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Development of a non-GMO tuberculosis vaccine, using *Lactobacillus* as a delivery vehicle

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Abstract

The long term goal of this study was to develop towards a non-genetically modified organism (non-GMO) as human mucosal vaccine against tuberculosis (TB), using lactic acid bacteria (LAB) as a delivery vehicle for surface anchored antigen-containing proteins. LABs have characteristics that make them excellent as delivery vehicle for vaccines: they are not associated with pathogenesis, they have been used in the food industry for centuries, they have known probiotic effects, and they are natural inhabitants of the human gastrointestinal tract (GIT) where they interact with immune cells in the mucosal surfaces. Additionally, LABs have the ability to survive the tough environmental conditions following an oral administrated vaccine, e.g. as the low pH values ranging from 1.8–3.

In this study, the widely studied antigens Ag85B and ESAT6 from the TB causative agent *Mycobacterium tuberculosis (Mtb)* were fused together to a 41 kDa protein, and various anchors were attached. The proteins were expressed in, and extracted from *E. coli* strains before being anchored to *Lactobacillus spp* and *B. subtilis*. The anchors used were peptidoglycan binding domains with a Lysine Motif (LysM domain). The two anchoring domains were tested; a single LysM domain from the *L. plantarum* protein Lp_3014 (extracellular transglycosylase), and the double LysM domains from the *L. plantarum* protein Lp_2162 (muropeptidase). To test the stability of the resulting constructs, *L. plantarum* loaded with recombinantly produced LysM containing Ag85-ESAT6-DC were subjected to 2% bile and pH values ranging from 1.8–6.5, showing that the antigen-containing proteins were able to resist degradation when incubated at low pH. This indicated that the protein-loaded bacteria would be capable to survive incubation at low pH values. However, the co-incubation of bile and the negative control (*L. plantarum* not displaying Ag85-ESAT6-containing protein) were revealing positive signals, thus implying that the positive signals achieved from co-incubating the protein-loaded bacteria and 2% bile could contain false positives, when analyzed by western blotting and flow cytometry. Incubation in bile should, therefore, be repeated using another method.

Previous studies have shown that LAB displaying the Ag85-ESAT6-DC on their surfaces creates promising immune responses. The current results, thus provide a promising starting point towards achieving the final goal; developing a non-GMO vaccine against TB, that elicits better and longer lasting immune responses than the now available BCG-vaccine.

Sammendrag

Det langsiktige målet for denne studien var å utvikle en ikke-genetisk modifisert organisme (ikke-GMO) til å bruke som en menneskelig vaksine mot tuberkulose, ved hjelp av melkesyrebakterier som overleveringsagenter for overflatefestede proteiner som inneholder antigenene. Melkesyrebakterier har egenskaper som gjør dem svært godt egnet som overleveringsagenter for vaksiner; de er ikke assosiert med patogenesitet, de har vært brukt i matindustrien i århundrer, de har kjente probiotiske effekter i tillegg til deres naturlige tilstedeværelse i det humane mage-tarm systemet, der de interagerer med immuncellene i slimhinnene. Melkesyrebakterier har i tillegg evnen til å overleve det utfordrende miljøet som følger en oralt administrert vaksine, f.eks. lave pH-verdier, i området 3–1.8.

I denne studien ble de bredt-diskuterte antigenene Ag85B og ESAT6 fra tuberkulosens utløsende agent, *Mycobacterium tuberculosis (Mtb),* sammensatt til et protein på 41 kDa tilsatt forskjellige ankere. Proteinene ble uttrykt i og ekstrahert fra *E. coli*-stammer før de ble ankret til *Lactobacillus spp* og *B. subtilis.* Ankerene som ble brukt var peptidoglucan bindende domener med et Lysine Motif (LysM domene). De to ankringsdomenene som ble testet; et enkelt LysM domene fra *L. plantarum*s protein Lp_3014 (extracellulær transglykosylase), og det doble LysM domenene fra *L. plantarum*s Lp_2162 (muropeptidase). For å teste stabiliteten til det resulterende konstruktet, ble *L. plantarum* ladet med det rekombinante produserte LysM inneholdende Ag85B-ESAT6-DC følgende inkubert i 2 % galle og pH-verdier som strekker seg fra 1.8–6.5. Dette viste at det antigen-inneholdende proteinet var i stand til å motstå degradering når den ble inkubert i lave pH-verdier. I motsetning viste den negative kontrollen (*L. plantarum,* ikke ladet med det antigeninneholdende proteinet), når inkubert i galle, positive signaler. Dette gav en indikasjon på at det oppnådde positive resultatet av den inkuberte proteinladede bakteriecellen i galle, muligens inneholdt falske positiver etter å ha blitt analysert med western blotting og væskestrømscytometri. Inkubering i galle burde derfor gjentas ved å bruke en annen metode.

Tidligere studier har vist at melkesyrebakterier ladet med antigenet A685B-ESAT6-DC på celleoverflaten oppnådde lovende immunresponser. De nåværende resultatene gir et lovende startpunkt mot å oppnå det optimale målet: å utvikle en ikke-GMO vaksine mot tuberkulose, som fremprovoserer bedre og lengre immunresponser enn den nå tilgjengelige BCG-vaksinen.

Abbreviations

2162-TB – the protein 2162-Ag85B-ESAT6-DC

3014-TB – the protein 3014-Ag85B-ESAT6-DC

BCG – Bacille Calmette-Guèrin

BSA – Bovine Serum Albumine

DC – Dendritic cells

FITC – Fluorescein isothiocyanate

GIT – Gastrointestinal tract

GRAS – Generally regarded as safe

LAB – Lactic acid bacteria

LysM – lysin Motif domain

MDR-TB – multidrug resistant tuberculosis

Mtb – Mycobacterium tuberculosis

NAG – N-acetylglucosamine

NAM – N-actelymuramic acid

Ni-NTA – Nikkel Nitrilotriacetic Acid

PBS – Phosphate Buffered Saline

PCR – The polymerase chain reaction

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TB – tuberculosis

XDR-TB – Extensively drug-resistant TB

CONTENTS

1. INTRODUCTION:

Tuberculosis (TB) is a single infections agent causing the second highest mortality worldwide, after HIV/AIDS (WHO 2014). After the successfully developed BCG-vaccine, based on weak or dead *Mycobacterium bovis,* the general assumption was that TB no longer posted a threat. Latest researches have revealed 80% effective protection in children, when given the BCG-vaccine. However, this protective effect is lost after reaching 25 years of age, and vaccination at this age causes no measurable protection against TB (Andersen & Doherty 2005). Another urgent issue is the development of a multi-resistant TB (MDR-TB). The development of MDR-TB has started the race to market the next TB-vaccine, hopefully resulting in a better and longer lasting immune response (Velayati et al. 2009; Wang et al. 2013).

The long-term goal of this study is to contribute to the development of a new non-GMO TB vaccine. This was done by exploiting two well-documented antigens from the causative agent of TB, *Mycobacterium tuberculosis* (*Mtb*). Using peptidoglycan anchors, the combined antigens were bound to harmless bacteria such as *Lactobacillus spp.* and *B. subtilis,* which hopefully could result in a non-GMO bacterial vaccine.

1.1 Tuberculosis and the further development of MDR-TB and XDR-TB

According to the World Health Organization tuberculosis (TB) fact sheet (WHO 2014), TB is a single infectious agent that causes the second highest number of mortality worldwide, after HIV/AIDS. In 2012, as many as 1.3 million people died from TB, and incidents are documented from all over the world, with the highest level of mortality in Asia.

The causative agent of TB is *Mycobacterium tuberculosis (Mtb),* which spreads through the air and affects the lungs. Typical TB symptoms are weight loss, night sweats and coughing. The drug-sensitive TB can be treated with a six months antibiotic cure, using the most powerful and commonly used drugs (first line anti-TB drugs), isoniazid and rifampicin (WHO 2013). Increased use of poor quality anti-TB drugs has led to the development of a multi-resistant (MDR) *Mtb,* causing the MDR-TB. MDR-TB escalates the need for second lined anti-TB drugs, which is limited or not available.

Searching for MDR-TB cases, revealed a new type of resistant *Mtb*, causing extensively drug-resistant TB (XDR-TB) and totally drug-resistant TB (TDR-TB) (Velayati et al. 2009; WHO 2013), which are resistant to all second line anti-TB drugs. The negative development of MDR-TB is difficult to control and the treatment equally difficult, because of the expensive and unavailable chemotherapy and second line anti-TB drugs. Importantly, one untreated and undetected individual could infect as many as 15 contacted individuals per year (WHO 2009).

1.2 The almost one-century old Bacille Calmette-Guèrin vaccine

Mycobacterium tuberculosis (Mtb) spread quickly through the air and infects large populations of people, causing TB, MDR-TB and XDR-TB. The treatment is expensive, limited and unavailable in some countries, making prevention against *Mtb* infection the best alternative to defeat new TB cases (Ottenhof & Kaufmann 2012). The best precaution available today is the Bacille Calmette-Guèrin (BCG) vaccine, which consists of weak or dead *Mycobacterium bovis.* In 80% of the cases, the BCG vaccine results in significant TB protection in infants, but the protective effect weakens in individuals reaching 25 years of age (Andersen & Doherty 2005). In addition, there is no protective effect measured after vaccination as adults. Despite limitations, the BCG vaccine is the only alternative available on the market today, and WHO considers BCG as an important tool for the treatment of TB, until an improved vaccine against TB is available at the market.
Of the 55 species of the genus *Mycobacterium*, half of these are known to cause diseases in humans (Ottenhof & Kaufmann 2012). Little is known about the interactions between host and pathogen, which precludes a targeted vaccine development and leads to a broad range of new vaccine approaches. Today, at least 15 new vaccine candidates are in the development pipeline, one of which was based on improving the already existing BCG vaccine by co-administration of dominant antigens such as Ag85B (Ottenhof & Kaufmann 2012). Other approaches include subunit vaccines, i.e. vaccines where several antigenic proteins are combined to elicit stronger immune responses (Ottenhof & Kaufmann 2012). An upcoming approach, not yet in the TB vaccine development pipeline, is the use of lactic acid bacteria (LAB) as a delivery vehicle for vaccines (Wells 2010; Wells & Mercenier 2008).

1.3 Lactic acid bacteria

Lactic acid bacteria (LAB) are gram positive bacteria with low GC content, and are characterized by their major product from the carbohydrate fermentation process, lactic acid. LABs are found in a broad range of food fermentation products from dairy, meat and vegetable, where they produce lactic acid to lower the food's pH and prevents spoilage (Daniel et al. 2011). LABs are not associated with pathogenic effects; some LAB genera, as *Lactobacillus* have a "Generally Regarded As Safe" status (GRAS), and are considered as probiotics. Probiotics are evaluated to give health benefits to the consumer, according to the Food and Agriculture Organization of the United Nations (FAO) and WHO (FAO/WHO 2001). Several strains of *Lactobacillus* are natural habitants of the human and animal's gastrointestinal tract (GIT), and have the ability to survive transportation through the GIT.

1.3.1 *Lactobacillus* as a delivery vehicle

The use of *Lactobacillus* as a delivery vehicle for orally administrated vaccines is conceivable because of their GRAS status, long record of safe consumption, food fermentative properties, and their ability to survive and even persist in the gastrointestinal tract (GIT). The mucosal surfaces in the GIT are the main entry sites for pathogens to enter into the body, and are therefore packed with immune cells (Lea 2006). To succeed as a delivery vehicle, the cells must survive the GIT environments and interact with the mucosal surfaces, which are in fact properties *Lactobacillus* possesses (Wells & Mercenier 2008). The mucosal route has the potential to stimulate both a systemic and mucosal immune response, by interacting with the inductive site of the mucosal immune system (see section 1.7 and Figure 1.4 for more details).

Recombinant lactic acid bacteria (rLAB) can be engineered to express targeting molecules or adjuvants, and has given promising secretory IgA- and antigen specific-immune responses (Daniel et al. 2011). rLAB, even though strain dependent, have revealed the potential to elicit immune responses (Wells & Mercenier 2008), and the possibility to sustain stress factors as bile and low pH values (Daniel et al. 2011; Kõll et al. 2008). These are important properties to possess when used as a delivery vehicle. The elicited immune response depends on the rLAB genera used, the antigen produced and the disease investigated (Daniel et al. 2011).

Lactobacillus engineered to produce and deliver antigens are one of the most commonly used LAB genera for mucosal vaccination.

The genus of *Lactobacillus* consists of more than 60 species with varying fermentation products, GC content and metabolisms (Wells & Mercenier 2008). This gives a broad range of alternatives used as a delivery vehicle. Many aspects need to be considered before the proper *Lactobacillus* strain is chosen as the delivery vehicle. Examples are: colonization abilities in human cavities (mouth, stomach, vaginal, small or large intestine), if they are "commercial" strains (daily used in industry, in the fermentation process) and assesses immune-adjuvant- and immune-stimulating capacities (Daniel et al. 2011). How cells are produced also affects the elicited immune response; in an *in vitro* study using *L. plantarum* as a delivery vehicle for antigens, only *L. plantarum* harvested in stationary phase, in contrast to exponential phase, gave a molecular response (Daniel et al. 2011). To elicit the proper immune response, delivery vehicles need to present the antigens at the proper inductive site (correct location) (Kim et al. 2012), and avoid immune tolerogenic environments at the mucosal surfaces. How immune cells are targeted and how they respond also affect the resulted immune response (Manicassamy & Pulendran 2011).

