by recording OD_{600} at one to four hours after protein induction of the cells.

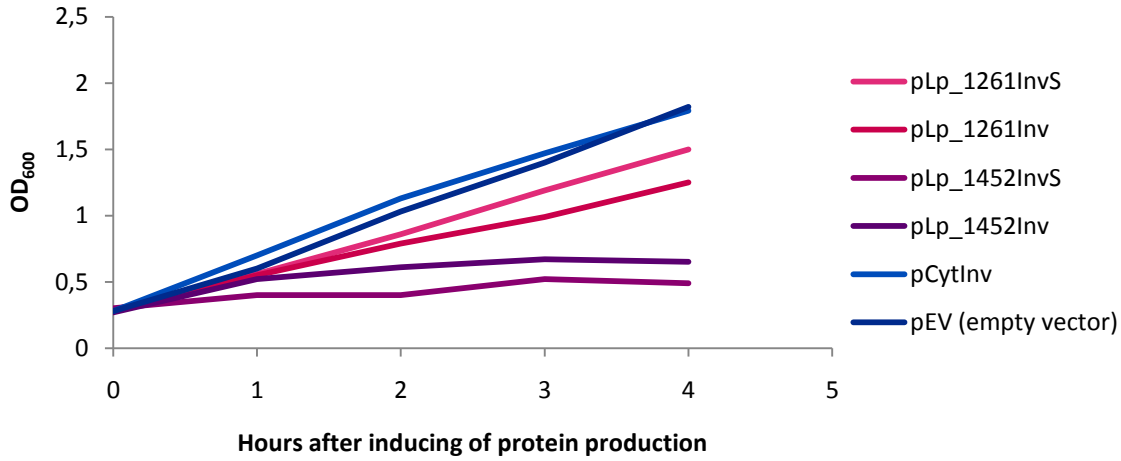


Figure 4.9: Growth of *L. plantarum* harbouring different constructs at 37°C. The cell density of the bacteria was measured by recording OD_{600} at one to four hours after protein induction of the cells.

Secretion and anchoring of invasin in *L. plantarum*

4.6 Translocation of invasin with lipo-anchor across the cell membrane

In the above section it was shown that the invasin proteins are successfully produced with all strains both at 30 and 37ºC and that the proteins could be detected over a period of at least 4 hours after induction. On the basis of the potential application of these strains in a human vaccine, secretion and anchoring of invasin was analyzed only at 37ºC.

The next step was to investigate invasin secretion by analyzing culture supernatants of *L. plantarum* cells harbouring *inv* containing plasmids. Although the proteins were designed to be anchored, an analyses of the culture supernatants makes sense, because shedding of lipoproteins is common; it was thus expected that invasin to some extent would be released from the cell surface (Antelmann et al. 2001; Tjalsma et al. 2008). Analyzing supernatants is a simpler procedure than detection of surface exposed invasin and was therefore performed before analysis of surface anchoring and exposure.

The secretion of invasin in the recombinant *L. plantarum* strains was analyzed by growing the cultures to OD_{600} ~0.3, inducing the strains with SppIP (25 ng/ml) and harvesting 1 to 4 hours after induction of protein production, as described in section 3.14. The proteins in the supernatants were precipitated with 16% TCA (see section 3.14.2 for more details) and analyzed by SDS-PAGE. Initially, secretion of invasin was investigated for cultures that were harvested one hour after induction. Based on the OD_{600}-value at the time of harvest, the amount of supernatant loaded onto SDS-PAGE gel originated from approximately the same number of cells.

Figure 4.10 show that cultures of *L. plantarum* cells harbouring the plasmids pLp_1261Inv, pLp_1452InvS or pLp_1452Inv have detectable amounts of invasin in the supernatant one hour after induction. The invasin protein was not detected in the supernatants of the *L. plantarum* strains containing the plasmids pCytInv or pLp_1261InvS, one hour after induction. *L. plantarum* containing the plasmid pLp_1261InvS was therefore examined more carefully by analysis of supernatant fractions obtained after culturing the cells for longer times. Figure 4.11 shows that invasin is present in the supernatant of *L. plantarum* cells carrying the pLp_1261InvS construct at two to four hours after induction, and that the amount increases over time.

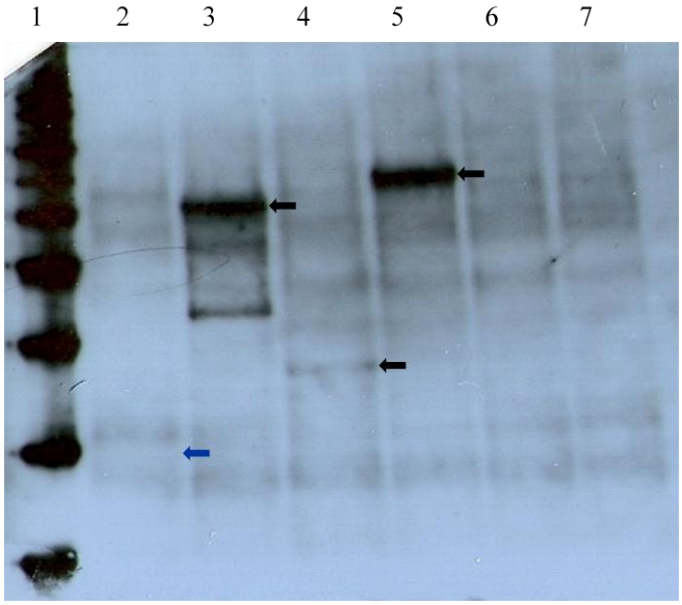


Figure 4.10: Invasin proteins in the culture supernatants. The picture show a western blot analysis of proteins in the supernatant obtained from *L. plantarum* strains harbouring various expression vectors 1 hour after induction of protein production. Size markers are shown in lane 1. The other lanes show proteins in the supernatant from *L. plantarum* strains harbouring the following plasmids: (2) pLp_1261InvS; (3) pLp_1261Inv; (4) pLp_1452InvS; (5) pLp_1452Inv; (6) pCytInv (no signal expected) or (7) pEV (empty vector; no signal expected). The arrows indicate the invasin protein; the size of the marked band corresponds to the expected size given in the legend of Figure 4.5. The approximate expected position of the invasin version from *L. plantarum* carrying the pLp_1261InvS construct is indicated with a blue arrow.

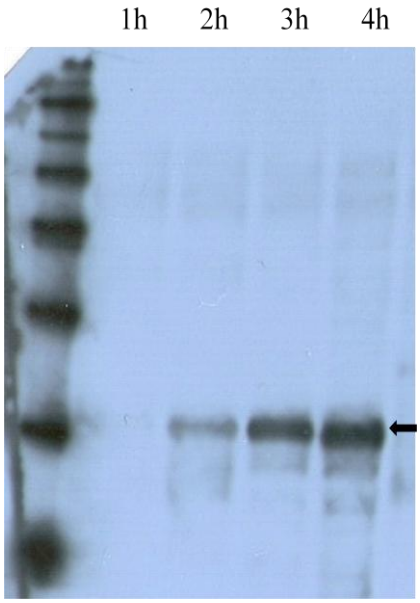


Figure 4.11: Invasin protein in the culture supernatant of *L. plantarum* harbouring the plasmid pLp_1261InvS. The picture show a western blot analysis of proteins in the supernatant obtained from *L. plantarum* harbouring pLp_1261Inv 1 to 4 hours (h) after induction of protein production. Size markers are shown in non-label lane. The arrow indicates invasin; the size of the marked band corresponds to the expected size given in the legend of Figure 4.5.

4.7 Detection of surface-anchored invasin

On the basis of the results for secretion of invasin, surface-anchoring of invasin was analyzed two hours after protein induction. Detection of invasin on the surface of *L. plantarum* is complicated and therefore, different strategies were used.

The use of immunofluorescence techniques is one method to detect surface exposed proteins. In this technique primary or secondary antibodies are conjugated to a fluorescent dye and fluorescence can then be visualized using fluorescence microscopy or flow cytometry. Other techniques that can be exploited are based on the use of mutanolysin to cleave MurNac-GlcNac linkages in cell wall peptidoglycan followed by analysis of the resulting cell wall fraction by western blotting. Finally, one may use western blotting-like procedure directly on cells (dot blot).

4.7.1 Detection of surface-anchored invasin by immunofluorescence

In order to analyze if invasin was present on the surface of the bacteria, the direct immunofluorescence method was examined. The primary antibody was specific for two epitopes on *Y. pseudotuberculosis* invasin (pAb invasin PAS Bleed #3, see section 2.3). The primary antibody has been conjugated with FITC (as described in section 3.18.1). This antibody will bind directly to invasin and the binding can be detected by fluorescence microscopy and flow cytometry.

The bacterial cells were stained approximately as described in 3.18.2, but optimization was necessary and several variants of the protocol were tested. One problem using direct immunofluorescence is a weak signal. Another known problem is the background signal, due to unspecific binding of the primary antibody. The optimizations were done to increase the signal without increasing the background signal. Since it is difficult to predict the optimal ratio between primary antibody and amount of cells several ratios were tested. When the antibody concentration was increased to detect a visible signal, background signal on the negative controls occurred. In an attempt to reduce the background signal different antibody concentrations and a BSA concentration gradient were tested. In addition, different incubation times, different incubation temperatures, incubation with or without shaking and different amount of washing step were tested. Most experiments did not yield any stained *L. plantarum* cells (data not shown). In some cases, minor fractions of stained cells were observed for

strains carrying constructs encoding lipo-anchored invasin but this was also the case for the negative controls (*L. plantarum* harbouring pCytInv or pEV, as well as wild-type *L. plantarum*) (Appendix, Figure A.1).

Since the experiments with FITC-labeled primary antibody did not yield satisfying results despite extensive optimization attempts, studies with a FITC labeled secondary antibody (Anti-Rabbit IgG (whole molecule)- FITC) were initiated. Secondary antibody conjugated with FITC would give a stronger signal than primary antibody conjugated with FITC because several molecules of secondary antibody will bind to one primary antibody, which binds to the antigen of interest (invasin). The cells, grown for two hours after protein induction, were stained approximately as described in section 3.18.3, but, again, extensive fine-tuning of the protocol was necessary. The optimal ratio between the amount of bacteria, primary antibody and secondary antibody was a challenge, and several different ratios were tested. The optimizations were done to decrease the background signal from the negative controls (*L. plantarum* harbouring pEV or pCytInv) without losing the signal from the strains carrying constructs encoding lipo-anchored invasin. In order to reduce the background signal, the bacteria were resuspended and washed with different concentrations of BSA (a gradient in the range 2 to 5%). Different incubation times with primary and secondary antibody, and different number of washing step in between antibody incubation were tested. Most experiments did not yield any strained *L. plantarum* cells, or the staining signal between *L. plantarum* strains harbouring constructs encoding lipo-anchored invasin and strains carrying constructs encoding pEV or pCytInv showed no significant differences (data not shown).

The optimization strategies described above did not remove the background signal, indicating unspecific binding of the primary or secondary antibody to the surface of *L. plantarum*. The primary antibody is from rabbit serum and polyclonal; it is therefore conceivable that some of the antibodies bind to other molecules than invasin. Therefore, a special pre-treatment of the primary antibody was tested (for details, see step 4 in section 3.18.3). In this treatment, the primary antibody was first incubated with cells of *L. plantarum* harbouring the empty vector (pEV) which does not produce invasin, with the aim of fishing out antibodies that bind unspecifically to the cells. The cells, grown for two hours after induction were stained with primary antibody or pre-treated primary antibody, followed by staining with secondary antibody, to compare the results. Interesting, the procedure with the pre-treated primary

antibody removed a significant portion of the background signal; therefore, this pretreatment of the primary antibody was done in the following experiments.