In an exciting review by Wells (2010), 27 different targeting vaccines using LAB as a delivery vehicle have reached the stage of animal model testing. One of which, uses *Lactobacillus acidophilus* as a delivery vehicle for the protective antigen (PA), yielding protection in mice against *Bacillus anthreacis* (Mohamadzadeh et al. 2009; Wells 2010).

1.3.2 *Bacillus subtilis* as a delivery vehicle

Other bacteria than LAB may also be considered as delivery vehicles and one of these are *B. subtilis*. *B. subtilis* is a gram positive bacterium, suitable as delivery vesicle in vaccine development against TB, due to the following advantages; *B. subtilis* is not associated with pathogenic activity, they are low in costs, thermo-stable, non-invasive and easily genetically manipulated (Amaguni & Tzipori 2012). *B. subtilis* is probiotic labeled and proven to be safe to humans (Cutting 2010). *B. subtilis* and *B. subtilis*' endospores have been used as antigen delivery vehicles in the human gut (Amaguni & Tzipori 2012), and researches have revealed the spore's ability to germinate in the gastrointestinal tract (GIT), providing an additional route for antigen delivery.

The delivery system (using *B. subtilis* or the endospore of *B. subtilis*) makes a better vaccine than the purified antigen alone, since the antigen displayed at the cell is presented directly to the immunization sites and generates a stronger immune response (Amaguni & Tzipori 2012). Similar reasoning applies to other bacterial delivery systems, including LAB.

1.4 The antigens of *Mycobacterium tuberculosis* used in this study

The sequencing of the *Mycobacterium tuberculosis (Mtb)* genome in combination with the progress in molecular studies of the bacterium resulted in the discovery of a broad range of antigens. In the following processes the most promising and relevant antigens from *Mtb* were combined to create a protein more suited to elicit a better immunity than the BCG-vaccine. Two of the most tested and promising antigens of *Mtb* are Ag85B and ESAT6, which are secreted upon infection (Cole et al. 1998).

The primary antigen discovered in vaccine-development was antigen 85B (Ag85B), and the first to be used in animal testing (Horwitz et al. 2000). A higher protective immunity against *Mtb* aerosol was discovered for recombinant BCG (rBCG) expressing and secreting Ag85B encoded protein, in contrast to the normal BCG (Horwitz et al. 2000). The Ag85 family (Ag85A, B and C) encodes for abundant secretory proteins of mycolyl transferases which takes part in cell wall mycolic acid synthesis. The family members are closely related, but only Ag85A and B are known to elicit human T-cell response (Takatsu & Kariyone 2003).

Another early identified T-cell targeted antigen was the Early Secreted Antigenic Target-6 (ESAT6) antigen, which unfortunately revealed no inherited immunity towards TB (Brandt et al. 1996). ESAT6 is part of a 23 membered multigene family in *Mtb*, and encodes a small (6 kDa) immunogenic protein. Expressed and purified ESAT6 as a vaccine gave a lower specific antigen response (Immunoglobin G, IgG) than Ag85B and BCG (Horwitz et al. 2000; Huygen et al. 1996; Kamath et al. 1998; Li et al. 1999). ESAT6 needed a stronger adjuvant, and combined with Ag85B, the enhanced immune response was stronger (Dietrich et al. 2006; Doherty et al. 2004). Ag85B-ESAT6, a 41 kDa fused protein, resulted in strong immune responses when given as a vaccine; either as DNA or as the encoded protein (Dietrich et al. 2006). The fused protein results in much higher protection in animal models (mouse and guinea pig) than the antigens given individually (Doherty et al. 2004). The fused protein also promotes high immune responses

against TB in mouse, guinea pig and non-human primate models (Langermans et al. 2005; Olsen et al. 2001; Olsen et al. 2004). Several other promising antigens are currently under investigation, including Rv2029c, RV1733 and RV0315 (Byun et al. 2012; Sutherland et al. 2013).

1.5 Anchoring heterologous proteins to vectors

As discussed in section 1.3.1 and 1.3.2, *Lactobacillus* and *B. subtilis* has many advantages as delivery vehicles, and are therefore often used as delivery vectors. Heterologous proteins can be displayed at the cell surface through various types of cell wall anchoring. The anchoring between the heterologous protein and the delivery vehicle needs to be as specific as possible, to ensure the attachment to a proper nonpathogenic bacterium. The protein must avoid degradation at the cell surface, and be presented as a structural and functional protein to immune cells in order to elicit an immune response.

Secreted proteins that anchor to the cell wall or membrane in gram positive bacteria can be divided into four main groups, using different anchoring mechanisms (Figure 1.1):

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

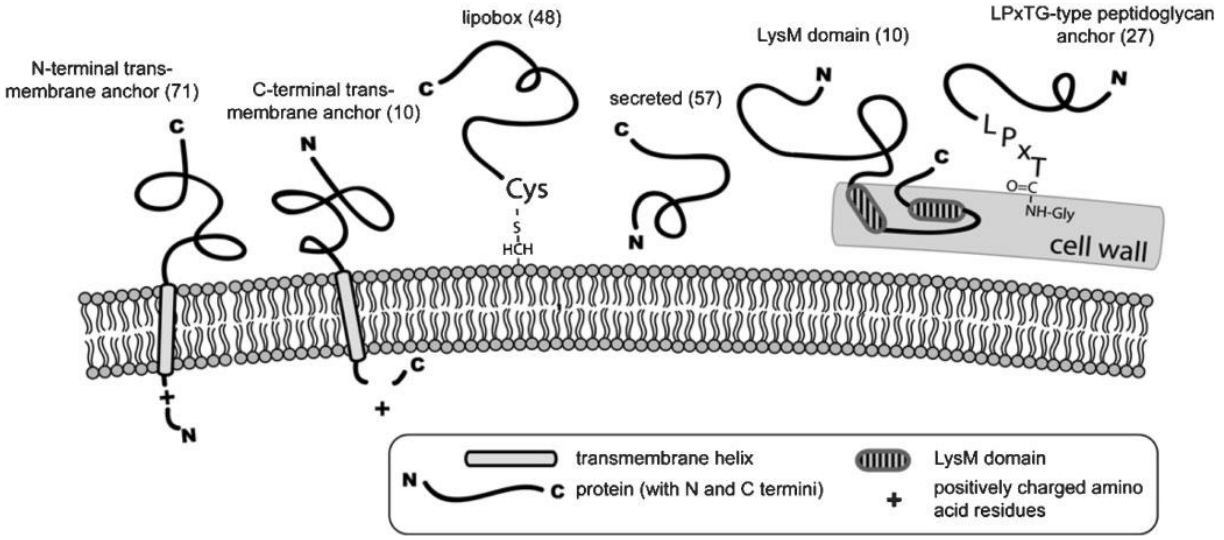

Figure 1.1: Illustration of different proteins secreted by *L. plantarum*, anchored to the cell wall or membrane. The predicted number of proteins secreted by *L. plantarum* containing each anchoring domain is given in parentheses. Furthest to the left in the picture are two transmembrane proteins, N- and C-terminally anchored to the cell membrane. Third from the left is the membrane-anchored lipoprotein, through its lipobox domain. Furthest to the right, are the cell wall anchors; LysM domains (non-covalently anchored), and the LPxTG (covalently anchored). For more information see the text. The picture is taken from (Boekhorst et al. 2006).

Transmembrane proteins are a large group of proteins that anchors either N- or C-terminal to the membrane in gram positive bacteria (Figure 1.1). The N-terminally anchored proteins lack the cleavage site in the signal peptide and are intact upon anchoring, while C-terminally anchored proteins are cleaved before anchored (Kleerebezem et al. 2003). Lipoproteins contain a C-terminal lipobox motif. The motif is recognized by an enzyme, that covalently anchors the mature proteins to the long-chain fatty acids that are inserted into the cell membrane, through a thioehter linkage (Figure 1.1) (Kleerebezem et al. 2003). LPxTG are a well-known group of proteins that are characterized by their C-terminal LPxTG domain, which are recognized by the sortase (srtA) enzyme. Upon recognition, srtA cleaves the domain and covalently anchors the protein to the peptidoglycan (Kleerebezem et al. 2003). Non-covalent cell wall binding proteins employ cell-wall binding domains such as the choline-binding domains, SH3 domains, SLH domains and LysM domains. The Lysine Motif (LysM) domains are well known peptidoglycan binding domains (Kleerebezem et al. 2003).

1.5.1 The peptidoglycan layer

The peptidoglycan layer is a thick and complex layer surrounding gram positive bacteria. The peptidoglycan layer varies in ultrastructure, and comprises a complex multilayered network containing peptidoglycan, polysaccharides and either teichoic acid or teichuronic acid (Ghuysen 1986). The sugar units in peptidoglycan are *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), and the repeating unit in the polysaccharide part is a disaccharide of these two sugars. NAG and NAM containing polysaccharides are cross-linked by peptide bridges (Ghuysen 1986). The peptide cross-linking bridges can vary chemically and may be branched (Figure 1.2).

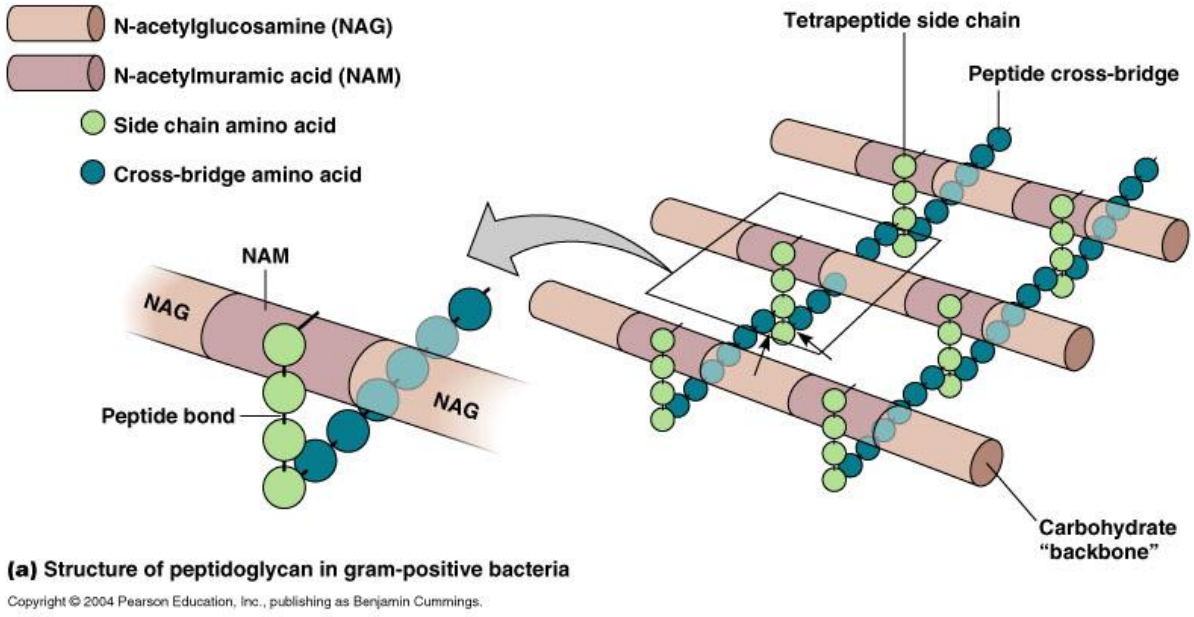

Figure 1.2: Schematic overview of the general peptidoglycan structure. The NAM and NAG monomers constitute glycan strains, also called the carbohydrates "backbones" of the structure (nude/purple). These are linked together by cross-bridged amino acids (blue). Side chains arise from the side chain amino acids (green), which appears as tetrapeptide chains. The picture is taken from (Biocourses 225).

It is the structural and compositional changes in the peptidoglycan layer (Figure 1.2) that contributes to the variation found in the cell walls of gram positive bacteria. Anchoring domains, such as LysM domains (see section 1.5.2) binds to these varying structures, possibly resulting in a specific bonding between protein and bacteria.

1.5.2 Lysine Motif (LysM) domain

The LysM domain is a specific protein domain that binds non-covalently to peptidoglycan and chitin. LysM domain-containing proteins are found in eukaryotes and prokaryotes, and a Pfam search on LysM domain results in more than 4000 hits (Buist et al. 2008). LysM domains are usually 44–65 amino acid residues in length, and multiple copies are separated by a conserved linker region, called PST (rich in proline, serine and threonine) (Ohnuma et al. 2007). The PST region is rather flexible and can vary in both length and composition. LysM domains can be located at the N-terminus or C-terminus of a protein, but are also found at internal positions. There is a broad range of LysM-containing proteins in nature, occurring for example in proteins involved in pathogenesis, bacterial cell wall-degrading enzymes, and proteins involved in plant recognition of symbiotic bacteria (Bosma et al. 2006). It has previously been pointed out that LysM domains could be used to anchor heterologous proteins to gram positive bacteria, for both medicinal and industrial purposes (Bosma et al. 2006).

LysM domains are most likely to interact with NAG monomers in the peptidoglycan layer (see section 1.5.1 and Fig. 1.2), because, by increasing the amount of added NAG monomers in the peptidoglycan through the elongation of carbohydrate ligands, increases the binding-affinity towards LysM (Buist et al. 2008; Wong et al. 2013). LysM domains binding-sites show conservation across phyla, and neither plant nor bacterial LysM-containing proteins discriminate between NAG-containing (hetero)polymers in peptidoglycan and the NAG homopolymer chitin (Wong et al. 2013). Additionally, increasing the number of LysM domains in a protein, proportionally increases the binding affinity towards peptidoglycan and chitin polymers (Wong et al. 2013).