L. plantarum strains harbouring the different constructs were harvested two hours after induction. After washing the cells, the cells were resuspended in 50 µl PBS containing 2% BSA and 10 µl pre-treated primary antibody, followed by incubation for 30 minutes at 4ºC. The cells were washed four times with PBS containing 2% BSA. After resuspending the cells in 50 µl PBS containing 2% BSA 0.2 µl secondary antibody conjugated with FITC was added followed by incubation for 15 minutes at 4ºC. The cells were washed four times with PBS containing 2% BSA, resuspended in 50 µl PBS containing 2% BSA and analyzed using fluorescence microscope (for more details, see section 3.18.3). The result is shown in Figure 4.12 and shows green spots on all of the *L. plantarum* strains containing constructs encoding lipo-anchored invasin except for *L. plantarum* harbouring the pLp_1452InvS construct. Strains carrying pCytInv or the empty vector (pEV) showed very few green spots. Some of the green spots on the figure could be from cell lysis. Especially, on the image of *L. plantarum* harbouring the pLp_1261Inv construct are several green spots present without any bacteria cell. Generally, these experiments were difficult to repeat and, therefore, other methods to detect surface-located invasin were tested. In the next step, the samples shown in Figure 4.12 were analyzed with flow cytometry.

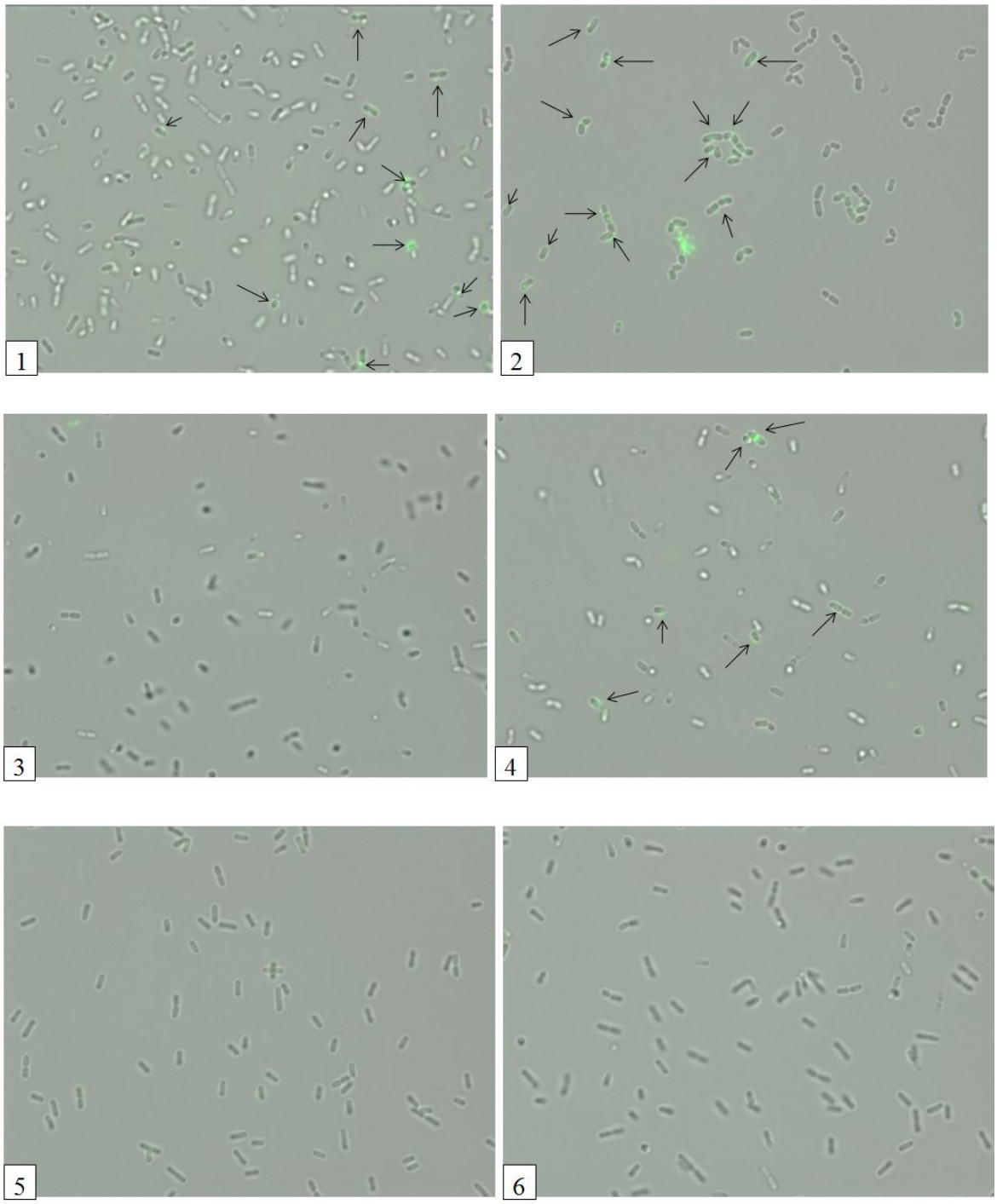


Figure 4.12: Detection of surface-anchored invasin. The images show *L. plantarum* strains harbouring the following constructs: (1) pLp_1261InvS; (2) pLp_1261Inv; (3) pLp_1452InvS; (4) pLp_1452Inv; (5) pCytInv or (6) pEV (empty vector). The cell amount was adjusted after OD_{600} at the harvesting time to stain approximately the same amount of cells. The images were achieved by using indirect immunofluorescence and analysis by fluorescence microscopy. The images represent one representative image of three random images that was taken of the different strains. The arrows demonstrated where there are green spots on the bacteria (manual inspection).

The flow cytometer detects bound fluorochrome-conjugated antibodies, such as secondary antibody conjugated with FITC, on cells. The result can be visualized by single-parameter histograms where the x-axis display the fluorescence intensity (FITC-A) and the y-axis display the number of events (hits/particles). More intense fluorescent signal (more fluorescence molecules on the surface of the cell) is indicated by a peak shift to higher fluorescent intensity (FITC-A). In theory, a sample containing no stained bacteria will yield one narrow peak at 0 (no fluorescence intensity).

The samples shown in Figure 4.12 were analyzed with flow cytometry and the result is shown in Figure 4.13. Figure 4.13 shows that generally, strains harbouring constructs encoding lipo-anchored invasin (no. 1-4) have a higher fluorescent intensity compared to strains carrying the negative controls (pCytInv and pEV; no. 5-6 respectively). Ideally, samples with *L. plantarum* strains harbouring the constructs pCytInv or pEV should have a narrow peak at 0, but instead the peaks are shifted to a higher FITC-A, especially for the *L. plantarum* strain containing pCytInv. This peak shift shows the present of stained bacteria on the strains carrying the negative controls. Generally, these experiments were difficult to repeat, and a significant difference between the strains containing constructs encoding lipo-anchored invasin and strains containing constructs encoding the negative controls were difficult to obtain.

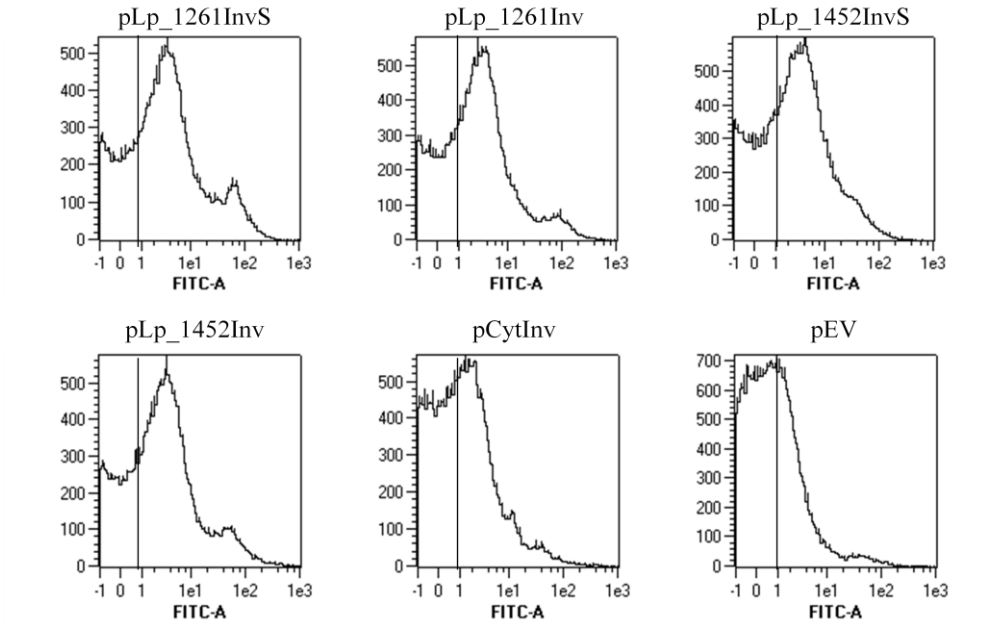


Figure 4.13: Flow cytometry analysis of various recombinant *L. plantarum* strains. The figure shows single-parameter histograms for cells of *L. plantarum* harbouring various constructs and stained with indirect immunofluorescence. The x-axis displays the fluorescent intensity (FITC-A) and the y-axis displays the number of events. The graphs represent *L. plantarum* harbouring the constructs: pLp_1261InvS, pLp_1261Inv, pLp1452InvS, pLp_1452Inv, pCytInv or pEV (empty vector). For each sample 10 000 cells were analyzed in the Flow cytometer.

In order to identify if invasin is more difficult to detect on the surface of *L. plantarum* than other surface-anchored proteins, *L. plantarum* harbouring another construct encoding two surface-anchored tuberculosis antigens (pLp_3050Ag85B-E6Cwa2 construct; (Tjåland 2011)) was studies, using appropriate antibodies. In this case, many stained bacteria were observed, while negative controls showed only few stained cells (data not shown). The primary antibody used in this study is monoclonal, which may explain why better signal-to-noise ratios could be obtained. Monoclonal antibodies are homogeneous with a defined specificity, which gives considerable less background signal. The primary antibody to invasin is polyclonal and it is not unusual that polyclonal antibodies give higher background signals (Lea 2006).

One possible explanation for the weak signals obtained when staining *L. plantarum* strains harbouring constructs encoding invasin could be that the primary antibody has insufficient access to epitopes on invasin. This can be caused by the epitopes being covered in the invasin structure. Therefore, *L. plantarum* containing the different constructs were incubated at 70ºC for 5 minutes before staining, to "open up" the invasin structure by protein unfolding. *L. plantarum* strains containing the various constructs and heat-treated in this manner were analyzed with fluorescence microscopy and three pictures were taken at random for every strain (see Appendix, Figure A.2). The result showed no significant difference between *L. plantarum* harbouring the constructs encoding lipo-anchored invasin compared to *L. plantarum* harbouring the constructs encoding pCytInv or pEV, except for *L. plantarum* containing the pLp_1452Inv construct, which showed considerably more stained bacteria than all other samples.

The weak signal from staining of the *L. plantarum* stains could also be due to the invasin proteins being buried in the cell wall, which could hinder binding of the antibodies. The samples were pre-treated with mutanolysin or mutanolysin together with lysozyme, prior to staining of the cells, to analyze if invasin was buried in the cell wall. Mutanolysin and lysozyme are enzymes that will cut specifically between the N-acetylmuramyl-β(1-4)-N-acetylglucosamine linkage (MurNac-GlcNac) of the cell wall polymer peptidoglycan-polysaccharide (Lichtman et al. 1992; Stan Tsai 1997). The challenge in these experiments was to remove some of the cell wall without lysis of the bacteria cells. A control for cell lysis is the *L. plantarum* strain harbouring pCytInv because this strain produces invasin but has no

signal for secretion or anchoring of invasin (invasin stays intracellularly). Cell lysis of the strain containing pCytInv would make the invasin protein available to react with the antibodies, resulting in green spots. The various recombinant *L. plantarum* strains were always threaded the same; therefore green spots on the *L. plantarum* harbouring pCytInv would indicate lysis of the other strain cells as well. To avoid cell lysis, different incubation times (20, 30 and 60 minutes) and concentration of the enzymes were tested.