L. plantarum is predicted to encode 223 extracellular proteins (some of which are shown in Figure 1.1), where 10 of these are predicted to contain one or more copies of the LysM domain (Boekhorst et al. 2006). Two of these proteins containing LysM domains; are the single LysM containing protein Lp_3014 and the double LysM domain containing protein Lp_2162 (Fig. 1.3) (Boekhorst et al. 2006).

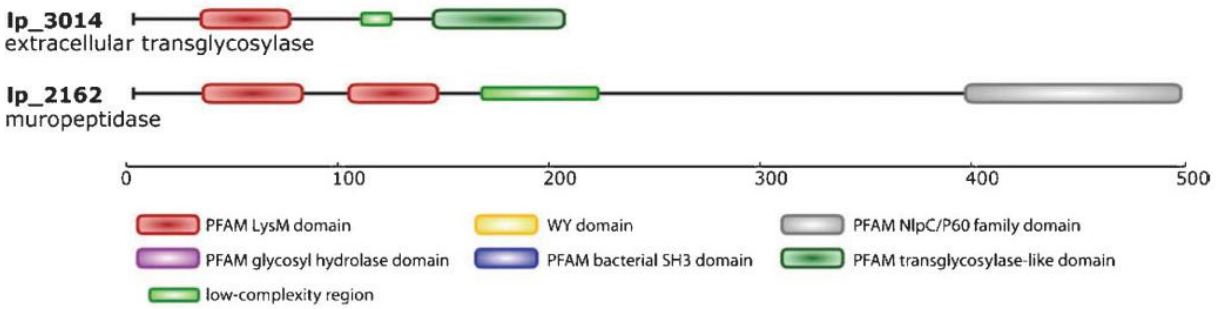

Figure 1.3: Illustrates two of the *L. plantarum* WCSF1 cells' extracellular proteins that contain LysM domain. The LysM domains are marked using red boxes and are located in the N-terminal of the proteins. The proteins differ in both sizes, expected functions and the number of LysM domains. See text for more information. The Figure is taken and altered from (Boekhorst et al. 2006).

Lp_3014 is predicted to be extracellular transglycosylase, whereas Lp_2162 is predicted to be muropeptidase (Boekhorst et al. 2006). The binding abilities of the LysM anchors are highly dependent on the proteins they are part of (Bosma et al. 2006), and some LysM domains even reveal strain specific binding affinities (Navarre & Schneewind 1999). The single and double LysM anchors from Lp_3014 and Lp_21621, respectively, were used in the present study to discover the better anchor for anchoring the heterologous protein to the peptidoglycan layer surrounding the gram positive bacterium.

1.6 Genetic modified organism

The use of genetic modified organism (GMO) in Norway has been administrated through the gene-technology law since its origination in 1993 (lastly altered in 2001) (Lovdata 2013). The law corresponds to the EU-regulations on GMO-use with some differences. In Norway a release of GMOs to the market has to be useful and in line with the principle of sustainable development. In addition Norway looks at each situation (product) individually, and Norwegians think of themselves as stricter than the EU regulations dictate (Biotekforum 2014). By this law; GMO is defined as plants, animals and microorganisms that have their genetic composition altered through gene- or cell-technology (Lovdata 2013). The objective function (§ 2) of the law states

(Lovdata 2013); legal provisions on genetically modified organisms also apply to substances and products consisting of or containing genetically modified organisms.

There is a natural skepticism towards GMO, mostly because of the unknown effects following the use or release of GMO in nature (The non-GMO project 2011). GMOs are banned in 60 countries, including Japan, Australia and the EU members. Due to skepticism and regulations, killed lactic acid bacteria (LAB) in vaccine delivery are considered advantageous over living LAB (Wells 2010; Wells & Mercenier 2008). Development of a non-GMO LAB-based vaccine will have an advantage; this may be achieved by loading non-GMO LAB with externally added vaccine molecules that contain anchoring domains such as LysM domains.

1.7 The human mucosal immune system

For a vaccine to elicit an immune response in the individual ingesting it, the vaccine must interact with the immune cells in the mucosal surfaces. The main entry into the body is through the mucosal surfaces covering the respiratory tract, the digestive tract and the urinary tract (Lea 2006). Mucosal surfaces are therefore packed with immune cells that must distinguish between pathogens, nutrition and the natural microflora of the gut. The most exposed being the digestive tract stretching from pharynx to the rectum, and the broad range of tasks corresponds to the varying immune systems found in these areas, whereas the most known is the gastrointestinal tract (GIT) (Lea 2006). The GIT stretches as a continuous long tube from mouth to colon, lined with mucus membrane (Richaud-Patin et al. 2005). Both the adaptive and the native immunity is present at the mucosal surfaces in the intestine, including particular structures as the payer's patches, epithelia and physical barriers (Lea 2006; Richaud-Patin et al. 2005) Antigens presented by live bacteria will interact with the immune cells located in these structures to elicit an immune response (Figure 1.4).

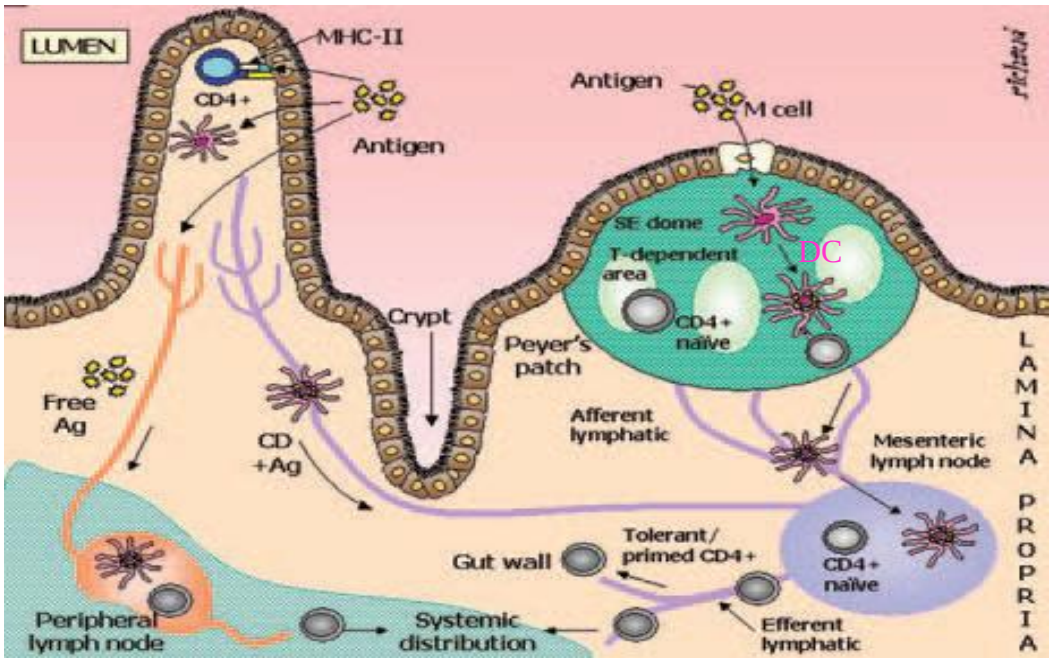

Figure 1.4: The immune defense line in the mucosal surfaces located in the intestine. The epithelial cells form a physical barrier between the gut lumen and lamina propria. Antigens in the lumen are picked up by M-cells or dendritic cells (DC), and are transferred into the payer patches where they meet T-cells. The intact antigen is then transferred to professional antigen-presenting cells (APC) which further activates the mesenteric lymph nodes. The activated lymphnodes migrate to the mesenteric lymph node, resulting in the started immune response. See text for more information. Figure modified from (Mowat 2003).

The physical barrier in the intestinal immune system consists of the gut epithelium, with tight junctions in between, only letting 2 kDa molecules pass. Beneath the epithelium line is the highly organized lymphoid tissue, with the common name GALT (Gut-associated lymphoid tissue). GALT is an organized tissue including the payer's patches, the lymphoid follicles and the mesenteric lymphnodes (Richaud-Patin et al. 2005). Antigens interacting with the mucosal surfaces in the intestine are either taken up by dendritic cells (DC) or Microfold (M)-cells. M-cells interact and activate the professional antigen-presenting cells (APC), as the DC and macrophages. Activated APC interact and activate strategically placed B- and T-cells in the payer's patches (Fig. 1.4). Any activated immune cells can migrate to mesenteric lymphnodes and induce an immune response (Figure 1.4).

1.7.1 Dendritic cells and the elicited immune response

Dendritic cells (DC) are found in an immature state in most tissues throughout the body, especially in T-cell rich areas (T-cells only recognize antigens processed and presented by antigen-presenting cells, APC). DC and B-cells are examples of professional APC, which ingest intruding microbes or particles by endocytosis, process these and present the antigens on their cell surfaces attached to major histocompatibility complex (MHC) class II molecules (Lea 2006). The resulting immune response depends on the processing of antigens, the antigen-presenting cell-type, and the type/amount of adhesion and co-stimulating molecules between activated APC and T-cell (Lea 2006). These variations determine the interaction with T-cells and subsequent T-cell activation (Lea 2006).

Depending on DC-surfaced expressed markers, three subpopulations of DC are found in the gut (Richaud-Patin et al. 2005), named myleoid, lymphoid and the double negative. DCs either receive antigens from M-cells or capture antigens in the gut lumen through their elongations between epithelial cells. Immune responses elicited by DC depends on how the DC interact with the vaccine, were in the GIT the DC interacts and the inductive signals from the surrounding microenvironment (Manicassamy & Pulendran 2011).

DCs are a heterogeneous, complex and multifunctional group of APCs, and they play a critical role in the activation of naive T-cells. Targeting DC is strategically to induce an antigen-specific immunity (Tyler et al. 2013), and have been obtained using a 12-mer DC peptide (Mohamadzadeh et al. 2009; Mohamadzadeh et al. 2010; Tyler et al. 2013). In a previous study, Mohamadzadeh (2009) revealed the increased survival rate by 75 % in mice challenged with *Bacillus anthracis* (see section 1.3.1), after oral treatment using antigens fused to DC-targeting peptide.

1.8 The outline of this study

The aim of this study was to contribute to the tuberculosis (TB) vaccine development, by developing methods for anchoring vaccine candidates to the peptidoglycan layer in gram positive bacteria using Lysine Motif (LysM) domains. In a previous study by Øverland (2013), it was shown that *lactobacillus* producing the Ag85B-ESAT6 antigen with a DC-targeting sequence fused to the C-terminus, anchored N-terminally to the bacterial surface gave promising responses.

Attachment of LysM domains to this antigen-containing protein would open up the opportunity to produce the protein in *E. coli*, purify it, and then anchor it to the peptidoglycan layer of the bacteria used as delivery vehicle, as for example lactic acid bacteria (LAB). The resulting LAB charged with antigen-containing proteins would then not be a GMO. Several LysM-containing fusion proteins were generated and their anchoring to various bacteria was studied.

Two anchors were tested in this study, the single LysM domain from the protein Lp_3014, and the double LysM domain from the protein Lp_2162, both naturally found in the secreted proteins from *L. plantarum*. *Lactobacillus* strains and *B. subtilis* are both capable of resisting the low pH in the stomach and the high bile concentrations in the intestine (Marteau et al. 1997) and both were therefore tested. In total, four species were assessed as carriers; *L. rhamnosus GG, L. gasseri, L. plantarum* (WCFS1) and *B. subtilis.*

The idea was to orally administrate the resulting antigen-charged strains, which would increase the need for the displayed protein to resist degradation during passage through the rough environments in the digestive tract (Wells 2010; Wells & Mercenier 2008). Therefore, the effects of environmental factors such as pH and bile, on the stability of the displayed antigens were tested in this study.

In summary, the following four experimental steps were carried out:

1. One or two LysM domains were attached N-terminally to the Ag85B-ESAT6-DC fusion protein (Øverland 2013), resulting in the two final fusion proteins; 3014_Ag85B-ESAT6-DC (called 3014-TB) and 2162_ Ag85B-ESAT6-DC (2162-TB). Both fusion proteins included a $(His)_7$-tag attached to the N-terminus of 3014 and 2162. Gene fragments encoding these fusion proteins were cloned into *E. coli* plasmids (pBAD) to produce the proteins in large quantities.
2. The possibility for *E. coli* strains to express 3014-TB and 2162-TB were evaluated. Following intracellular production, attempts were made to purify the proteins from cell extract using the His-tag t the N-terminus.
3. The final fusion proteins were further anchored to the cell surfaces of *Lactobacillus* and *B. subtilis*, using the N-terminal attached LysM domains. Following the display of 3014-TB and 2162-TB on the bacterial cells, the displayed proteins were visually semi-quantified

using flow cytometry and western blots. Following the semi-quantification, the two fusion proteins displayed at the cell surface were compared.

4. The stability of the cells charged with either 3014-TB or 2162-TB, and the LysM domain binding strength were tested by subjecting the bacteria to conditions resembling those in the digestive tract, namely low pH and the presence of bile. Storage stability was also assessed.