In experiments with only mutanolysin, the greatest difference in number of stained cells between strains carrying constructs encoding lipo-anchored invasin and strains carrying constructs encoding pCytInv or pEV was from a pre-treatment of 50 U/ml mutanolysin incubated for 20 minutes, prior to staining of the cells (see section 3.14.3 for more details) (the result is shown in Figure A.3, in the appendix). Still, this result showed no significant difference between *L. plantarum* harbouring constructs encoding lipo-anchored invasin compared to *L. plantarum* harbouring pCytInv or pEV. However, the result did indicate that *L. plantarum* containing the pLp_1261Inv or pLp_1452Inv constructs could have more stained bacteria than the other strains.

In experiments with *L. plantarum* harbouring various constructs, pre-treated with mutanolysin and lysozyme, the staining results showed many stained bacteria for *L. plantarum* containing constructs encoding lipo-anchored invasin, but the same was observed for *L. plantarum* containing the intracellular version of invasin (pCytInv). Further optimization led to a procedure where the cells were treated with 60 U/ml mutanolysin and 15 mg/ml lysozyme for 30 minutes. This result showed no significant difference between *L. plantarum* containing constructs encoding lipo-anchored invasin compared to *L. plantarum* harbouring pCytInv or pEV. However, the result did indicate some more stained bacteria for *L. plantarum* containing the pLp_1261Inv or pLp_1452Inv constructs; the same as the experiment with pre-treatment of 50 U/ml mutanolysin for 20 minutes.

All in all, the immunofluorescence-based methods indicate surface-anchoring of invasin. Figure 4.12 indicates that *L. plantarum* harbouring the pLp_1261InvS, pLp_1261Inv or pLp_1452Inv plasmid have surface-anchored invasin, and the result in Figure 4.13 indicates that *L. plantarum* harbouring the pLp_1452InvS plasmid also have surface-anchored invasin. The results were promising, but few significant differences between the strains carrying

constructs encoding lipo-anchored invasin and strains carrying the pCytInv or pEV constructs (negative controls) were observed, and no conclusive evidence was obtained. Therefore, other analytical methods were tested as well.

4.7.2 Detection of surface-anchored invasin by isolating the cell wall fraction

L. plantarum has a thick cell wall composed primarily of peptidoglycan which has glycan chains with units of N-acetylglucosamine (GlcNac) and N-acetylmuramic acid (MurNac) in a β-1,4 linkage. Cell wall fractions of *L. plantarum* strains may be analyzed and this provides another method to detect if invasin is located in the cell wall. This method was performed as described in 0 and involved incubation of the cells with mutanolysin to cleave the MurNac-GlcNac linkage of the cell wall polymer peptidoglycan-polysaccharide (Lichtman et al. 1992). Despite repeated attempts to optimize the method, with different concentrations of mutanolysin and incubation times, every experiment yielded an invasin signal in the negative control (*L. plantarum* harbouring pCytInv). Figure 4.14 shows a typical result. Invasin bands with expected sizes are observed for all invasion expressing strains, including negative control (lane 5).

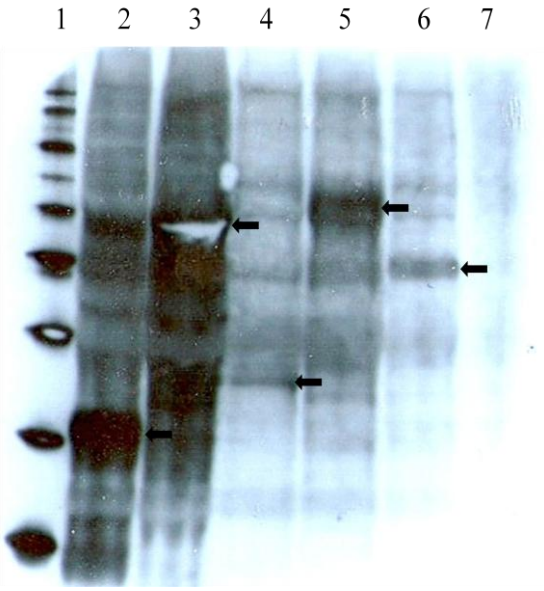


Figure 4.14: Detection of surface-anchored invasin by analysis of cell wall fractions. The picture shows a western blot analysis of the cell wall fraction obtained from *L. plantarum* strains harbouring various expression vectors. The size marker is located in lane 1. The other lanes show extracts from *L. plantarum* strains harbouring the following plasmids (predicted molecular mass of the invasin protein in parenthesis):
2, pLp_1261InvS (~29kDa); 3, pLp_1261Inv (~60kDa); 4, pLp_1452InvS (~37kDa); 5, pLp_1452Inv (~68kDa); 6, pCytInv (~52kDa); 7, pEV (empty vector; no signal expected).
The arrows indicate invasin. After harvesting the cells two hours after induction of protein production, the cells were treated with mutanolysin as described in section 3.14.3. The solution was precipitated with TCA (see section 3.15.2 for more details). The amount of protein extracts from each strain was adjusted according to the OD_{600}-value at the time of harvest to ensure that the protein extracts loaded onto the SDS-PAGE gel (see section 3.15) were from approximately the same amount of cells. Western blotting was performed as described in section 3.16.

4.7.3 Detection of surface located invasin with cell dot-blot

Since the methods described above did not give a conclusive result, a third method was tested, namely the cell dot-blot method (procedure described in section 3.17). Various recombinant *L. plantarum* strains were harvested four hours after induction of protein production. The samples were suspended in volumes of buffer that were proportional to the different OD_{600}-values for the sample at the harvesting time, to make sure that approximately the same amount of cells was studied. The cell suspensions were applied to a membrane and incubated with antibodies. After hybridization with the primary and secondary antibody (see section 3.17 for details) a positive result will give black spots on the film, indicating that invasin is anchored on the surface of the bacteria.

The challenge using the cell dot-blot method was to avoid a background signal. Optimization was necessary and several variants of two different protocols were tested. The method was optimized by changing of buffer, membrane, incubation time, concentration of antibodies and BSA, and the number of times the membrane was washed. Most experiments yielded a totally black membrane (data not shown), or if it was possible to see the spots, all the spots were black, i.e. positive (data not shown). Generally, it was difficult to see a significant difference between the *L. plantarum* strains carrying constructs encoding lipo-anchored invasin and the controls.

One optimized experiment yielded a quite good dot-blot, which is shown in Figure 4.15. The figure shows two degrees of black, dark black spots that represent cells and lighter black spots around the dark spots that represent the buffer signal. The figure shows a difference between *L. plantarum* harbouring the constructs encoding lipo-anchored invasin and *L. plantarum* containing the pCytInv or pEV constructs and the wild-type bacterium (negative controls). However, the negative controls all had quite dark spots and are certainly not really negative. Ideally, the negative controls would not show any visible spots. In addition, it seems like wild-type *L. plantarum* (lacking plasmid) has a black spot in the undiluted sample similar to the positive control. Generally, results from this dot-blot procedure were difficult to repeat and thus hardly conclusive.

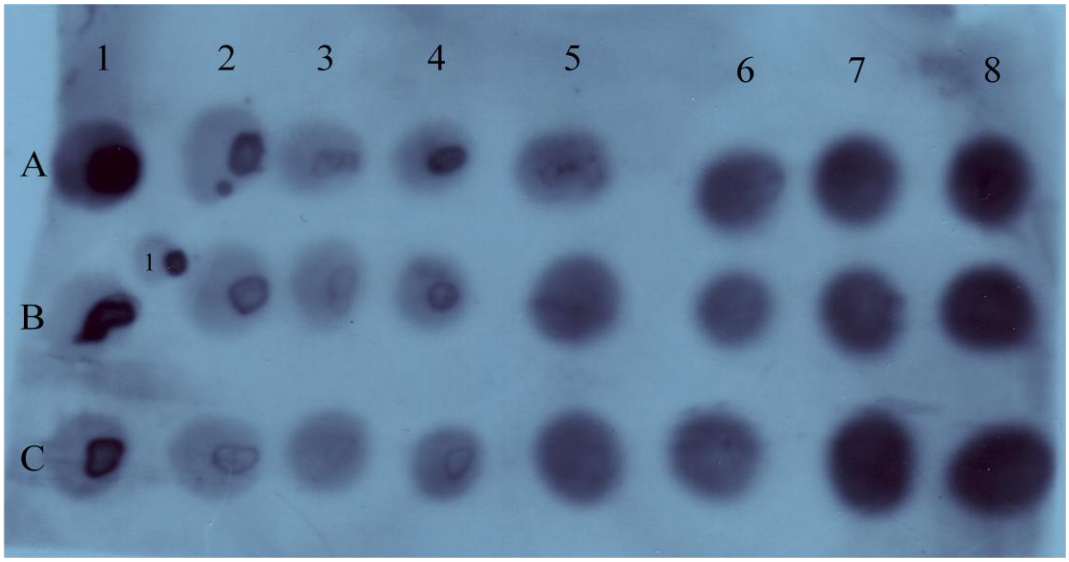


Figure 4.15: A cell dot-blot of different *L. plantarum* strains. The cell dot-blot was performed on a PVDF membrane (Procedure described in section 3.17, 1 µl/ml primary antibody and 0.05 µl/ml secondary antibody were used). All samples are intact cells suspended in TES-buffer, except for lane 1, which contains proteins from glass bead disrupted *L. plantarum* cells harbouring pCytInv (positive invasin control). Cells were harvested 4 hours after induction and applied undiluted (A), as well as diluted 5 (B) and 25 (C) times. The resuspension volume adjusted to the OD_{600}-value at the time of harvest to make the number of cells similar in all samples. The spots contain 2 µl of undiluted (A) or diluted (B, C) cell suspension of *L. plantarum* harbouring the following plasmids: (2) pLp_1261InvS, (3) pLp_1261Inv, (4) pLp_1452InvS, (5) pLp_1452Inv, (6) pCytInv, (7) pEV (empty vector) and (8) wild-type of *L. plantarum*.

Internalization of *L. plantarum* strains by Caco-2 cells

4.8 Analysis of internalization of *L. plantarum* strains by Caco-2 cells

Although the experiments described in section 4.7 did not convincingly show the presence of anchored invasin on the surface of *L. plantarum* cells, they do provide strong indications that this indeed may be the case. Therefore, the internalization of these bacteria into human intestinal cells was analyzed. The studies were performed with non-polarized Caco-2 cells which are known to have β_1-integrin receptors on their apical sides and which do not have microvilli. Thus, these cells to some extent resemble M-cells and can be used as a model for those. Invasin binding to the β_1-integrin receptor normally results in internalization of the bacteria, but it requires a high density of invasin molecules on the bacterial surface (Leo & Skurnik 2011).

First a preliminary experiment was performed to analyze the internalization of *L. plantarum* strains harbouring various constructs into Caco-2 cells. The various recombinant *L. plantarum* strains were harvested two hours after protein induction and stained with either FITC or CFSE (the bacterial cells were stained green) (see section 3.21) to compare the two staining

methods. After staining of the bacterial cells *L. plantarum* harbouring various constructs were incubated with Caco-2 cells for one hour. After the incubation, the samples were washed and then fixated on microscope slides. The slides were stained with Hoechst stain to visualize the nuclei to the Caco-2 cells (the Caco-2 cells were stained blue) (done by Charlotte Kleiveland). The samples were analyzed using Confocal Laser Scanning Microscopy (CLSM). The CLSM analysis was done by Lene Olsen and Charlotte Kleiveland. The result showed that the *L. plantarum* strains were better visualized in the microscope with CFSE compared to FITC. Since this was a preliminary experiment only a few images were taken of some of the recombinant *L. plantarum* strains. Figure 4.16 shows the images of *L. plantarum* harbouring the pLp_1261Inv or pLp_1452Inv, to demonstrate that the number of bacteria cells incubated with Caco-2 cells was observed to differ between the recombinant strains. In addition, it was difficult to determine if the bacteria were outside or inside the Caco-2 cells because the membrane to the Caco-2 cells was difficult to detect. Therefore optimization of the procedure was necessary.

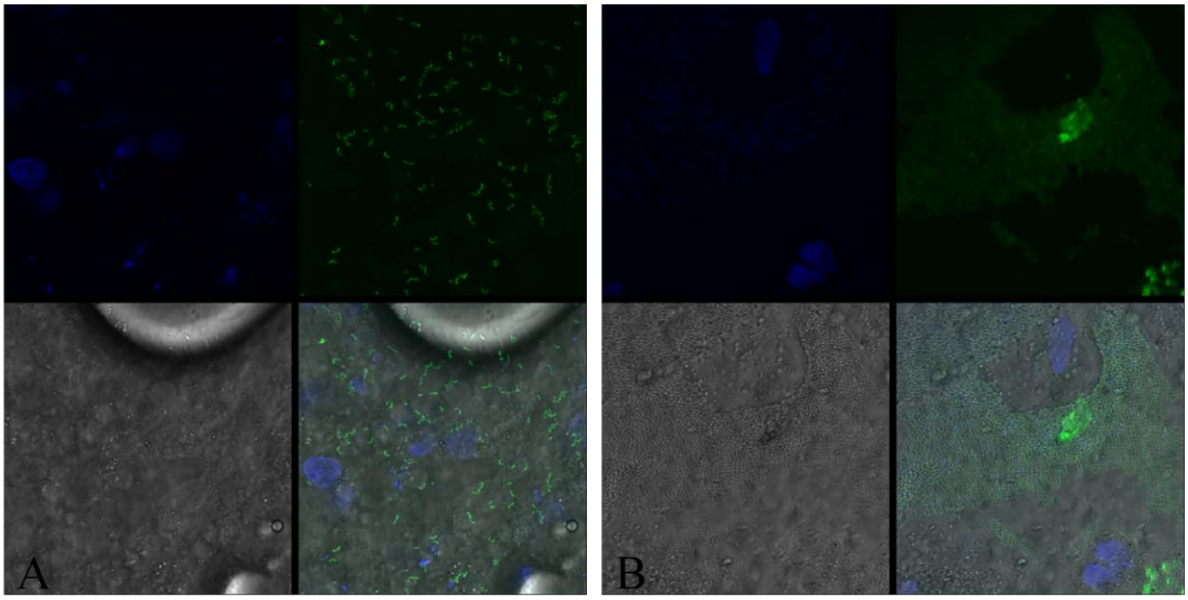


Figure 4.16: CLSM images of Caco-2 cells and *L. plantarum* harbouring the different invasin constructs: (A) pLp_1261Inv or (B) pLp_1452Inv. The recombinant *L. plantarum* strains were harvested two hours after pheromone induction and the bacterial cells were stained green with CFSE. The various *L. plantarum* strains were incubated with Caco-2 cells at 37ºC for one hour. The sample was fixed on microscope slides and the Caco-2 cells were stained blue with Hoechst stain. The images shows only the blue channel, only green channel, no color, and green and red channel together (down in right corner) The images show that the number of bacterial cells in the samples differs and that it was difficult to detect bacterial cells inside Caco-2 cells.

In order to compare the Caco-2 internalization for the different recombinant *L. plantarum* strains it was necessary to add approximately the same amount of viable bacterial cells to each

sample with Caco-2 cells. The experiment depicted in Figure 4.16 shows that the relation between OD_{600} and viable cells varied between cultures of *L. plantarum* harbouring the different constructs. Figure 4.16B shows a considerably higher amount of bacteria (green) compared to image A despite the fact that, judged by OD_{600}, the same amount of bacteria was added. Therefore, the relation between OD_{600} and CFU/ml was checked for *L. plantarum* harbouring all the different constructs. The results showed that for the *L. plantarum* strains harbouring the pLp_1452InvS or pLp_1452Inv constructs the amount of viable cells (CFU) decreased over time after induction (Appendix, Figure A.4). *L. plantarum* harbouring the other constructs increased in amount of cells. The relation between OD_{600} and CFU/ml for *L. plantarum* containing empty vector (pEV) (Appendix, Figure A.5) was used to calculate the amount of cells for all constructs except pLp_1452InvS and pLp_1452Inv for which the graph in Figure A.4 in the Appendix was used.

Internalization of recombinant *L. plantarum* strains into Caco-2 cells were difficult to observe because it was difficult to discriminate between bacteria inside or outside Caco-2 cells in the microscope. In order to analyze the internalization of the bacteria, one other possible method is the gentamicin survival assay (Innocentin et al. 2009). In theory, the gentamicin (an antibiotic) will kill non-internalized bacteria while bacteria inside the Caco-2 cells are protected from the antibiotic and would therefore survive the treatment. Gentamicin was chosen as the most optimal antibiotic because this antibiotic cannot permeate mammalian cells (Critchley-Thorne et al. 2006). To be able to apply this method, first, concentrations of gentamicin were tested to find appropriate amount of gentamicin that is needed to kill *L. plantarum*. It was found that 400 µg/ml gentamicin killed *L. plantarum* without harming the Caco-2 cells.

The gentamicin survival assay was performed as described in section 3.23. In short, the cells were harvested two hours after induction of invasin production, washed and resuspended in 1.5 ml RPMI 1640 medium with L-glutamine and 10% fetal calf serum (FCS). To find the start concentration of bacterial cells before incubation with Caco-2 cells, 100 µl of the suspensions were spread out on MRS plates. The rest of the suspensions (1.4 ml) were incubated with Caco-2 cells in microplates for 3 hours (based on the protocol of Innocentin et al. (2009)). After adding antibiotic (400 µg/ml gentamicin), the cells were incubated over night (~15 hours). The cells were then washed with PBS, and 300 µl PBS containing 0.1%

triton was added in order to cell lyse the Caco-2 cells. Subsequently, the entire solution with Caco-2 cells was spread out on agar-plates (100 µl on three different plates). The result yield no significant differences between *L. plantarum* strains carrying constructs encoding lipo-anchored invasin and *L. plantarum* harbouring the pCytInv or pEV constructs (negative controls). Some optimization of the procedure was done in order to see if it was possible to detect a difference between the various recombinant strains, but none gave a better result. The best results received in this study was with the procedure described above and start amounts of ~10^8 and ~10^6 bacteria, yielding ratios between Caco-2 cells and bacteria at 1:5000 and 1:50 respectively. The results are reported in Table 4.2 and 4.3 and show a very low survival percents, high standard errors and no significant difference between the recombinant strains; *L. plantarum* strains carrying constructs encoding lipo-anchored invasin did not show an increased internalization into Caco-2 cells compared to the negative controls. As a control, the triton treatment was tested on *L. plantarum* cells to examine if 0.1% triton could induce cell lysis of *L. plantarum* cells, but *L. plantarum* harbouring the different constructs survived treatment with 0.1% triton (data not shown).

Table 4.2: The gentamicin survival assay performed with a start amount of ~10^8 bacteria, giving a ratio between Caco-2 cells and bacteria at ~1:5000. Percent survival is calculated after how many bacteria cells survived compared to the start amount, and is given with the standard error.

Constructs	Start amount CFU/ml	Average amount of survivors CFU/ml	Percent survival/ml
pLp_1261InvS	$4.1 * 10^8$	1900	$6.6 * 10^{-4} \pm 2.4 * 10^{-4}$
pLp_1261Inv	$2.8 * 10^8$	450	$2.3 * 10^{-4} \pm 1.4 * 10^{-4}$
pLp_1452InvS	$1.7 * 10^8$	40	$3.3 * 10^{-5} \pm 1.7 * 10^{-5}$
pLp_1452Inv	$4.5 * 10^8$	280	$8.8 * 10^{-5} \pm 1.2 * 10^{-5}$
pCytInv	$1.2 * 10^8$	1700	$2.1 * 10^{-3} \pm 1.5 * 10^{-3}$
pEV (empty vector)	$1.8 * 10^8$	1180	$9.1 * 10^{-4} \pm 4.2 * 10^{-4}$

Table 4.3: The gentamicin survival assay performed with a start amount of ~10^6 bacteria, giving a ratio between Caco-2 cells and bacteria at ~1:50. Percent survival is calculated after how many bacteria cells survived compared to the start amount, and is given with the standard error.

Constructs	Start amount CFU/ml	Average amount of survivors CFU/ml	Percent survival/ml
pLp_1261InvS	$9.6 * 10^6$	340	$3.5 * 10^{-3} \pm 2.2 * 10^{-3}$
pLp_1261Inv	$2.5 * 10^6$	55	$2.1 * 10^{-3} \pm 1.5 * 10^{-3}$
pLp_1452InvS	$2.4 * 10^6$	0	0
pLp_1452Inv	$3.8 * 10^6$	0	0
pCytInv	$2.0 * 10^7$	305	$1.5 * 10^{-3} \pm 6.5 * 10^{-4}$
pEV (empty vector)	$2.2 * 10^7$	120	$5.5 * 10^{-4} \pm 2.3 * 10^{-4}$

The gentamicin survival assay is based on recovery of viable bacteria after internalization by Caco-2 cells. In this assay an incubation time of ~18 hours was used, this could be too long and *L. plantarum* cells could lyse before the solution is spread on agar-plates. Therefore, this assay was complemented with a microscopic evaluation of *L. plantarum* containing the different constructs; in this method, the step of lysing the Caco-2 cells is omitted.

The various recombinant *L. plantarum* strains were harvested 1 ½ hour after induction, stained with CFSE (see section 3.21) and then treated with Biotin (see section 3.22). In this method the CFSE is transferred into the bacteria and binds to intracellular proteins, which makes all bacteria green, while biotin binds to the surface of the bacteria. After the treatment, the bacteria were incubated with Caco-2 cells for 1 hour and then with antibiotic (400 μg/ml gentamicin) for another hour to kill bacteria outside Caco-2 cells. After the gentamicin treatment TRITC was added to the Caco-2 cells. The TRITC will bind to biotin and is visualized as a red color. Thus, bacteria inside Caco-2 cells will be green, due to the CFSE staining, but the bacteria outside will react with TRITC and become yellow/orange, due to mixing of the green CFSE signal and the red TRITC signal. After the TRITC staining, the internalization of the *L. plantarum* strains was analyzed with CLSM by Lene Olsen and Charlotte Kleiveland and representative microscopy images are shown in Figure 4.17.

Figure 4.17 shows only some of the recombinant *L. plantarum* strains because only a few images were taken. The negative controls are not included in the figure because of lack of images but the samples were observed in the microscope. For *L. plantarum* harbouring the pCytInv or pEV constructs (negative controls), some bacteria were observed bound to Caco-2 cells but in clearly lower numbers than for strains carrying constructs encoding lipo-anchored invasin. *L. plantarum* harbouring the pLp_1261InvS construct (image A and B in Figure 4.17) was observed to be the strain where most bacteria bound to the Caco-2 cells and one Caco-2 cell had two green spots inside that could possibly be bacteria. *L. plantarum* harbouring the pLp_1261Inv construct was observed to bind many Caco-2 cells and one bacterium (image C in Figure 4.17) was observed to be both green and orange; could be penetrating the Caco-2 cell membrane. *L. plantarum* harbouring the pLp_1452InvS construct was observed to bind well to the surface of Caco-2 cells (image D in Figure 4.17) but no bacteria were observed to be inside the Caco-2 cells. *L. plantarum* harbouring the pLp_1452Inv construct was not analyzed in the microscope because of loss of the bacterial pellet during the staining

treatment. Interestingly, in this experiment strains containing constructs encoding lipo-anchored invasin had increased affinity for the Caco-2 cells than strains without lipo-anchored invasin. It must be noted that this experiment was done only once.