2. MATERIALS

2.1 Laboratory consumables

Laboratory consumables	Supplier
Centrifugal device (10K WCO)	Pall
CL-Xposure™ Film	Thermo
Scientific	
Cuvettes	
Disposable cuvettes, 1.5 ml	Brand
Eppendorf tubes	Axygen
Regular 1.5 m	
Sequencing tubes	
FastPrep® tubes and lids	Fisher Scientific
Nunc tubes, 15 ml and 50 ml	Nunc
Sterile filters, 0.22 µn pore size	Millex GP
Syringes, 10ml - 50 ml	Plastipak
Ultracel® 10 K	Amicon® Ultra
Various glass equipment	Labsystems
Waterbaths	

Instruments	Supplier
Authorized Thermal cycler	Eppendorf
Bench top UV Transilluminator	UVP

BioLogic LP	Bio-Rad
iBLOT™	Invitrogen
Centrifuges:	
Eppendorf centrifuge, 5415 R	Eppendorf
Eppendorf centrifuge, 5430 R	Eppendorf
Table centrifuge	Biofuge Pico, Heraeus
Vacuum centrifuge	Maxi Dry Lyo, Heraeus
Avanti™ centrifuge J-25	Beckman Coulter
Electrophoresis electricity supplier	
Gene Pulser II	Biorad
Photo equipment:	
Gel Doc machine	Bio-Rad
Qubit ™ Fluorometer	Invitrogen
PowerPak 300	Bio-Rad
SNAP i.d. Protein detector system	Millipore
Steri-Cycle CO_2 Incubator	Thermo-electron Corporation

2.2 Software

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

2.3 Chemicals

Chemicals	Supplier
Acetone, C_3H_6O	Merck
Agar	Merck
Agarose	
SeaKern® LE agarose	Lonza
Nusieve® GTG® agarose	Lonza
Ampicillin	Merck
Arabinose, $C_5H_{10}O_5$	Sigma-Aldrich
Calsium chloride, $CaCl_2$	Merck
Chloroform, $CHCl_3$	Merck
Disodium hydrogen phosphate, Na_2HPO_4	Merck
DTT (DL- Dithiothreito)	Sigma
EDTA, $C_{10}H_{16}N_2O_8$	Merck
Erythromycin, $C_{37}H_{16}N_2O_8$	Merck
Ethanol, C_2H_5OH	Sigma-Aldrich
Ethidium Bromide, EtBr	Biotech
Fructose, $C_6H_{12}O_6$	Sigma-Aldrich
L-arabinose	Sigma-Aldrich
L-arginine	Sigma
Glucose, $C_6H_{12}O_6$	Merck
Glycerol, $C_3H_8O_3$	Merck

Glycine, $C_2H_5NO_2$	Duchefa Biochemie
Guanidium HCl	Sigma-Aldrich
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma
Magnesium chloride, MgCl	Aldrich
Magnesium sulfate, $MgSO_4$	Sigma
Ni-NTA His-Bind® Superflow	Qiagen
Oxidized Glutathion	Sigma
Phenylmethanesulfonyl fluoride (PMSF)	
PolyEthylenGlycol, 1500 (PEG1500)	Merck-Schuchardt
Potassium chloride, KCl	Merck
Potassium dihydrogen phosphate, KH_2PO_4	Merck
Sodium chloride, NaCl	Merck
Sodium hydroxide, NaOH	Merck
Reduced Glutathion	Sigma
Trichloroacetic acid (TCA), $C_2HCl_3O_2$	Sigma
Tris-base, $C_4H_{11}NO_3$	Sigma
Tris-HCl, $C_4H_{11}NO_3$ x HCl	Sigma
Tween20	Sigma-Aldrich
B-mercaptoetanol	Sigma-Aldrich

2.4 Proteins, enzymes and related standard solutions

Protein/enzymes	Supplier
5X In-Fusion HD Enzyme Premix	Clontech
Antibodies	
Penta - His™ Antibody, BSA free	Quagen
HRP-Rabbit Anti-Mouse IgG	Daco
ESAT6 Mouse mcAb (ab26246)	Abcam
Bovine serum Albumin (BSA)	Sigma
Lysozyme	Sigma
Protein standards	
BenchMark™ Protein Ladder	Invitrogen
MagicMark™	Invitrogen
Phusion High Fidelity DNA polymerase	Finnzymes
Q5™ High-Fidelity 2x Master Mix	NEB
Restriction buffer	
NEbuffer 3	NEB
NEbuffer 4	NEB
Restriction enzymes	
BglII	NEB
EcoRI	NEB
HindIII	NEB
NdeI	NEB
SalI	NEB
T4 DNA ligase	NEB
VWR RED Taq DNA polymerase Master Mix	Finnzymes

2.5 Nucleotodes and DNA

DNA	Supplier
dNTP-mix, 10mM	NEB
DNA standard	
GeneRuler™ 1kb DNA Ladder	Fermentas

2.6 Primers

The primers used in this study are shown in Table 2.1 and 2.2. The primers sequences are shown in Table 2.1 while the primers are described in Table 2.2.

Table: 2.1. Primer sequences. The red colour indicates restrictions sites. The use of these primers is summarized in Table 2.2.

Name	Sequence	Restriction site in sequence
3014AG85R	CCTGGACGACTAAAGTCGACAGTTGAACTCGTTGAAGTGGT	SalI
3014 F	GGAGTATGATTCATATGAAAAAACTTGTAAGTACAATCGTAACTAC	NdeI
3014HisF	TCATCATCACAGATCTGACTCAACTTACACCGTTAAGAGC	BglII
3014 R	GACGACTAAAGTCGACAAGGGCCCAAGCAGCC	SalI
Ag85DCHisR	GCCAAGCTTCGATTCTTATGGCCGTTGTGGCGT	HindIII
Ag85Fus3014F	CAACGAGTTCAACTGTCGACTTTAGTCGTCCAGGTT	SalI
Lp_3014AGF	TTGGGCCCTTGTCGACTTTAGTCGTCCAGGTTTGCC	SalI
Lp_2162_MSP_F	GGAGTATGATTCATATGTCACAAGCACATACAAC	NdeI
Lp_2162_SVAG_R	CCGGGGTACCGAATTCTTATGGCCGTTGTGGCG	EcoRI
P2162LS_R	GACGACTAAAGTCGACGCCGGTTACAGAACCAGT	SalI
P2162_SV_R	CTGGACGACTAAAGTCGACACCTGTTTTTTGACT	SalI
P2162_USP_F	CATCATCATCATCACGCTGCTGCTGCTAGATCTGCCTCAATCACTGTAAAAGC	BglII
SeqAg85F	GCCGGTAGTTCAGCGAT	
Seq852F	CCGCGGGTGGTCATAAT	

Table 2.2. Description of the application of the various primers. See Table 2.1 for primer sequences

Name	Description
3014AG85R	Reverse primer, for the amplification of the gene fragment encoding the LysM domain, which is part of the p_3014 (pBAD-derivative).
3014F	Forward primer, for the amplification of the gene fragment encoding the LysM domain, which is part of the Lp_3014 (pSIP-derivative).
3014HisF	Forward primer, for the amplification of the gene fragment encoding the LysM domain, which is part of the p_3014 (pBAD-derivative).
3014R	Reverse primer, for the amplification of the gene fragment encoding LysM domain, which is part of the Lp_3014 (pSIP-derivative).
Ag85DCHisR	Reverse primer, for the amplification of the gene fragment encoding the Ag85-ESAT6-DC antigen in pBAD-derivatives.
Ag85Fus3014 F	Forward primer, for the sequencing the fusion between the gene fragment of LysM domain (*3014)* and *Ag86B-ESAT6*.
Lp_3014AGF	Forward primer, for the amplification of the gene fragment encoding Lp_3014_Ag85B-ESAT6-DC (pSIP-derivatives).
Lp_2162_MSP_F	Forward primer, for the amplification of the gene fragment encoding Lp_2162 (pSIP-derivatives).
Lp_2162_SVAG_R	Reverse primer, for the amplification of the gene fragment encoding Ag85B-ESAT6-DC (pSIP-derivatives).
P2162LS_R	Reverse primer, for the amplification of the gene fragment encoding p_2162_L (pBAD-derivatives).
P2162_SV_R	Reverse primer, for the amplification the gene fragment encoding p_2162 (pBAD-derivatives).
P2162_USP_F	Forward primer, for the amplification the gene fragment encoding p_2162_S (pBAD-derivatives).
SeqAg85F	Forward primer, for the internal sequencing of the gene fragment *Ag85B-ESAT6,* reaching the pBAD vector.
Seq852F	Forward primer, for the sequencing of the pBAD vector, downstream of the inserted gene fragment encoding the protein 3014-TB

2.7 Bacterial and plasmids

Tables 2.3 and 2.4 list the bacterial strains and plasmids used in this study, respectively.

Table 2.3. Bacterial strains.

Strain	Source
***Escherichia coli* Top10 cells**	Invitrogen
***Lactobacillus plantarum* WCFS1**	(Kleerebezem et al. 2003)
***Bacillus subtilis* WB800N**	MoBiTech, Germany
Lactobacillus brevis SMZ20556	ATCC
Lactobacillus curvatus	DSMZ
***L. rhamnosus* GG**	ATCC 53103
Lactobacillus acidophilus	ATCC
Lactococcus lactis subs. Lactis	(Park et al. 2013)
***Pediococcus acidolactis* LMGT2351**	LMGT *
***Carnobacterium pisicola* UI49 LMGT 2332**	LMGT *
***Pediococcus pentosaceus* LMGT 2001**	LMGT
L.gasseri ATCC33323T	(Azcarate-Peril et al. 2008)
***Enterococcus faecium* LMGT 2749**	LMGT *
***L. sakei* Lb790**	(Sørvig et al. 2003)

*Strains collected at LMGT, Laboratory of Microbial Gene Technology. Dep. Chemistry, Biotechnology and Food Science, NMBU.

Table 2.4 Plasmids

Plasmids	Description	Source
Lp_2162_SMSP	pSIP-derivative, for the production of 2162-TB_S in *L. plantarum* WCFS1.	This work
Lp_2162_LMSP	pSIP-derivative, for the production of 2162-TB_L in *L. plantarum* WCFS1.	This work
Lp_3014_Ag85B_ESAT6-DC	pSIP-derivative, for the production of 3014-TB in *L. plantarum* WCFS1.	This work
pBAD_2162_SUSP	pBAD-derivative, for the production of 2162-TB_S in *E. coli.*	This work
pBAD_2162_LUSP	pBAD-derivative, for the production of 2162-TB_L in *E. coli.*	This work
p_3014Gagp24	pSIP-derivative, for the retrieval of the gene encoding 3014.	(Urdal 2013)
p_1261_Ag65B-E6	pSIP-derivative, for the retrieval of the *Ag85B-ESAT6-DC* gene fragment.	(Øverland 2013)
pBAD	Empty vector (pBAD-derivative not harboring any target-gene)	Invitrogen
pBAD3014_Ag85B_ESAT6-DC	pBAD-derivative, for the production of 3014-TB in *E. coli*	This work
pBAD_Ag85B_ESAT6	pBAD-derivative, for the production of Ag85B-ESAT6 in *E. coli*	This work
pBAD_3014_Ag85B_ESAT6	pBAD-derivative, for the production of 3014_Ag85B-ESAT6, without the DC-peptide in *E. coli*	This work
pBAD_2162_Ag85B_ESAT6	pBAD-derivative, for the production of 2162_Ag85B-ESAT6, without the DC-peptide, in *E. coli*	This work
pBAD_Elu	pBAD-derivative, not harboring the gene fragment encoding 3014-TB or 2162-TB	G. Mathiesen Unpublished

2.8 Kits

Kits	Supplier
iBlot™ Dry Blotting system	Invitrogen
Blotting roller	
iBlot™ Gel Transfer Stack, Mini and Regular	
iBlot™ Cathode stack, top	
iBlot™ Anode stack, bottom	
iBlot™ Disposable sponge	
iBlot™ Filter paper	
iBlot™ Gel Transfer Device	
Mini Trans - Blot® Electrophoretic Transfer Cell	Bio - Rad
2 gel holder cassettes	
4 fiber pads	
Modular electrode assembly	
Blue cloning unit	
Lower buffer chamber	
Lid with cables	
NucleoSpin® Plasmid Miniprep Kit	Macherey-Nagel
NucleoSpin® Plasmid Coloumns	
Collection tubes, 2ml	
Resuspension Buffer A1	
Lysis Buffer A2	
Wash Buffer AW	
Wash Buffer A4	
Elution Buffer AE	
Rnase A	
NucleoSpin® Extract II	Macherey-Nagel
NucleoSpin® Extract II Coloumn	

Collection tubes, 2 ml
Binding Buffer NT
Wash Buffer NT3
Elution Buffer NE

Novex® NuPAGE® SDS-PAGE Gel Systems — Invitrogen
- NuPAGE®Bis-Tris Gels 10 % 8 cm x 8 cm x 1mm, 10 wells
- NuPAGE® LDS Sample Buffer (4x)
- NuPAGE® Reducing agent (10x)
- NuPAGE® Mops Running Buffer

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

QIAamp® DNA Mini Kit — Qiagen

Collection tubes (2ml)
QIAamp® ATL Buffer
QIAamp® Elution Buffer (AE)
QIAamp® Lysis Buffer (AL)
QIAamp® Mini Spin Column
QIAamp® Proteinase K solution
QIAamp® Wash Buffer 1 (AW1)
QIAamp® Wash Buffer 2 (AW2)

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

SNAP i.d.® Protein detection system — Millipore
SNAP i.d. Single Well Blotter

SNAP i.d. Spacer

SNAP i.d. Blot Roller

Supersignal® West Pico Chemiluminiscent Substrate — Thermo Scientific

Luminol/Enhancer Solution

Stable Peroxide Solution

2.9 Mediums and agars

Mediums	Suppliers

2 X TY medium

16 g Bacto™ Tryptone — BD (Becton, Dickinson and Company)

10 g Bacto™ yeast extract — BD (Becton, Dickinson and Company)

5 g NaCl

dH_2O to 1 liters

autoclaved 121^0C for 15 min

BHI (Brain-Heart-Infusion) medium — Oxoid

37g BHI

dH_2O to 1 liters

Sterilized in autoclave for 15 minutes at 115^0C

HS medium

10 ml 10 x Spizizen's salt

2.5 ml 20 % (w/v) glucose

5 ml 0.1 % (w/v) L - tryptophan

1 ml 2 % (w/v) casein

5 ml 10 % (w/v) yeast extract (Difco)

10 ml 8 % (w/v) arginine, 0.4 % histidine

66.5 ml dH_2O

All components were autoclaved separately, and the tryptophan solution was sterile filtrated

LB medium

10 g Bacto™ Tryptone	BD (Becton, Dickinson and Company)
5 g Bacto™ yeast extract	BD (Becton, Dickinson and Company)

10 g NaCl

dH_2O to 1 liter

autoclaved for 15 minutes at 115^0C

LS medium

10 ml 10 x Spizizen's salt

2.5 ml 20 % (w/v) glucose

0.5 ml 0.1 % (w/v) L - tryptophan

0.5 ml 2 % (w/v) casein

5 ml 2 % (w/v) yeast extract (Difco)

0.25 ml 1M $MgCL_2$

0.05 ml 1M $CaCl_2$

80 ml dH_2O

All components were autoclaved separately, and the tryptophan solution was sterile filtrated

MRS (de Man, Rogosa, Sharpe) medium Oxoid

52 g MRS

dH_2O to 1 Liter

Sterilized in autoclave for 15 minutes at 115 °C

MRSSM medium

5.2 g MRS
17.1 g Sucrose (500 mM)
2.0 g $MgCl_2$ x 6 H_2O (100 mM)
dH_2= to 100 ml, and sterile filtrated using 0.2 µm pore size

S.O.C medium

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

0.057 g NaCl
0.019 g KCl
0.247 g $MgSO_4$
60 ml dH_2O
Sterilized in autoclave for 15 min at 115^0C
After cooling down to room temperature, 1 ml 2 M glucose (sterile filtered, with pore size 0.2 µl) was added and sterile dH_2O was added to 100 ml

Appropriate antibiotics were added to both liquid media and plates, as described in section 3.1.

2.10 Buffers and solutions

Buffers/ Solutions	Content
0.5 M EDTA	186.1 g EDTA-Na_2 1 L dH_2O

1x PBS (Phosphate Buffered Saline), pH 7.4	8 g/l NaCl 0.2 g/l KCl 1.44 g/l Na_2PO_4 0.24 g/l KH_2PO_4 dH_2O to 1L
0.1 M $NaPO_4$ (Sodium Phosphate buffer), pH 7.4	3.1 g $NaH_2PO_4 * H_2O$ 10.9 g Na_2HPO_4 dH_2O to 1L
50x TAE	242 g Tris Base 57.1 ml acetic acid 100 ml 0.5 EDTA, pH 8 dH_2O to 1L
1x TBS (Tris Buffered Saline), pH 7.4	150 mM NaCl 25 mM Tris HCl, pH 7.4 dH_2O to 1L
TTBS	TBS 0.05 % (v/v) Tween 20
1 M Tris HCl	121.1 g Tris Base 42 ml HCl dH_2O to 1L

3. METHODS

3.1 Growth of bacteria cultures

Bacterial cultures were grown in appropriate liquid mediums or agar plates. To select for bacteria containing plasmids with antibiotic resistance, the appropriate antibiotic was added.

Escherichia coli cultures were grown in either LB or BHI media. Liquid cultures were incubated at 37°C and shaking at 200 rpm, while agar plates were incubated at 37°C whiteout shaking. Bacteria containing derivatives of pBAD-plasmids were selected for using the final concentration of 200 µg/ml ampicillin. Bacteria containing derivatives of pSIP-plasmids were selected for using the final concentration of 200 µg/ml erythromycin.

Bacillus subtilis were grown in liquid 2xTY medium at 37°C and shaking at 200 rpm, while 2xTY agar plates in 37°C without shaking.

Lactobacillus spp were grown in liquid MRS mediums or MRS agar plates at 37°C, without shaking. Bacteria containing pSIP-plasmids derivatives were selected for using the final concentration of 10 µg/ml erythromycin.

3.2 Long term storage of bacteria

Bacterial cultures were long-time stored in 20% (v/v) glycerol at –80°C, to prevent cells from disruption at low temperatures.

Materials:

87% (v/v) glycerol

1.5 cryovial

Bacteria culture

Medium (BHI, LB, 2xTY and MRS)

Procedure:

Bacterial cultures were grown using appropriate media and conditions, overnight. 1ml culture was mixed with 300 µl 87% (v/v) sterile glycerol in a 1.5 ml cryvial, and mixing, the vial was stored at –80°C

Stored glycerol stocks were used to start cultures by using toothpicks to scrape off some ice, which was transferred to tubes containing appropriate media and antibiotics.

3.3 Isolating plasmids from bacteria cultures

Bacterial cultured plasmids were isolated with the NucleoSpin® Plasmid miniprep Kit.

Materials:

NucleoSpin® Plasmid miniprep Kit, Macherey-Nagel (see materials, section 2.8).

Procedure:

The manufactured protocol from the supplier was followed for plasmids isolations.

1. 1–5 ml of an overnight bacterial culture was centrifuged at 11,000 x g for 30 sec in standard eppendorftubes, before the supernatant was discarded.
2. The cell pellet was re-suspended in 250 µl Suspension Buffer (A1).
3. After adding 250 µl Lysis Buffer (A2), the tube was inverted 6–8 times and incubated at room temperature for 5 min, or to the lysate appeared clear.
4. After adding 300 µl Neutralization Buffer (A3), the tube was inverted 6–8 times, and centrifuged at 11,000 x g for 5 min at 24°C, to pellet the precipitate.
5. A NucleoSpin® Plasmid column was placed in a collection tube and the supernatant to the column. The column was centrifuged at 11,000 x g for 1 min, and the flow-through was discarded. The column was replaced into the collection tube.
6. 600 µl Wash Buffer (A4) was added to the column and then centrifuged at 11,000 x g for 1 min. The flow-through was discarded and the column replaced into the empty collection tube.
7. The collection tube was centrifuged at 11,000 x g for 2 min to remove remaining ethanol, before the collection tube was discarded.

8. The column was placed into a clean Eppendorf tube, and 50 µl Elution Buffer (AE) was added before incubated at room temperature for 1 min and centrifuged at 11,000 x g for 1 min.
9. The eluted DNA was stored at –20°C

3.4 Genomic DNA isolation

Genomic DNA from *Lactobacillus plantarum* was isolated using QIAamp® DNA Mini Kit.

Material:

QIAamp® DNA Mini Kit, Qiagen (see material, section 2.8)

Procedure:

1. 1–5 ml of an overnight bacterial culture was centrifuged at 7500 rpm for 10 min in standard eppendorftubes before the supernatant was discarded.
2. The cell pellet was then re-suspended in 180 µl ATL buffer.
3. 200 µl proteinase K was added to the mixture and vortexed for 15 sec, before incubating the mixture at 56°C to lyse completely.
4. The tube was centrifuged briefly to remove drops from the lids inside.
5. To retrieve DNA free from RNA, 4 µl RNase (100mg/ml) was added before vortexed 15 sec and incubated at room temperature for 2 min. Drops from the inside of the lid were removed by briefly centrifugation.
6. After adding 200 µl Buffer AL and vortexing for 15 sec, the reaction mixture was incubated for 10 min at 70°C.
7. 200 µl 96% (v/v) ethanol was added and the sample was vortexed for 15 sec.
8. After adding the sample to a QIAamp® Mini Spin Column in 2 ml collection tubes, the samples were centrifuged at 8000 rpm for 1 min. The QIAamp® Mini Spin Column was then replaced in a new clean collection column, and the collection tube containing the filtrate was discarded.

9. 500 µl Wash buffer (AW1) was added and the collection tube was centrifuged at 8000 rpm for 1 min. The collection tube was discarded and the QIAamp® Mini Spin Column was replaced in a new clean collection tube.
10. After adding 500 µl Wash buffer (AW2), the collection tube was centrifuged at 8000 rpm for 3 min. The collection tube was discarded and the QIAamp® Mini Spin Column was replaced in a new clean collection tube.
11. Potential buffer AW2 carryover was removed by centrifugation at 13, 000 rpm for 1 min.
12. The QIAamp® Mini Spin Column was placed in a clean 1.5 ml eppendorftube, and the collection tube containing the filtrate was discarded.
13. After adding 200 µl Elution buffer (AE) to the column, the tube was incubated at room temperature for 5 min. The sample was centrifuged at 8000 rpm for 1 min.
14. The eluate was saved and the QIAamp® Mini Spin Column was replaced in a new 1.5 ml eppendorftube. The column was added 200 µl Elution buffer (AE), incubated at room temperature for 5 min and centrifuged at 8000 rpm for 1 min.
15. The eluate from step 13 was mixed with the eluate from step 14, and used directly.

3.5 The polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a biochemical method that relies on thermo-cycling and a thermostable polymerase to amplify specific DNA fragments. PCR is divided into three steps, denaturation, annealing and extension. In the first step, double-stranded DNA (dsDNA) is denatured by heating, which causes separation into two single-stranded DNA (ssDNA) threads. These ssDNA threads acts as templates for subsequent polymerase-catalyzed DNA synthesis. Two oligonucleotides are then annealed to the ssDNA sequences when the temperature is lowered, functioning as primers for the thermostable polymerases, which subsequently incorporate deoxynucleotides (dNTPs), synthesizing the DNA strands. In the thermal cycling device, these three steps are repeated, to give a high amount of the specific DNA fragment.

3.5.1 PCR reactions with Q5™ High-Fidelity DNA Polymerase

Materials

Primers (see material, section 2.6)

Q5™ High-Fidelity DNA Polymerase Master Mix

Procedure:

1. Reactions were set up according to the protocol from the supplier of the Q5™ High-Fidelity DNA Polymerase. Reactants were mixed in clean PCR tubes according to Table 3.1. During preparation of the reaction mixtures, the tubes kept on ice at all times.

Table 3.1 Q5 High-Fidelity PCR reactants

Reactants	volumes (µl)	Final Concentrations
5X Q5 Reaction Buffer	10	1 X
10 mM dNTP	1	200 µM
Forward primer	1	0.1–10 µM
Reversed primer	1	0.1–10 µM
Template DNA	Variable	<1.000 ng/50 µl
Q5 High-Fidelity DNA Polymerase	0.5	0.02 U/µl
dH_2O	To 50	

2. The tubes were placed in the thermal cycler, and the program was set according to Table 3.2.

Table 3.2 Q5 High-Fidelity cycling program

Program	Temperature (°C)	Time	Cycles
Initial denaturation	98	30 sec	1
Denaturation	98	10 sec	25
Annealing	50 - 72 *	30 sec	25
Elongating	72	20- 30 sec/kb	25
Final elongating	72	2 min	1

***** The annealing temperature was adapted to the melting temperature of the primers used in the reactions. The melting temperature of the primers was calculated using the TM-calculator, and the annealing temperatures used were lower than the calculated melting temperature.

3.5.2 PCR with the VWR® Taq DNA Polymerase Master Mix

PCR with the VWR Taq DNA polymerase Master Mix was used to identify correct plasmids by colony PCR. The VWR Taq DNA polymerase Master Mix contains an inert red dye and stabilizer, so that the PCR product can be applied directly to the gel without the need of a loading dye.

Materials:

Primers (see material, section 2.6)

Taq Master Mix RED

Procedure:

1. Reactions were set up according to the protocol from the supplier of the VWR® Taq DNA polymerase Master Mix. Reactants were mixed in clean PCR tubes according to Table 3.3. During preparation of the reaction mixtures, the tubes were kept on ice at all times.

Table 3.3 Red Taq PCR reactants

Reactants	volumes (µl)	Final Concentrations
2.0 X Taq Master Mix	25	1 X
Forward primer	1	0.1–10 µM
Reversed primer	1	0.1–10 µM
dH_2O	23	
Template DNA	Variable	Variable

2. The tubes were placed in the thermal cycler, and the program was set according to table 3.4.

Table 3.4 Red Taq cycling program

Program	Temperature (°C)	Time (minutes)	Cycles
Initial denaturation	94	3	1
Denaturation	94	1	30
Annealing	55*	2	30
Elongating	72	3	30
Final elongating	72	10	1

***** The annealing temperature was adapted to the melting temperature of the primers used in the reactions. The melting temperature of the primers was calculated using the TM-calculator, and the annealing temperatures used were lower than the melting temperature.

3.6 Agarose Gel Electrophoresis

DNA fragments can be separated according to size using agarose gel electrophoresis. An electric charge over the gel causes the negatively charged DNA fragments to migrate towards the positive pole. The smaller fragments meet less resistance from the gel pores and therefore migrate faster, separating them from the slower migrating larger fragments. Ethidium bromide was added to the agarose gel, in order to visualize the fragments after UV light exposure, and a DNA ladder was used to determine the size of DNA fragments.