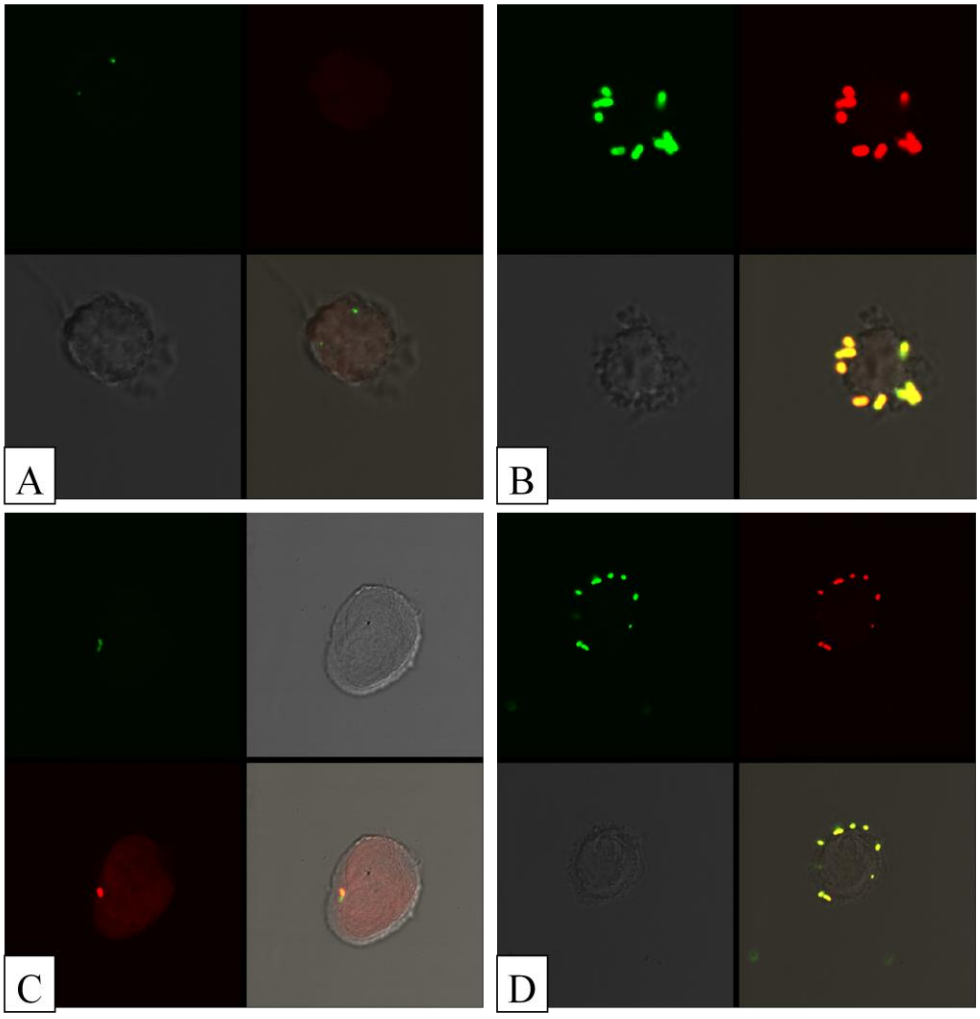


Figure 4.17: CLSM images of Caco-2 cells and *L. plantarum* harbouring different invasin constructs. (A) and (B) pLp_1261InvS, (C) pLp_1261Inv and (D) pLp_1452InvS. The bacteria are stained green with CFSE and treated with Biotin, incubated with Caco-2 cells and then with antibiotic and stained with TRITC. This made bacteria located outside Caco-2 cells yellow/orange while bacteria inside Caco-2 cells stained green. The antibiotic treatment should ideally kill all bacteria outside Caco-2 cells. The images shows only the green channel, only red, no color, and green and red channel together (down in right corner)

5. DISCUSSION

Construction of invasin expression vectors

The production and cell-wall anchoring of an M-cell binding protein in *L. plantarum* WCFS1 utilizing the pSIP-system could be a new way of delivering mucosal vaccines. In this study invasin was used since this protein binds to receptors on M-cells; first step was to anchor the invasin protein to the surface of *L. plantarum* to hopefully in the end achieve binding and uptake to M-cells (Palumbo & Wang 2006). *L. plantarum* WCFS1 was selected as host because (1) it is a GRAS bacterium that is persistent in the GI tract (Seegers 2002), (2) its genome sequence is known (Kleerebezem et al. 2003), (3) gene expression systems are available (Sørvig et al. 2003), and (4) the research group where this project was carried out has much experience with this bacterium (e.g. Mathiesen et al. 2008).

5.1 Selection of an invasive protein and a suitable anchoring strategy

Invasin from *Y. pseudotuberculosis* is an M-cell binding protein. It has been shown that invasin binds to β_1-integrin on the apical surface of M-cells and can mediate uptake of bacteria (Palumbo & Wang 2006). Harms et al. (2009) and Critchley-Thorne et al. (2006) have shown that recombinant *E. coli* expressing invasin could efficiently enter cells that expressed β_1-integrin. Suzuki et al. (2006) demonstrated that a non-invasive *Shigella* mutant that expressed invasin became an effective invasive *Shigella* vaccine. Invasin gives the impression of being an attractive choice for enhancing immune responses to bacteria, since it delivers the antigen to M-cells, trigger transport of the antigen-expressing bacteria across the epithelial barrier into organized lymphoid structures. However, invasin could also be a virulence factor and insertion in *L. plantarum* may introduce safety issues. Alternatively, invasin may have desired effects only and the invasin-expressing bacteria can be used to deliver protective antigens.

The 5 domains that binds the β_1-integrin receptors and promote uptake of the bacterium are in the C-terminal end of invasin, and the last 2 C-terminal domains comprise the minimal invasin fragment required for binding (Hamburger et al. 1999). Since the binding site of invasin is in the C-terminal end, it is necessary to select an anchor that is fused to the N-terminal end. Lipo-anchors were selected as the most suitable alternative since lipoproteins

are covalently anchored to the cell membrane via their N-terminal part (Kovacs-Simon et al. 2011). So far, lipoproteins have only rarely been utilized for surface display of proteins (Samuelson et al. 2002). Lipo-anchors from two lipoproteins previously found extracellularly (Fredriksen, unpublished) in *L. plantarum*, where the length of the two lipo-anchors differed substantially, were selected to anchor invasin. It was desirable to have a signal peptide and anchor that were homologous (i.e. from *L. plantarum* itself), since this limits the use of foreign DNA and may lead to a more efficient secretion (Mathiesen et al. 2009). The results from Lipo1.0 predicted that the lipoproteins, Lp_1261 and Lp_1452, were cleaved by Spase II. Even though LipoP is trained on target sequence from Gram-negative bacteria, it has been shown to predict lipoproteins with >90% overall accuracy in Gram-positive bacteria (Rahman et al. 2008), therefore it was reasonable to assume that the predicted anchors of these lipoproteins were suitable as lipo-anchors in this study. In addition, the Lp_1452 lipoprotein has a predicted function as a peptidylprolyl isomerase, which is involved in extracellular folding of secreted proteins (Tjalsma et al. 2000; Wahlstrøm et al. 2003). This is interesting since the anchor most likely will have a length that makes invasin surface exposed. The Lp_1261 lipoprotein has a predicted function as ABC transporter and a shorter anchor sequence that probably will keep invasin more buried in the cell wall.

5.2 The plasmids

The cloning process was challenging, but using the In-Fusion cloning strategy, which is based on homologous recombination, the five plasmids, pLp_1261InvS, pLp_1261Inv, pLp_1452InvS, pLp_1452InvS and pCytInv were successfully constructed.

The constructed expression plasmids have an antibiotic resistance gene against erythromycin to achieve stable maintenance of the plasmids. In a vaccine, an antibiotic resistance gene should not be included because this gene can be transferred to other species e.g. pathogenic bacteria (Detmer & Glenting 2006). Accordingly, the need to identify and develop alternatives to antibiotic resistance marker genes becomes apparent in the development of the vaccine. A food-grade variant of the pSIP-system has very recently been constructed where the *L. plantarum* WCFS1 alanine racemase gene (*alr*) is the selection marker instead of an antibiotic marker (Nguyen et al. 2011). Even with food-grade selection markers, the release of genetically modified bacteria to the environment raises safety concerns. It is essential to make sure that the bacterial vaccine does not survive outside the human body, because the

consequences of spreading live bacterial vaccines into the environment are unknown and of a considerable concern. To overcome this challenge it is possible to use the strategy published by Steidler et al. (2003) where an essential gene for bacterial survival was replaced. They exchanged the *thyA* gene with the expression cassette for human IL-10. This made the *L. lactis* strain dependent on thymidine or thymine to grow and survive; thus it would not be able to survive outside the human body (Steidler et al. 2003).

Intracellular invasin production in *L. plantarum*

5.3 Intracellular production of invasin with lipo-anchor

The invasin proteins with the Lp_1261 or the Lp_1452 lipo-anchor were successfully produced intracellularly. The presence of invasin in cell free extracts was detected 1 to 4 hours after induction of protein production at both 30 and 37ºC (Figure 4.5, Figure 4.6 and Figure 4.7). The western blot analysis in Figure 4.5 indicated that *L. plantarum* strains carrying constructs encoding lipo-anchored invasin produce more invasin than *L. plantarum* harbouring the construct encoding the intracellular version of invasin (pCytInv). The difference of invasin production could be related to the fact that *L. plantarum* harbouring various constructs contains different versions (length) of invasin and anchor. Since the proteins differ, the production of them would also be expected to be different. Sørvig et al. (2003, 2005) observed that the expression efficiencies (at the protein level) obtained with the SIP-system differed from protein to protein in a rather unpredictable manner. Figure 4.6 and Figure 4.7 further show that the invasin bands from *L. plantarum* strains containing constructs encoding lipo-anchored invasin are approximately the same size throughout all the hours that were tested. In contrast, the *L. plantarum* strain containing the pCytInv construct (without anchor), the invasin band is gradually accumulated in size over time. Still, after four hours when the invasin production has increased over time for *L. plantarum* harbouring the pCytInv construct, the intensity on the band is still weaker than for *L. plantarum* carrying constructs encoding lipo-anchored invasin that do not increase significant over time (Figure 4.5). This indicates that the production of the lipo-anchored invasin proteins is better than production of the invasin protein without anchor.

The observation that the intracellular amounts of invasin from strains harbouring constructs encoding lipo-anchored invasin do not increase over time (Figure 4.6 and Figure 4.7) may indicate that the lipo-anchored invasin proteins are transported out of the cell. Because, as

long as the protein is produced the amount of protein inside the cell will increase except if the protein is secreted out of the cell, or degraded at the same speed as production. The lipo-anchored invasin proteins could potentially be proteolytically degraded intracellularly to a higher degree than invasin without anchor, but then proteolytic products should have been visible in the western blot. Proteolytic products are normally detected below the main band in the western blot. Even when no such bands are detected there could be proteolytic products present, because invasin could be degraded in the C-terminal end where the primary antibody recognize epitopes on invasin (see section 2.3), which makes the proteolytic products non-detectable. However, all in all the analysis of intracellular invasin levels indicate that invasin is exported, meaning that it also may become lipo-anchored.

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

L. plantarum with the pSIP system are normally grown at 30ºC (Sørvig et al. 2003), but because of the potential application of the various recombinant *L. plantarum* strains in a human vaccine, evaluation of the strains at 37ºC as well, was important. Figure 4.8 and Figure 4.9 shows that the induced cultures of *L. plantarum* harbouring the various constructs grew slightly faster at 30ºC; the recombinant strains grow nearly as well at 37ºC as at 30ºC. The growth rates of the various recombinant strains showed major differences, which were similar at 30 and 37ºC. The fact that the growth rate of the *L. plantarum* strains containing construct encoding lipo-anchored invasin decreased substantially after induction compared to *L. plantarum* harbouring pEV or pCytInv constructs, indicates that secretion and anchoring of the invasin protein could be stressful for the bacteria. This decrease in growth rates after induction of the target protein production in a recombinant strain compared to the wild-type bacterium has been observed previously (Bolhuis et al. 1999; Lulko et al. 2007; Mathiesen et al. 2008). In addition, there seems to be a clear difference between the two lipo-anchor versions, especially at 37ºC. *L. plantarum* harbouring the Lp_1452 anchor constructs hardly grew. In addition, *L. plantarum* harbouring the Lp_1452 anchor constructs the amount of viable cells (CFU) decreased over time after induction (Appendix, Figure A.4). This decreasing of viable cells may be because the Lp_1452 constructs contain a quite long anchor which can cause problems, for example by affecting the folding of the protein. *L. plantarum* harbouring the pLp_1261InvS plasmid is the strain with highest growth compared to the other strains carrying constructs encoding lipo-anchored invasin. This strain has the shortest lipo-anchor-invasin protein that could be less stressful to (produce and) secrete and anchor for the

bacteria. However, if bacterial growth is just dependent on size of the invasin protein, it would be expected that *L. plantarum* harbouring pLp_1452Inv construct would grow slowest, but this was not the case. Therefore, the size of the invasin protein is not the only reason for slow growth. Based on these results it is reasonable to assume that the Lp_1261 anchor is a better choice than the Lp_1452 anchor, at least in terms of growth viability of the recombinant bacteria.

Secretion and anchoring of invasin in *L. plantarum*

5.5 Translocation of invasin with lipo-anchor across the cell membrane

Translocation of invasin in *L. plantarum* cells harbouring *inv* containing plasmids across the membrane is interesting because it indicates that the protein is potentially anchored. Since shedding of lipoproteins is normal (Antelmann et al. 2001; Tjalsma et al. 2008), it was expected that invasin to some extent would be released from the cell surface. Therefore, presence of invasin in culture supernatants was analyzed.

For *L. plantarum* harbouring the plasmids pLp_1261Inv, pLp_1452Inv or pLp_1452InvS, the invasin proteins were detected in the supernatant fraction one hour after induction of invasin production (see Figure 4.10). *L. plantarum* encoding the pCytInv construct showed no invasin protein in the supernatant, which reduce the possibility that detected invasin in the supernatant, is the result of cell lysis. It should be noted though that, *L. plantarum* containing constructs encoding lipo-anchored invasin may be more stressed as indicated by the growth analysis (Figure 4.8 and Figure 4.9), which could lead to increased cell lysis for these strains. Since the *L. plantarum* strains containing the Lp_1261 lipo-anchor constructs grow quite well, the result from these constructs is more reliable than the result from the strains harbouring the Lp_1452 lipo-anchor constructs. For *L. plantarum* harbouring the plasmid pLp_1261InvS the protein was not detected in the supernatant one hour after induction (see Figure 4.10). The reason for this is unclear. One possible explanation is that *L. plantarum* harbouring the pLp_1261InvS construct has an invasin version that leads to less shedding, compared to the other recombinant strains. Since only two different lipo-anchors were used it is difficult to conclude if these are the best alternative. However, both lipoproteins were predicted by SignalP 3.0 (Bendtsen et al. 2004) to have the typical N-terminal, H domain and C-region for a signal peptidase cleavage site, and since all the anchored proteins are secreted; it seems that the signal peptides from Lp_1261 and Lp_1452 are functional.

Choosing signal peptides that give high secretion efficiency is challenging since optimal secretion requires an optimal combination between the signal peptide and the target protein (Brockmeier et al. 2006; Mathiesen et al. 2009). One of the goals in the present study was to anchor the protein, not just to secrete it. Testing of several signal peptides could result in higher secretion efficiency with more surface-anchored invasin protein, which could be essential for internalization of the bacteria. However, high secretion efficiency is a disadvantage for internalization of *L. plantarum* if the invasin protein is just secreted and not anchored. Because secreted invasin molecules that are not attached to the bacteria can bind and occupy integrin receptors, making the receptors less available for surface-anchored invasin on *L. plantarum*.

5.6 Detection of surface-anchored invasin

Most proteins involved in invasion are anchored to the surface of the bacteria (Niemann et al. 2004). Invasin has to be surface-anchored to *L. plantarum* cells to achieve internalization by M-cells or other β_1-integrin exposing cells. A strategy with two lipo-anchors and two invasin-versions with different lengths was utilized to hopefully achieve cell-membrane anchoring of invasin with the binding site at four different distances away from the anchoring site (two anchor lengths times two invasin lengths). The length of the target proteins can influence how exposed the proteins are on the surface of the bacteria.

In order to evaluate surface-anchoring of invasin, immunofluorescence techniques were used. Staining of the bacterial cells with the direct immunofluorescence method was challenging, since only one primary antibody will bind to each invasin molecule. Therefore, if few invasin molecules were on the surface of the bacteria it would be difficult to see the fluorescent signal from the FITC stained antibody. With direct immunofluorescence a low percentage staining has been shown. McCarthy & Culloty (2011) showed that only 10-35% of *E. coli* cells were stained with direct immunofluorescence, depending on the detection method and antibody used. The antibody concentration is a relevant factor for staining of bacteria; significant difference in the number of cells stained with different concentration of the antibody was shown (McCarthy & Culloty 2011). This matches the observations described in this thesis (data not shown).

In theory, an indirect immunofluorescence method will give stronger fluorescent signals than direct immunofluorescence, because several FITC molecules will bind to each primary antibody that binds to invasin. The problem with this method was background signals, which was most likely not caused by technical problems. The primary antibody to invasin (pAb invasin PAS Bleed #3) is from rabbit serum and serum contains different antibodies against different antigens. Only 5-10% of antibodies in serum are specific for the immunogen used in the immunization (Lea 2006). Therefore, it is not unusual that use of polyclonal antibodies leads to unspecific interactions, leading to a background signal.

A difference between *L. plantarum* carrying constructs encoding lipo-anchored invasin and *L. plantarum* harbouring the intracellular invasin (pCytInv) or empty vector (pEV) constructs (Figure 4.12) was observed. Despite that this difference was only seen clearly once, the result indicates that three of the strains, *L. plantarum* harbouring the plasmids pLp_1261InvS, pLp_1261Inv or pLp_1452Inv, have invasin on the surface. *L. plantarum* harbouring the pLp_1452InvS construct showed no stained cells. This result indicates that *L. plantarum* encoding pLp_1452InvS do not have invasin on the surface. Interestingly, this is the same strain that grows slowest (Figure 4.9) and the amount of viable cells (CFU) decreased over time after induction (Appendix, Figure A.4). The invasin protein from *L. plantarum* containing the pLp_1452InvS construct is detected intracellularly and in the culture supernatant. It could be that for *L. plantarum* harboring the pLp_1452InvS construct secretion and anchoring is more stressful than for *L. plantarum* harbouring the other constructs; therefore the invasin protein was not detected on the surface of this strain.

For the *L. plantarum* strain containing the pCytInv construct, very few weakly stained bacteria were seen. The stained bacteria could be due to cell lysis, because this bacterium produces invasin intracellularly; after cell lysis the invasin protein from this strain could react with the fluorescent antibodies. Some cell lysis could also occur for *L. plantarum* strains harbouring the constructs encoding lipo-anchored invasin. Especially, on the image of *L. plantarum* harbouring the pLp_1261Inv construct it seems like there could be some cell lysis, because there are several green spots present without any bacteria cell. The observation of some green *L. plantarum* cells harbouring empty vector (pEV), indicates that some of the fluorescent signal is background signals. The images in Figure 4.12 show that not all bacteria

are stained. This was as expected, since others have shown that not all bacteria are stained with the use of this method (Cortes-Perez et al. 2007).

The same samples examined in Figure 4.12 were analyzed using flow cytometry. Figure 4.13 shows the same difference between *L. plantarum* harbouring the constructs encoding lipo-anchored invasin and *L. plantarum* harbouring the negative controls, except that this analysis also indicates anchoring of invasin for *L. plantarum* harbouring the pLp_1452InvS plasmid. Because *L. plantarum* strains containing constructs encoding lipo-anchored invasin (no. 1-4 in Figure 4.13) several bacteria had a higher fluorescent intensity (FITC-A) than *L. plantarum* strains harbouring the negative controls (no. 5-6 in Figure 4.13). The fluorescence signals from *L. plantarum* strains carrying constructs encoding lipo-anchored invasin did not show a very narrow distribution of the fluorescence intensity, which indicates that the cells vary in the amount of expressed invasin protein (Nhan et al. 2011). It should also be noted that background signals were detected, since *L. plantarum* harbouring the pEV construct did not yield a narrow peak at 0 as expected after flow cytometry analysis. The peak was shifted towards some fluorescent signal, but not as much as for the *L. plantarum* strains containing constructs encoding lipo-anchored invasin. Therefore, it is not likely that the entire fluorescent signal observed for the strains expressing anchored invasin is a background signal. In addition, others have shown a negative control that was not a narrow signal at 0, but a signal surrounding 0, and that the signal from analyzed samples were shifted towards more fluorescence intensity (FITC-A) (Nhan et al. 2011). *L. plantarum* harbouring the pCytInv construct shows a quite high peak at 0, so many bacteria are not stained, but it also shows a high peak with a higher fluorescent signal (FITC-A), which indicates that some bacteria were stained. Invasin resulting from cell lysis would most likely be washed away in the staining procedure, but it could be some remains in the samples. The results from Figure 4.12 and Figure 4.13 are promising and provide a strong indication that invasin is surface-anchored.

Since the above methods gave weak signals and reproducibility problems, they could not provide more than indications concerning the possible surface anchoring of invasin. Therefore, additional strategies for invasin detection were tested. The reason for the weak signal from staining of the bacterial cells could be due to the primary antibody having problems reaching the epitopes on invasin; caused by the epitopes being covered in the invasin 3D-structure. Therefore, the cells were incubated at 70ºC for 5 minutes before staining

to “open up” the invasin structure. This gives the antibodies better access to the epitops, which could lead to better binding. The result showed no significant differences between the *L. plantarum* stains and no increased signal strength. The result could indicate that the epitopes is not covered in the invasin structure, but it could also indicate that the method did not work; the invasin structure was not unfolded or cell lysis occurred.

Another hypothesis could be that the burying in the cell wall itself shields invasin from the primary antibody. The various recombinant *L. plantarum* strains were pre-threaded with mutanolysin or mutanolysin and lysozyme before staining of the cells. Interestingly, the results obtained were best for *L. plantarum* strains harbouring the pLp_1261Inv or pLp_1452Inv construct. Since these strains consist of the invasin version with all 5 domains and are thus expected to protrude further out of the cell membrane, these observations may be taken to indicate that the weak signals indeed are caused by invasin being buried in the cell wall.

In order to get a stronger indication or a confirmation of invasin being surface exposed other methods were tested. Isolation of cell wall fractions of *L. plantarum* strains harbouring various constructs was tested. Invasin is among the cell wall-associated proteins if invasin is detected in the cell wall fraction from *L. plantarum* strains carrying lipo-anchored constructs and not in the cell wall fraction from *L. plantarum* containing the pCytInv construct. Every experiment with this method yield an invasin signal in the negative control (pCytInv, no 5 in Figure 4.14). Therefore the observed invasin signal from strains expressing anchored invasin could be because of cell lysis.

Another method tested was the cell dot-blot. Unfortunately, the experiments gave often high background levels, but one cell dot-blot gave results (see Figure 4.15). This dot-blot showed a difference between *L. plantarum* containing constructs encoding lipo-anchored invasin and *L. plantarum* containing intracellular invasin (pCytInv) or empty vector (pEV), and the wild-type *L. plantarum* (without plasmid). However, all the negative controls had quite dark spots, while no visible spots were expected. In addition, it seemed like wild-type *L. plantarum* has a black spot in the undiluted sample similar to the positive control. This sample could be contaminated or some of the proteins on the surface of *L. plantarum* could have reacted with

the antibodies. Since the strains harbouring the negative controls did not give a clear negative result, the result from the cell dot-blot was uncertain.