Materials:

1x TAE buffer

10 mg/ml Ethidium Bromide

SeaKem® LE Agarose

DNA ladder

Loading dye

Procedure:

1. For preparing a 1.2 % Agarose gel, 12 g SeaKem® LE Agaros was dissolved in 1 L 1x TAE. The solution was autoclaved at 115°C for 15 min and stored at 60°C.
2. The gels were made by mixing 60 ml solution and 1 µl 10mg/ml ethidium bromide, and then poured into a moulding tray with well combs.
3. After approximate 20 min the combs were removed, and the now harden gel was transferred to electrophoresis chambers and covered with 1x TAE buffer.
4. 0.1 volume dye was added to each sample before loaded onto the gel, using an appropriate DNA ladder. The gel was run at 90 V for 15–40 min.

3.7 DNA purification and extraction from agarose gels

DNA was extracted from agarose gels using the NucleoSpin® Extract II Kit following the provided protocol for extraction and purification.

Materials:

NucleoSpin® Extract II Kit, Macherey-Nagel (see materials, section 2.8)

Procedure:

1. 200 µl Binding buffer (NT) was added to each 100 mg agarose gel. The sample was incubated for 5–10 min at 50°C and vortexed every 2–3 min, until the gels were completely dissolved.
2. After placing the NucleoSpin® Extract II column into the collection tube, the sample was loaded and centrifuged at 11,000 x g for 1 min. The flow-through was discarded and the column replaced into the collection tube.
3. 700 µl Wash buffer (NT3) was added and the sample was centrifuged at 11,000 x g for 1 min. The flow-through was discarded and the column replaced into the collection tube.
4. The Wash buffer (NT3) was completely removed using centrifugation at 11,000 x g for 2 min.
5. After placing the columns into clean eppendorftubes, the DNA yield was increased with the addition of 15–50 µl Elution buffer (NTE) and incubation for 1 min. The sample was centrifuged at 11, 000 x g for 1 min.
6. The eluted DNA was stored at –20°C.

3.8 Restriction enzyme digestion of DNA

Restriction enzyme digestion is a method used to digest double-stranded DNA at specific sequences, called restriction sites. Restriction enzymes can break the DNA chains in several ways, one resulting in the formation of blunt ends, while the other results in the formation of sticky ends. The blunt end is formed by cutting at the exact same location in both DNA strands, while sticky ends are formed by cutting at different locations, which causes an overhang of bases.

Materials:

10 x BSA (if needed)

10 x restriction buffers (see material, section 2.4)

DNA to be cut

Restriction enzymes (see material, section 2.4)

Procedure:

1. Reactants were mixed according to Table 3.5 in Eppendorf tubes.

Table 3.5 Restriction mixtures for restriction enzymes

Reactants	**Volumes (µl)**
Template	15–25
Restriction enzymes	2
NE Buffers	5
BSA	5
dH_2O	To 50

2. After incubation for 2 hours at 37°C, the samples were run on agarose gels, separating the fragments according to their sizes.

3.9 Determination of DNA concentration

The DNA concentration of plasmids and PCR fragments after purification were determined using the Qubit® dsDNA BR Assay Kit.

Materials:

Qubit® dsDNA BR Assay Kit (see material, section 2.8)

Qubit™ fluorometer

Procedure:

1. The Qubit™ Reagent was diluted with Qubit™ Buffer by a factor of 1:200 in a 200 µl stock solution.
2. Two Qubit™ standards of 10 µl were each mixed with the 190 µl stock solution. The mixture was vortexed and incubated at room temperature for 2 min.
3. 1 µl of each sample were mixed with 199 µl stock solution, using vortex and incubation at room temperature for 2 min.
4. The the Qubit™ Fluorometer was equilibrated using prepared standards. The DNA concentration in each sample was determined.
5. The samples were chilled on ice and stored at –20°C.

3.10 Ligation of DNA fragments

DNA fragments can be ligated by DNA ligases. In this study Quick T4 DNA ligase was used. This ligase can join both blunt and sticky ends of DNA fragments through the formation of a phosphodiester bond between the 5' phosphate- and 3' hydroxyl-ends of the DNA fragments. The ligation buffer supplies ATP and Mg^{2+} to the solution, which is needed for the ligase to be active.

Materials:

2x Quick Ligation Reaction Buffer

DNA to be ligated (fragment and insert)

Quick T4 DNA Ligase

Procedure:

1. 50 ng vector was mixed with 3x ng of the insert in PCR tubes. The total volume was adjusted to 10µl.
2. 10 µl 2x Quick Ligation Reaction Buffer were added to the solution.
3. 1 µl Quick T4 DNA Ligase was added to the solution and incubated at room temperature for 5 min.

3.11 Preparation of competent bacterial cells

Competent bacterial cells can take up plasmids from the extracellular environment, resulting in genetically modified bacteria. Transformation, i.e. uptake of DNA, is usually achieved by applying an electric pulse or a heat shock. Bacterial cells become (or can be made) competent in the exponential phase, and is therefore harvested in this phase

3.11.1 Preparation of electro-competent *Lactobacillus plantarum* WCFS1

Electro-competent *L. plantarum* were made by growing them in a medium containing high amounts of glycine. The glycine is incorporated into the cell wall, replacing L-alanine, leaving the cell wall more permeable and favoring plasmid uptake. The procedure was done according to the protocol described by Aukrust et al. (1995).

Materials:

20% glycine (w/v)

1 mM $MgCl_2$

2x MRS medium

MRS medium

PEG 1500

TEN buffer

Procedure:

1. 10 ml MRS containing *L. plantarum* cultures were incubated in 30°C overnight, and used to create dilution series.
2. In 12 tubes with 10 ml MRS; 1 ml overnight culture was transferred to the first tube, and mixed well. Then 1 ml was transferred further to the next tube, and so on. These tubes were incubated in 30°C.
3. The samples that reached OD_{600} = 0.5–0.7 were used further to make electro-competent cells.
4. After transferring the sample to a 50 ml Nunc tube containing; 20 ml 2xMRS, 5 ml 20% glycine and 5 - 10 ml dH_2O (Total volume 40 ml), the sample was incubated in 30°C until OD_{600} = 0.7 was reached.

5. The cells were cooled down with ice before centrifuged at 5200 rpm for 5 min at 4°C. After discarding the supernatant, the pellet was washed with 10 ml TEN buffer and centrifuged at 5200 rpm for 5 min at 4°C.
6. The supernatant was discarded and the pellet washed using 40 ml 1 mM $MgCl_2$. The solution was centrifuged at 5200 rpm for 5 min.
7. The supernatant was discarded and the pellet washed using 5 ml 30 % PEG. The solution was centrifuged at 6000 rpm for 10 min at 4°C.
8. The cells were re-suspended in 400 µl 30% PEG and divided into cold 40 µl tubes, before the tubes were stored at –80°C.

3.11.2 Preparation of chemically competent *Escherichia coli* TOP10 cells

Chemically competent *E. coli* TOP10 cells were prepared as follows:

Materials:

Ice-cold 0.05M $CaCl_2$

Ice-cold 0.05M $CaCl_2$, with 15% glycerol

LB medium

E. coli TOP 10 cells

Procedure:

1. A single colony of *E. coli* TOP10 was used to inoculate 5 ml LB medium, followed by overnight growth at 37°C.
2. After diluting the culture to OD_{600} = 0.1 in 200 ml LB, the culture was incubated at 37°C to OD_{600} = 0.4.
3. In new centrifuge tubes, the cultures were incubated on ice for 10 min, before centrifuged at 4000 rpm for 5 min, at 4°C.
4. After removing the supernatant, the pellet was re-suspended in 15 ml ice-cold 0.05 M $CaCl_2$, followed by centrifugation at 4000 rpm for 10 min at 4°C.
5. Step 4 was repeated.

6. After removing the supernatant, the pellet was re-suspended in 10 ml ice-cold 0.05 M $CaCl_2$ with 15% glycerol and incubated in an ice bath for 10 min.
7. 200 µl portions of competent cells were pipetted into sterile 1.5 ml eppendorftubes and immediately stored at –80°C.

3.12 Electroporation of *Lactobacillus plantarum* WCFS1

Electro-competent *L. plantarum* WCFS1 cells were electroporated to enhance the uptake of plasmids.

Materials:

Electroporation cuvettes

MRSSM

Procedure:

1. 40 µl containing competent *L. plantarum* cells were mixed with 5 µl of purified plasmid.
2. The electroporator Gene Pulser® II and Pulse Controller plus were used with the parameters:
   Tension 1,5 kV
   Capacity 25 mF
   Resistance 400 W
3. The 2 mm cuvette was placed in the electroporator for application of the electric pulse.
4. 950 µl of ice cold MRSSM was added to the cuvette before transported into eppendorftubes.
5. The tubes were incubated for 2–4 hours at 37°C, before plated on appropriate plates containing appropriate antibiotics.

3.13 Transformation of chemical competent *Escherichia coli*

The uptake of extracellular plasmids by chemical competent *E. coli* was enhanced through heat shock.

Materials:

BHI agar plates containing 200 µl erythromycin

Plasmid solution

S.O.C medium

Chemically competent *E. coli* TOP10 cells, 200 µl portions

Procedure:

1. A portion of the electro-competent *E. coli* Top10 cells were mixed in 14 ml Falcon 2059 Polypropylene Round Bottom tube, with 1–5 µl of the plasmid. The mixture was inoculated on ice for 30 min.
2. The cells were heat shocked at 42°C for 1 min and immediately chilled on ice for 2 min.
3. 250 µl room tempered S.O.C medium were added to the cells, and the solution was incubated at 37°C, shaking for 1–4 hours.
4. The cells were spread on agar plates and incubated at 37°C overnight.

3.14 Protein production *Escherichia coli*

In this study, cells were lysed by combining lysozyme treatment and sonication to retrieve intracellular proteins. Lysozymes are enzymes that degrade the peptidoglycan layer in the cell wall, leaving the cell more exposed and weakened. Lysozymes disrupt the link between *N*-acetylglucosamin (NAG) and *N*-acetylmuramic acid (NAM), which causes the cell wall to break and contributes to cell lysis. Sonication uses a high frequency sound wave that causes cells to lyse. The probe's mechanical energy results in shock waves that spread throughout the suspension in short multiple pulses, preventing the suspension from overheating.

Materials:

100 mg/ml Lysozyme

$NaPO_4$ Buffer

PMSF

Protease inhibitor cocktails

Sonics, Vibracell

Procedure:

1. Bacterial cultures that were grown at 18°C, were induced by adding arabinose to a final concentration of 0.02% at OD_{600} = 0.5–0.7.
2. The cultures were induced and incubated overnight at 18°C. The cells were then harvested through centrifugation at 8000 rpm for 10 min at 4°C. While keeping the pellet on ice, the supernatant was discarded.
3. The cell pellet was re-suspended in 15 ml $NaPO_4$ buffer and was centrifuged at 7000 rpm for 10 min at 4°C. The supernatant was then discarded.
4. The cell pellet was re-suspended in 15 ml $NaPO_4$ buffer and was added lysozyme to the final concentration 0.1 mg/ml. 2 µl PMSF or 1 protease inhibitor cocktail tablet was added and the solution was incubated on ice for 20 min.
5. The solution was sonicated for 20–30 min, 5 sec ON/OFF, with 33% amplitude.
6. The solution was centrifuged at 7000 rpm for 10 min, before pellet and supernatant was divided into two separated tubes.

3.15 Protein purification

Ni^{2+}-NTA (Nickel Nitrilotriacetic Acid) chromatography was used to purify the proteins in this study, since these proteins all had His-tags. NTA has four chelating sites, which allows a stable interaction between Ni^{2+} and the column matrix. This leaves two free sites to interact with functional groups on proteins. If 7 histidine residues (a His-tag) are linked to either the N- or C-terminus of a protein, such a protein will have a high affinity interaction with Ni^{2+}-NTA (Figure 3.1).

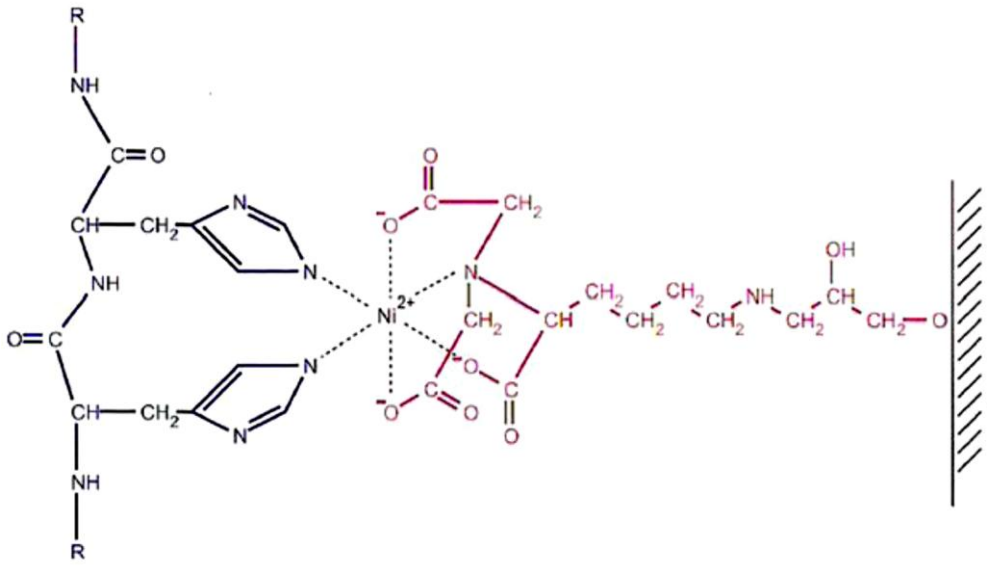

Figure 3.1: Interaction between Ni^{2+}-NTA matrix of the column and the $(His)_6$ residues in the proteins. The interaction point between the column matrix and the His-tag (located either N- or C- terminally) is the Ni^{2+}, which stabilizes the interaction. See text for more information. The picture is taken from (Bævre Heggset 2005).

The interaction between the column matrix and the His-tagged proteins separates these proteins from the rest of the proteins in the sample (Fig. 3.1), which normally pass right through the column. After running the sample through the column, proteins retained in the column can be eluted with buffers containing either imidazole (competing to bind the chelating binding sites), or with changing pH (leading to a structural change in the proteins). In either way the His-tag containing proteins are eluted from the column, often in a highly pure state.

Materials:

20% Ethanol

Buffer A: 100 mM Tris HCl pH 7.4.

Buffer B: 100 mM Tris HCl pH 7.4, 500 mM Imidazole

Ni-NTA superflow column

Procedure:

1. The column was packed with Ni-NTA superflow beads, and buffer A was run through the system with a 1.5 mg/ml flow rate. Care was taken to avoid bubbles in the system.
2. The sample was run through the system after the entire system contained buffer A. The high peak that was detected on the computer's digital analyzed chromatogram, after the sample was added, contained proteins that failed to bind to the column, and was collected.
3. After the "flow-through peak" was finished, the buffer was changed from buffer A to B.
4. Peaks detected after the buffer-switch was collected. Proteins that bind to the column are expected to end in this peak.

3.16 Gel electrophoresis of proteins

Polyacrylamide gel electrophoresis (PAGE) separate proteins accorded to their size. In this study, Invitrogen's NuPAGE® Novex Bis-Tris electrophoresis system was used. The LDS Sample Buffer contains lithium dodecyl sulphate, which is a Sodium Dodecyl Sulfate (SDS) analogue that interferes with non-covalent stabilizing interactions in the proteins, resulting in denaturation and introducing a fixed negative charge on each residue. Disulphide bonds are reduced (broken) by the addition of a reducing agent such as dithiothreitol (DTT). The negatively charged and denatured proteins are then separated by applying an electric field, where the proteins migrate towards the positive pole. The smaller proteins meet less resistance and migrate faster than the larger proteins, causing the separation. Protein sizes are determined using suitable standards ("ladders"). Proteins are visualized by the Gel Doc™ EZ system, which images stain-free gels placed on a stain-free tray. In western blotting (see section 3.22), specific proteins are visualized using antibodies.

Materials:

NuPAGE ® LDS Sample Buffer (4x)

NuPAGE ® Novex Bis – Tris Gels

NuPAGE ® Reducing Agent (10x)

Procedure:

1. After adding 7 µl LDS Sample Buffer and 3 µl Reducing Agent to a 10 µl protein sample, the mix was heated to 90°C for 5 min.
2. The electrophoresis chamber was assembled, using a proper amount of 1x TAE buffer in both the inner and outer chamber.
3. The heated samples were applied to the wells and run at 300 V for 15 min.
4. The chamber was disassembled and the gels were released before washed in a plastic tray containing dH_2O.
5. After 5 min in dH_2O, the gel was placed on a stain-free tray and analyzed by Gel Doc™ EZ system.

3.17 Concentrating proteins in a solution

Ultrafiltration tubes contain a semi-permeable membrane with a chosen defined Molecular Weight Cut-Off (MWCO). MWCO normally approximates 50% of the size (expressed in kDa) of the protein of interest, to achieve the highest potential protein recovery (which is approximately 95%). Centrifugation forces the solution through the membrane, only withholding molecules larger than the MWCO (GE Health Care 2014).

Materials:

Amicon concentrating tubes

MWCO filter (3 kDa)

Procedure:

1. After adding the protein samples to the MWCO tubes, the tubes were centrifuged at 45000 rpm for 30 min or more, until the desire amount of buffer solution remained.
2. The remaining solution was transferred to Eppendorf tubes and stored at 4°C.

3.18 Measuring protein concentrations

Bio-Rad Protein Assay is a dye-binding assay to determine protein concentrations in sample solutions. The Bio-Rad Protein Assay Dye Reagent Concentrate, when added to the protein sample, changes colour from red to blue. The variation in colour is measured using a spectrophotometer (Bio-Rad Laboratories).

Materials:

20 mM Tris HCl pH 8.0

Bio-Rad Protein Assay Dye Reagent Concentrate

Procedure:

1. Three samples of 799 µl 20 mM Tris HCl pH 8.0 was mixed with 1 µl of the protein samples each. 200 µl Protein assay dye reagent concentrate was added and the sample was vortexed.
2. All samples were incubated at room temperature with the protein assay dye reagent concentrate for 5 min, letting the dye attach to proteins in the samples, before measuring the concentration using the Bradford micro program.

3.19 Recovery of proteins from inclusion bodies

The formation of inclusion bodies captures unfolded proteins within the cell, where they are found in a relatively pure state. Denaturing will improve extraction of proteins from the interior of the cell. In this denatured state, the protein's primary structure (the amino acid sequence) is exposed to the extracellular material, potentially revealing pre-hidden structures needed to purify the protein (Bævre Heggset 2005). This purifying process is divided into the denaturing part and the refolding part.

Materials:

6M Guanidium Hydroklorid

10 mM 2-β-mercaptoetanol (β-ME)

25 mM DTT

20 mM Tris-HCl pH 8.0

8M Urea

Procedure:

1. *E. coli* cultures were grown at 30^0C to OD_{600}=0.5–0.7 was reached. The cultures were then induced with 0.2% arabinose and incubated for 12 hours.
2. Steps 2–6 in section 3.14 were followed. The resulting cell pellets were diluted in 8 ml 20 mM Tris HCl pH 8.0 (Bævre Heggset 2005) and divided into 8 Eppendorf tubes.
3. The Eppendorf tubes were centrifuged at 13. 000 rpm for 10 min at 4º C, and the supernatant discarded.
4. The Eppendorf tubes were labeled 1–8, and added the different denaturants:
    1. 6 M Guanidium Hydroklorid
    2. 6 M Guanidium Hydroklorid + 25 mM DTT
    3. 6 M Guanidium Hydroklorid + 10 mM β-ME
    4. 6 M Guanidium Hydroklorid + 10 mM β-ME + 25 mM DTT
    5. 8 M Urea
    6. 8 M Urea+ 25 mM DTT
    7. 8 M Urea+ 10 mM β-ME
    8. 8 M Urea+ 10 mM β-ME + 25 mM DTT

    All eppendorftubes were added 10 µl 1 M Tris HCl pH 8.0 and 0.0137 g NaH_2PO_4.

5. The Eppendorf tubes were incubated at room temperature for 2 hours (occasionally vortexed) and centrifuged for 10 min at 13000 rpm. Both the supernatants and the pellets were analyzed by SDS-PAGE, to reveal the amount of protein dissolved into the supernatant.
6. Based on these results 8 M urea with no further additions was selected for the solubliziation of inclusion bodies.

The denatured protein was purified by Ni-NTA beaded column, according to section 3.15, the only difference being that buffer A and B contained 8 M urea to keep the protein in the denatured state during purification.

Protein refolding

Two approaches were used to refold the denatured proteins, both derived from (Bævre Heggset 2005). Both methods depend on the removal of denaturants (8 M urea); Dialysis rapidly changes the proteins' environment from denaturing to neutral. The proteins are kept in this neutral condition for a while. Refolding solutions change the environment from denaturing to neutral in a gradual manner, removing denaturants slowly. Depending on the proteins' nature, one of the methods could possibly result in soluble proteins, rather than aggregated proteins (Tsumotoa et al. 2003).

Dialysis

Materials:

SnakeSkin® Dialysis Tubing

Spectra/Por® Closures

Procedure:

1. 1 ml purified denatured proteins in denaturing buffer B (100 mM Tris HCl, pH 8, 500 mM imidazole and 8 M Urea), was transferred into the SnakeSkin® Dialysis Tubing. The protein solution was kept within the SnakeSkin® Dialysis Tubing using Spectra/Por® Closures on both ends of the SnakeSkin.
2. The SnakeSkin® Dialysis Tubing was then left in a dialysis buffer for two days, containing 200x volume 20 mM Tris HCl pH 8.
3. The SnakeSkin® Dialysis Tubing was then transferred to a new dialysis buffer, with 200x volume 20 mM Tris HCl pH 8 and incubated for 3 days.
4. The proteins were concentrated in 20 mM Tris HCl pH 8 using MWCO tubes (section 3.17), before analyzing by Native acrylamide gel.

Refolding proteins in refolding-solution

Materials:

0.9M arginine
0.5mM oxidized glutathione
5mM reduced glutathione
50mM Tris HCl pH 8.0
dH_2O

Procedure:

1. 100 ml sample of the refolding solution was made following (Bævre Heggset 2005). The refolding solution was divided on two 100 ml beakers, both containing 50 ml of the refolding solution.
2. 1 ml of the denatured and purified protein sample in denaturing buffer B (100 mM Tris HCl, pH 8, 500 mM imidazole and 8 M Urea), was poured into one of the 100 ml beakers and incubated for 30 min at 4°C.
3. After the incubation, the remaining 50 ml refolding solution was poured into the 100 ml beaker, and incubated at 4°C for 3 days. The proteins were concentrated using MWCO tubes (see section 3.17) in 20 mM Tris HCl pH 8, before analyzing by Native acrylamide gel (section 3.20).

3.20 Gel Electrophoresis with Native acrylamide gels

Native PAGE gels separate proteins under non-reducing and non-denaturing conditions, maintaining the protein's secondary structure and charge natural density. The proteins are separated by a charge to mass ratio based on the proteins natural pI (isoelectric point) (Sino Biological Inc. 2004).

Materials:
NativeMark™ Unstained Protein Standards
NativePAGE™ Cathode Buffer Additive (2x)
NativePAGE™ Gel
NativePAGE™ Running buffer (20x)
NativePAGE™ Sample Buffer (4x)

dH_2O

Procedure:

1. Reactions were set up according to the protocol from the supplier. 2.5 µl NativePAGE™ Sample Buffer (4x) were mixed with the appropriate amount of protein sample in an Eppendorf tube, because the protein concentration was so low, 15 µl of the protein samples were used. dH_2O was added to a final volume of 20 µl.
2. 50 ml of the NativePAGE™ Cathode Buffer Additive (2x) was mixed with 950 ml dH_2O, and 50 ml of the NativePAGE™ Running buffer (20x) was mixed with 950 ml dH_2O.
3. The NativePAGE™ Running buffer was placed in the outer room, while NativePAGE™ Cathode Buffer Additive was placed in the inner room.
4. The samples were applied into the wells, and the gel run on 150 V for 1.5–2 hours.

Coomassie staining of Native gels

Materials:

Brilliant Blue R concentrates Electrophoresis Reagent

0.1 % (v/v) Coomassie
Brilliant Blue R 250
50 % (v/v) methanol
10 % (v/v) acetic acid

Destaining solution

10 % (v/v) acetic acid
10 % (v/v) ethanol

dH_2O

Procedure:

1. The Native acrylamide gel was washed in pre-heated dH_2O for 1 hour, in plastic trays and shake.

2. The gel was incubated in pre-warmed Brilliant Blue R concentrates Electrophoresis Reagent for 1 hour, shaking.
3. After removing the Brilliant Blue R concentrates Electrophoresis Reagent, the Destain solution was added to the gel. The tray was incubated for 1 hour, with shaking.
4. The Destain solution was removed and the gel washed with dH_2O until the bands were visible. The gel was transferred to the white tray before analyzing by the Gel Doc™ EZ system.

3.21 Anchoring LysM domain-containing proteins to gram positive bacteria

Proteins containing LysM domains, produced in *E. coli*, were incubated with bacterial cells to result in proteins being anchored to the peptidoglycan layer.

Materials:

BHI

MRS

Phosphate buffered saline (PBS)

Table centrifuge

Procedure:

1. Bacterial cells were grown in appropriate media (*Lactobacillus spp.* in MRS and *B. subtilis* in BHI), to reach the exponential phase. The cells were harvested through centrifugation, and the supernatant discarded.
2. The pellet was washed twice in PBS, and centrifuged for 2 min in between. The supernatant was discarded after each wash.
3. The cell pellet was diluted in 1 ml PBS, to the final OD_{600}=0.5. The solution was centrifuged at 5000 x g at 4°C for 3 min before the supernatant was discarded.
4. The pellet were re-suspended in 1 ml of protein extracts (see Result section for more details) and incubated at room temperature for 1 hour (with occasionally vortexing).
5. The sample was centrifuged for 3 min, at 5000 x g at 4°C. The supernatant was discarded, and the cell pellet was diluted in 1 ml PBS.

6. The sample was centrifuged for 2 min, at 5000 x g at 4°C. The supernatant was discarded, and the cell pellet was diluted in 1 ml PBS.
7. Step 6 was repeated 2 times.
8. The supernatant was discarded and the pellet was analyzed further using flow cytometry (section 3.23) or western blot (see section 3.22).