The most frequently used strategy to surface-anchor proteins in Gram-positive bacteria is the use of a C-terminal cell wall anchor that includes a conserved LPXTH motif, and most experiences with detection of surface-anchored proteins are based on this anchor (Samuelson et al. 2002). Few other studies have used lipo-anchors to surface-anchor a protein and detection of invasin when it is anchored to the cell membrane with a lipo-anchor has been difficult to confirm in other experiments. Acheson et al. (1997) linked invasin together with a cell membrane (DppE) lipoprotein in *B. subtilis*. DppE is secreted and will most likely remain attached to the cytoplasmic membrane by a lipoprotein-anchor together with the invasin protein (InvS). When binding to the surface was examined, the antibody bound to both wild-type *B. subtilis* and *B. subtilis* with the DppE-Inv192 fusion protein to approximately the same extent. Acheson et al. (1997) showed that when most of the cell wall was removed by lysozyme, it was a fourfold increase in antibody binding to the recombinant strain compared to the wild-type. The result indicated that the DppE-inv192 protein was attached to the cell membrane but the protein was covered by the thick cell wall of the *Bacillus*. This could be the reason for the weak staining signal from *L. plantarum* containing constructs encoding lipo-anchored invasin, but since some difference between the strains were detected, all invasin proteins is most likely not entirely buried in the cell wall.

The time of harvest could have affected the amount of surface-anchored invasin. *L. plantarum* harbouring the different constructs were always harvested two hours after induction of gene expression to analyze surface anchoring. Harvesting of the strains after two hours was chosen because a considerable amount of invasin was detected in the culture supernatant at that time. Since the strains harbouring the Lp_1452 lipo-anchor constructs decreased in amount of viable cells, the harvesting of the cells could not be to long after induction of the target protein. It is possible that higher amounts of surface-anchored invasin could be obtained if longer culturing times were used. This is something that could be interesting to analyze. Tjåland (2011) showed that the Lp_1261 anchor used to anchor another protein gave a stronger staining signal when the *L. plantarum* strain was harvested 24 hours after induction compared to 2 hours. This indicates that the weak signal from invasin on the surface could be due to that the cells were harvested at a too early time point. Alternatively, it could be that the

invasin proteins are just secreted and never anchored to the cell membrane, thus all invasin proteins ends up in the growth medium. The staining signals achieved in Figure 4.12 and 4.13 could be due to invasin on its way out of the cell instead of anchored invasin.

Another method that could be used to detect invasin on the bacterial surface is to use indirect immunogold labeling combined with electron microscopy, but this method will most likely not give a strong signal if invasin is buried in the cell wall.

Despite considerable uncertainty, all results combined indicate anchoring of invasin on *L. plantarum*. An indirect way to analyze surface-anchoring of invasin is to analyze if *L. plantarum* harbouring constructs encoding lipo-anchored invasin enhance internalization by Caco-2 cells with β_1-integrin.

Internalization of *L. plantarum* strains by Caco-2 cells

5.7 Analysis of internalization of *L. plantarum* strains by Caco-2 cells

L. plantarum carrying constructs encoding lipo-anchored invasin are hopefully going to be used in a vaccine where they could promote internalization of the bacteria by M-cells. Since M-cells are very difficult to generate, a non-polarized human Caco-2 intestinal cell line was used to study internalization of the invasion-expressing strains. Because analysis of various recombinant *L. plantarum* strains after incubation with Caco-2 cells was unknown territory, direct visualization of the cells was performed to get an overview of challenges to overcome. The results in Figure 4.16 show that analysis of the relations between OD_{600} and CFU/ml for the strains was necessary, in addition to a better staining method to discriminate between bacteria inside or outside Caco-2 cells.

The gentamicin survival assay was used to estimate bacterial survival. After incubation with Caco-2 cells and gentamicin treatment, cell lysis of the Caco-2 cells and subsequent counting of viable bacteria (CFU) were performed. Ideally, only bacteria protected from the antibiotic inside Caco-2 cells will survive and grow on the agar-plates. Innocentin et al. (2009) demonstrated that *L. lactis* expressing invasin genes (from *L. monocytogenes* or *Staphylococcus aureus*) showed a clear difference in uptake (about 1000 fold higher uptake rate) in the gentamicin survival assay compared to the wild-type, but the wild-type showed also some uptake. Guimaraes et al (2005) showed in a gentamicin assay that the invasiveness

of *L. lactis* expressing internalin (InlA) from *L. monocytogenes* was higher (about 100 fold) invasiveness compared to the wild-type. In this study, the wild-type was also shown to demonstrate some uptake. However, the difference is not significant with or without an invasin gene when internalization of Caco-2 cells was examined. For example, Guimaraes et al. (2005) showed that *L .lactis* expressing internalin (InlA) was only marginally more efficient for DNA delivery (about 1% of the Caco-2 cells had the delivered gene).

In the present study, the gentamicin assay yield no significant differences between the *L. plantarum* containing the negative controls (pCytInv and pEV) and *L. plantarum* carrying constructs encoding lipo-anchored invasin (shown in Table 4.2 and 4.3). It is known from previous studies that addition of too many bacteria may lead to formation of biofilms in which the bacteria are protected from the antibiotic treatment although they are not internalized (Innocentin et al. 2009). Therefore, adding of more bacteria to the Caco-2 cells would probably not give a better internalization result. The amount of bacteria that survived in this experiments was very low, which indicates that the internalization was very low or absent.

The gentamicin survival assay is based on recovery of viable bacteria after internalization by Caco-2 cells, which is not representative if the bacterial cell lyse inside the Caco-2 cells before the incubation is over. *L. plantarum* is assumed not to survive inside Caco-2 cells for a long time; therefore it could be that the gentamicin survival assay did not show any internalization of the bacteria because the incubation time was too long. This assay was complemented with microscopic evaluation, using staining techniques. The staining method was set up in such a way that the bacteria inside Caco-2 cells stayed green while bacteria outside turned into a yellow-orange color. In the microscopic evaluation, more bacteria were observed bound to Caco-2 cells when *L. plantarum* encoded anchored invasin plasmids; the strains had increased affinity for the Caco-2 cells, but it should be noted that this experiment was done only once, which makes the results uncertain. However, the result indicates that invasin promotes binding to Caco-2 cells. The experiment did not convincingly show that *L. plantarum* strains promoted internalization, since only three bacteria were observed to probably be inside Caco-2 cells (compared to several hundred bacteria in the samples, combined). These three bacteria were *L. plantarum* harbouring the pLp_1261InvS or pLp_1261Inv constructs (Figure). *L. plantarum* containing these constructs have a better

result than *L. plantarum* containing the Lp_1452 anchor, which showed no bacteria inside Caco-2 cells. This is in line with the rest of the result in this thesis.

Internalization of *L. plantarum* strains carrying constructs encoding lipo-anchored invasin into Caco-2 cells was not shown, which could be because the invasin proteins are not anchored to the cell-membrane, or they could be anchored but to buried in the cell wall to manage to react with β_1-integrin receptors. One other reason could be that few invasin proteins are one the surface of the bacterial cells since invasin binding to the β_1-integrin receptor requires a high density of invasin molecules on the cell surface (Leo & Skurnik 2011). Alternatively, it could be the Caco-2 cells used here did not have β_1-integrins receptors on the apical surface. Non-polarized Caco-2 cells are supposed to express β_1-integrin but this was never tested; lack of β_1-integrin receptors could be the reason for lack of uptake. The present of β_1-integrin receptors could be checked by using β_1-integrin specific antibody on the Caco-2 cells. If the problem is that there are no β_1-integrins on the surface of the Caco-2 cells, other cells need to be used to analyze internalization. Options include the use of an M-cell/FAE model, where Caco-2 cells are co-cultured with Peyer's patch lymphocytes or a Raji human lymphocyte cell line (Corr et al. 2008).

5.8 Conclusions and perspectives

This thesis describes successful cloning, production and secretion of invasin together with the lipo-anchors from the lipoproteins Lp_1261 and Lp_1452 in *L. plantarum*. In addition, the result showed that invasin was most likely lipo-anchored. Internalization of *L. plantarum* harbouring the construct encoding lipo-anchored invasin into Caco-2 cells was not convincingly shown, but more experiments are needed to reach a final conclusion. Of the four anchor constructs described in this thesis is it not recommended to go further with the two constructs that had the anchors from Lp_1452 (pLp_1452InvS and pLp_1452Inv) because these strains have problems with growth and survival, and because there were no indications whatsoever that *L. plantarum* harbouring these constructs enhanced internalization of the bacteria. The two *L. plantarum* strains harbouring anchors from Lp_1261 (pLp_1261InvS and pLp_1261Inv) are more promising, especially pLp_1261InvS. Based on the result accumulated so far, invasin produced by the pLp_1261InvS containing strain is likely anchored (image 1 in Figure 4.12), and leads possibly to internalization (the CLSM images

may indicate some Caco-2 internalization; Figure 4.17). Furthermore, it is the strain with best growth (Figure 4.9) and intracellular invasin production (Figure 4.5).

The results showed that better experimental methods and alternative strategies to obtain M-cell translocating of lactobacilli should be considered. For example, it could be that invasin is anchored to the cell membrane, but that lipo-anchoring is not the best option due to shedding and burial of invasin inside the cell wall. It is recommended to exchange the anchor to another N-terminal anchor (such as N-terminal transmembrane anchor or anchor from other lipoproteins) which could lead to increased surface display of invasin. The lipo-anchors have been exchanged with other N-terminal anchors recently and the results are promising (Lasse Fredriksen, unpublished). It would be interesting to see if this would give a better exposing of invasin and subsequently a better internalization result.

Targeting of M-cells does not necessary have to be with invasin from *Y. pseudotuberculosis*; there are other invasive proteins that could be used. The results in this thesis do not indicate that the invasin protein should be replaced, but if it should appear that an alternative to invasin is required, other potential candidates are available. For example, the *Shigella* species have an invasin complex (IpaB, IpaC and IpaD) which is secreted upon host cell contact and seems to exploit β_1-integrin receptors (Palumbo & Wang 2006). Other receptors on M-cells that can be exploited includes glycoprotein 2 which recognizes FimH. FimH is a component of type I pili and transcytosis of FimH expressing bacteria through M-cells has been shown (Hase et al. 2009). However, invasin from *Yersinia* is the most studied invasive protein and it appears that it is the most common invasive protein used for therapeutic delivery application.

When it comes to using *Lactobacillus* as a delivery-vehicle for therapeutic proteins, it is an attractive strategy to target M-cells in order to achieve a better immune response. Although the results in this thesis did not convincingly show specific *L. plantarum* internalization into Caco-2 cells this strategy is still useful. Other anchoring strategies or other invasive proteins could be utilized instead which could lead to increased uptake *in vivo*. It should be noted that the fact that we were able to express and secrete invasin in itself is a major step forward and that several strategies for improved surface-display still can be tested. On the basis of the result described here and the general picture merging from the literature, there certainly is no reason to abandon the overall strategy underlying the present study.

Assuming that invasin gives increased internalization of the *L. plantarum*, *L. plantarum* must then also express an antigen against the target disease to hopefully get a successful initiation of the mucosal immune responses. To achieve this, the need for a different selection marker than antibiotics becomes apparent. One alternative to the antibiotic marker is the *L. plantarum* WCFS1 alanine racemase gene (*alr*) as the selection marker (Nguyen et al. 2011). In addition, it could be that constitutive expression of the antigens is more practical in a vaccine than the present inducible expression. Such optimized invasive *L. plantarum* strains may be ideal oral vaccines since they are non-pathogenic, and target M-cells to deliver antigens directly to mucosal immune initiation sites.

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APPENDIX

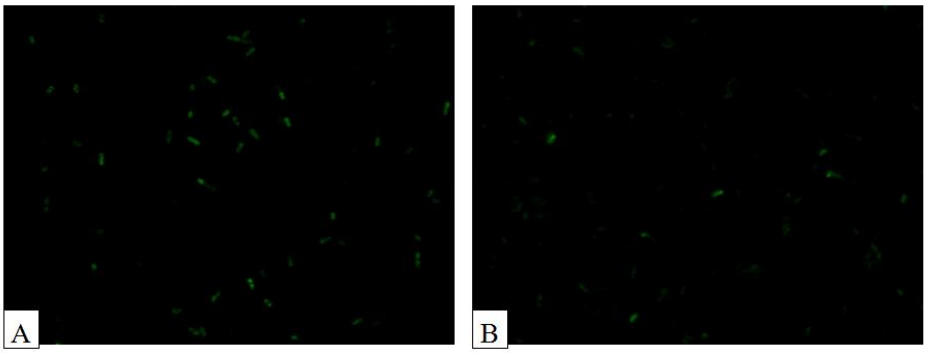

Figure A.1: (A) *L. plantarum* harbouring the pLp_1261InvS construct and (B) wild-type of *L. plantarum*, both stained with primary antibody conjugated with FITC to detect surface exposed invasin. Since the amount of cells is unclear and most likely not the same in the two images, the result is inconclusive.

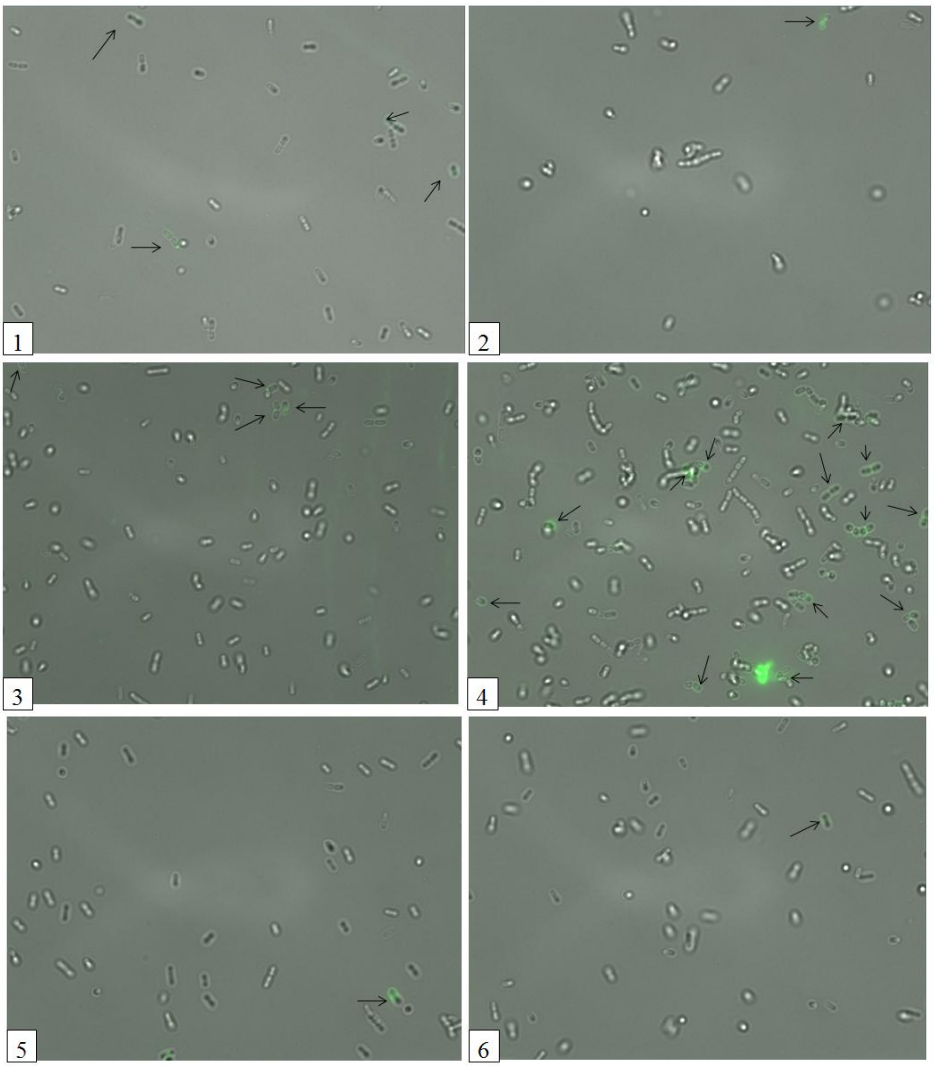

Figure A.2: Detection of surface-anchored invasin. The images show *L. plantarum* strains harbouring the following constructs: (1) pLp_1261InvS; (2) pLp_1261Inv; (3) pLp_1452InvS; (4) pLp_1452Inv; (5) pCytInv or (6) pEV (empty vector). The strains were incubated at 70ºC for 5 minutes before staining of the cells. The images were achieved by using indirect immunofluorescence and analysis by fluorescence microscopy. The images represent one representative image of three random images that was taken of the different strains. The arrows demonstrated where there are green spots on the bacteria (manual inspection).

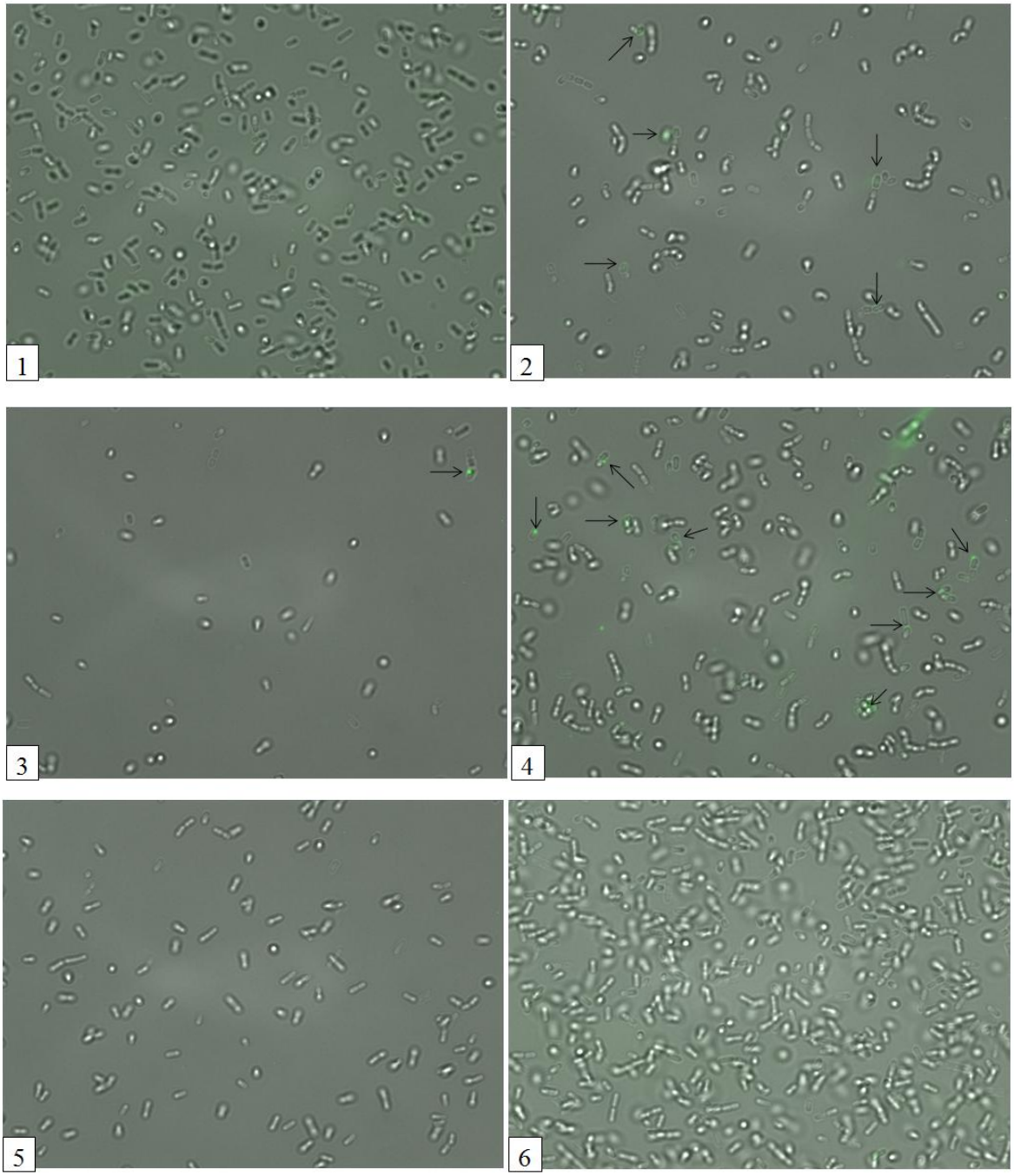

Figure A.3: Detection of surface-anchored invasin. The images show *L. plantarum* strains harbouring the following constructs: (1) pLp_1261InvS; (2) pLp_1261Inv; (3) pLp_1452InvS; (4) pLp_1452Inv; (5) pCytInv or (6) pEV (empty vector). The cell amount was adjusted after OD_{600} at the harvesting time to stain approximately the same amount of cells. The strains were treated with 50 U/ml mutanolysin and incubated at 37°C for 20 minutes before staining of the cells. The images were achieved by using indirect immunofluorescence and analysis by fluorescence microscopy. The images represent one representative image of three random images that was taken of the different strains. The arrows demonstrated where there are green spots on the bacteria (manual inspection).

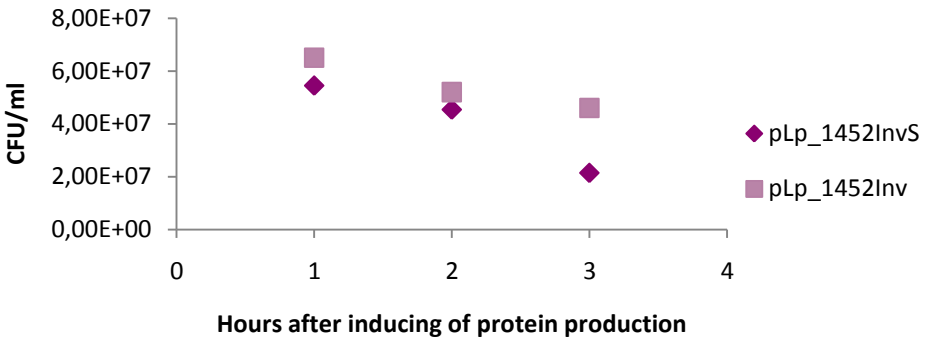

Figure A.4: CFU/ml, 1 to 3 hours after induction of invasin production, for *L. plantarum* harbouring the pLp_1452InvS or pLp_1452Inv constructs. The figure shows the average results from three parallels and each point represent the mean CFU/ml from 6 plates.

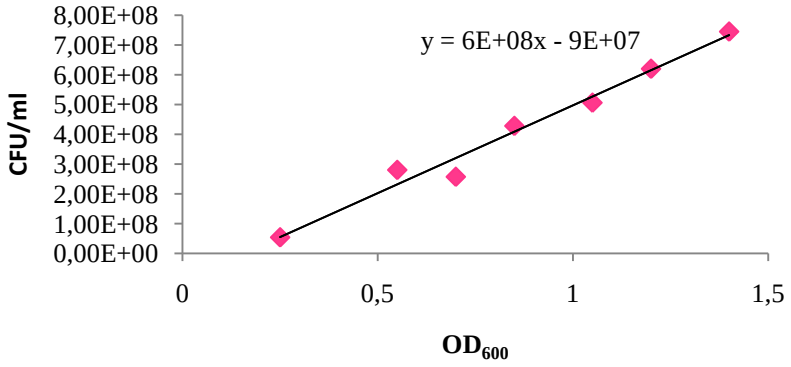

Figure A.5: The relationship between OD_{600} and CFU/ml for *L. plantarum* harbouring empty vector (pEV). This relationship was used to calculate approximately the same number of cells based on the OD_{600}-value at the time of harvest for *L. plantarum* harbouring the pLp_1261InvS, pLp_1261Inv, pCytInv or pEV constructs.