3.22 Western blot analysis

In blotting, proteins separated according to their size on electrophoresis gels, are transferred to a nitrocellulose membrane, by applying an electric current. To detect proteins of interest with specific epitopes, a very sensitive detection method is used, called antibody hybridization. The membrane has a high affinity towards proteins and to prevent unspecific binding, the membranes are blocked before primary antibodies are added. The protein of interest is probed with the primary antibody that binds to a specific epitope on the protein. Unbound primary antibodies are washed away before the secondary antibodies are added. The secondary antibodies are linked to a reporter enzyme, which produces a detectable signal when introduced to the proper substrate. In this study chemiluminescent detection was used (see section 3.22.3)

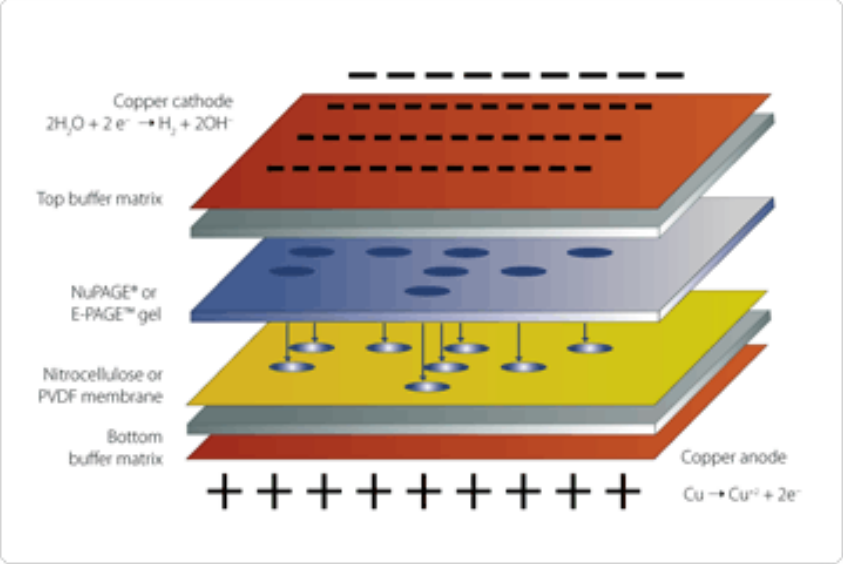

Figure 3.2: A schematic illustration of the layers in the iBlot system; transferring proteins from the gel to the membrane. The Figure is taken from "iBlot Dry Blotting system user manual" (Life technologies™).

Materials:

Blotting roller

iBlot™ Anode stack, bottom

iBlot™ Cathode stack, top

iBlot™ Disposable sponge

iBlot™ Dry Blotting system

iBlot™ Filter paper

iBlot™ Gel transfer device

iBlot™ Gel Transfer Stack, Mini

Procedure:

1. SDS-PAGE gels (see section 3.16) were washed in dH_2O for at least 5 min.
2. The membrane containing anode stack (Figure 3.2) was placed in the gel transfer device, placing the gel on top of the membrane.
3. A wet filter paper with dH_2O was placed on top of the gel, and air bubbles trapped between the layers were removed with the blotting roller.
4. The cathode stack containing the copper electrode sited upwards on top of the filter paper.
5. The disposable sponge was placed in the lid and the transfer device was closed.
6. Program 3; with the settings 23 V and 7 minutes. After finishing the program, the membrane was removed from the transfer device and incubated in dH_2O for at least 5 min.
7. The membrane was then run through an antibody hybridization process using the SNAP i.d. immmuneblotting system (see section 3.22.1).

3.22.1 SNAP i.d. immunodetection

The SNAP i.d. ® detection system was used to probe all western blots. In this process, the antibodies are introduced to the membrane-bound proteins, hybridizing to the proteins containing specific epitopes. To speed up the method and give a high-quality protein blot, the SNAP i.d. ® detection system uses vacuum to pull the reagents into the membrane. This is a contrast to the traditional immunodetection system, which relies on diffusion to help antibodies into the

membrane (Figure 3.3).

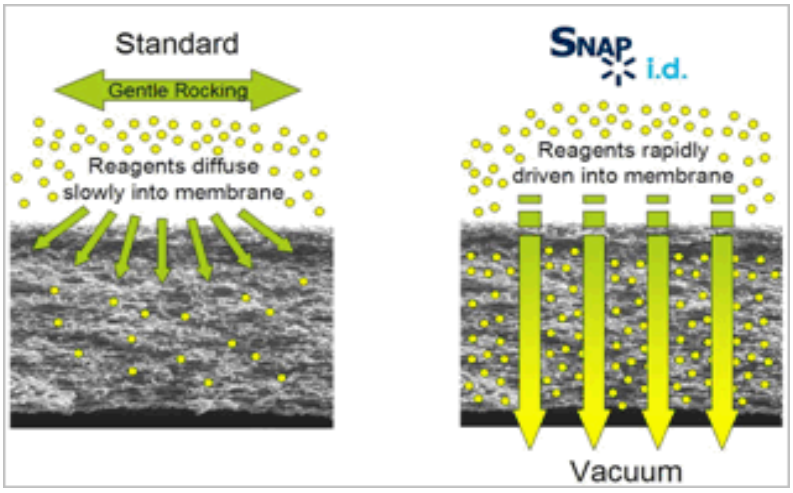

Figure 3.3: Schematic illustration of the difference between immunodetection systems using vacuum and systems based on diffusion. The standard method relies on diffusion to slowly distribute the reagents, while the SNAP i.d. method uses a strong pulling force driven by the vacuum, to rapidly drive the reagents into the membrane. The picture is taken from "SNAP i.d.™ Protein Detection System" (Komabiotech 2008).

Materials:

Blocking Buffer (TTBS/ 1% BSA)

Primary antibodies:

ESAT6 Mouse mcAb (ab26246)

Penta - His™ Antibody, BSA free

Secondary antibodies HRP – Rabbit Anti – Mouse IgG

SNAP i.d. Detection System

TTBS

TTBS/ 0.5% BSA

Procedure:

1. The Blot Holder was wetted using dH_2O before placing the blotted membrane inside the Blot Holder, protein faced down. A wet filter paper was placed at the top, and the holder was firmly closed and placed in the SNAP i.d. Protein Detector system device.
2. 30 ml Block buffer was added, and the vacuum turned on immediately. The vacuum was turned off when the well was empty.
3. 3 ml TTBS/ 0.5% BSA with 2 µl/ml primary antibody was incubated in the well for 10 min.
4. The well was washed with 3x 10 ml TTBS with the vacuum on continuously; when the well was empty the vacuum was turned off.
5. 3 ml TTBS/ 0.5% BSA with 0.04 µl/ml secondary antibody was incubated on the well for 10 min.
6. Step 4 was repeated.
7. The membrane was removed from the blot holder and placed in an appropriate detection agent (section 3.22.2).

3.22.2 Chemiluminiscent detection of protein

Chemiluminiscence was used to visualize proteins on the membranes, labeled with the secondary antibody. The secondary antibody contains horseradish peroxidase (HRP), which oxidizes the appropriate substrate when added to the reaction solution. This results in an emission of light which is captured on an x-ray film.

Materials:

CL-Xposure™ Film
Developer solution
Fix solution
Luminol/Enhancer
Stable Peroxide Buffer
Solution SuperSignal® West Pico Chemiluminiscent Substrate

Procedure:

1. 3.3 ml of Luminol/Enhancer Solution was mixed with 3.3 ml of Stable Peroxide Buffer to prepare the substrate working solution.
2. After incubating the membrane in a substrate working solution for 5 min, in plastic trays, the membrane was placed in plastic foiled covered lightproof film cassette, leaving the protein-side of the membrane facing upward.
3. In the dark room, a CL-Xposure™ Film was cut in an appropriate size and placed on top of the membrane, before closing the cassette for 1–15 min, depended on the strength of the chemiluminiscens signal.
4. After incubation in developer solution for 2 min, the film was incubated in the fix solution for additional 2 min.
5. The film was then washed with water and air dried.

3.22.3 Stripping and re-probing the nitrocellulose membrane from western blot analysis

Materials:

Restore™ Western Blot Stripping Buffer

TTBS

Procedure:

1. The previously blocked, probed and developed nitrocellulose membrane from western blot (see section 3.22, 3.22.1 and 3.22.2) was stored in TBS (4°C), before the membrane was stripped.
2. The blot was incubated in Restore™ Western Blot Stripping Buffer for 30 min at room temperature.
3. The blot was washed with TTBS and new antibody hybridization and detection was performed (see section 3.22.1 and 3.22.2).

3.23 Detection of surface antigens using FITC-labelled secondary antibodies and flow cytometry

Proteins of interest contain specific epitopes that are recognized by primary antibodies, which again, are recognized by secondary antibodies (e.g. anti-mouse IgG). In this experiment, the secondary antibodies were labeled with the fluorochrome fluorescein isothiocyanate (FITC). FITC-labeled cells can be detected using flow cytometry.

Flow cytometry (Figure 3.4) detects the fluorescence and the scattered light from a single cell that passes (one at a time) through a laser beam. The light scatters in all directions, and are monitored by detectors. Light scattered forward gives information about the cells' size, while light scattered sideways gives information about the cells' intracellular properties, as the granularity of the cell. The laser beam is also called an excitation light, which has the possibility to excite antigens containing fluorochormes (Lea 2006); the potential resulting fluorescence is detected by a mirror- and filter-system. By combining the data collected, a cell population may be analyzed.

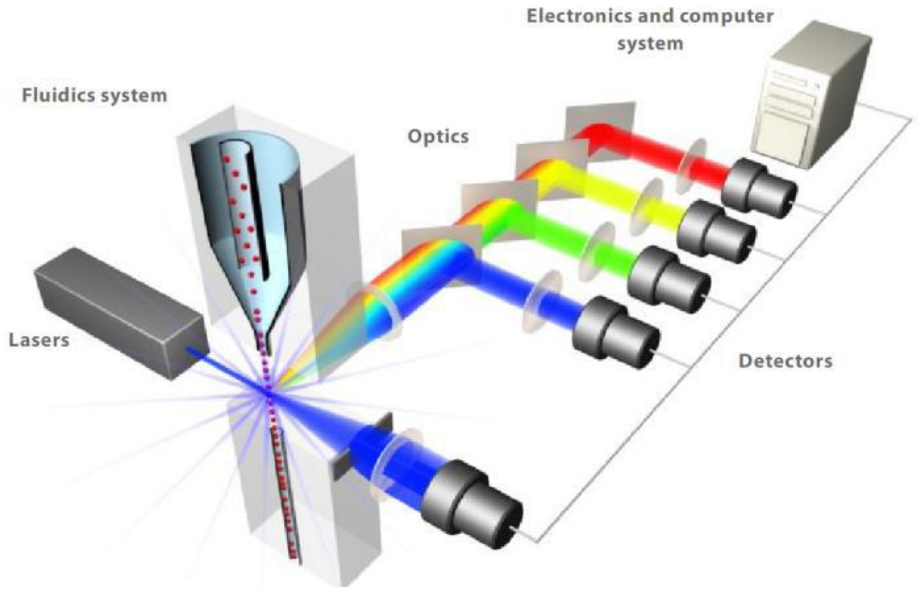

Figure 3.4: The schematic illustration of the principles of a flow cytometry. The cells are led through a laser beam, one by one, and the information retrieved from the analysis of the cells depends on the scattered light from the cell surface. The optics gathers and directs the light, and the computer system converts the data retrieved from the detectors into digital data that are analyzed. The picture is taken from (Tjåland 2011)

Materials:

PBS

PBS/ 2% BSA

Primary antibody ESAT6 Mouse mcAb (ab26246)

Secondary antibody Anti – Mouse IgG (FITC)

Procedure:

1. Bacterial cells where prepared and charged with LysM-containing proteins as described in section 3.21, step 1–8.
2. After re-suspending the cells in 50 µl PBS / 2% BSA and 0.4 µl of primary antibodies, the solutions were incubated for 30 min on ice. The solutions were then centrifuged at 5000 x g, for 2 min, at 4°C.
3. The samples were washed with1 ml PBS/ 2% BSA and centrifuged for 2 min at 5000 x g at 4°C and the supernatant discarded.
4. Step 3 was repeated 2 times.
5. After re-suspending the cells in 50 µl PBS / 2% BSA and 0.8 µl of secondary antibodies, the solutions were incubated for 30 min on ice. The solutions were then centrifuged at 5000 x g, for 2 min, at 4 °C.
6. The samples were washed as in step 3 and 4.
7. The stained pellets were re-suspended in 100 µl PBS before immediately analyzed by MacsQuant® Analyzer (Miltenyi Biotech) and MacsQuantify™ software.

3.24 Protein production in *Lactobacillus plantarum*

Materials:

Erythromycin 10 µg/ml

Inducing peptide SppIP

MRS medium

PBS buffer

Procedure:

1. Overnight cultures of *L. plantarum* harboring the appropriate plasmid were grown in 10 ml MRS medium containing the final concentration of 10 µg/ml Erythromycin at 37°C.
2. The overnight cultures were diluted in 50 ml pre-warmed MRS medium containing the final concentration of 10 µg/ml Erythromycin, to OD_{600}=0.1 followed by incubation at 37°C.
3. Before the cultures reached an OD_{600} of 0.3, they were induced by adding the inducing peptide, SppIP, to a final concentration of 25 ng/ml.
4. The cultures were incubated for 2 or 5 hours and chilled on ice for 10 min, for the cells to stop growing.
5. The cells were centrifuged at 5000 x g for 10 min at 4°C, and the supernatant discarded.
6. The pellets were re-suspended in 1x PBS, and diluted to a final OD_{600} = 0.5, before a pre-stained protocol for flow cytometry was followed (see section 3.23), or the cell pellet was disrupted using glass bead for subsequent western blot analysis (see sections 3.25 and 3.22, respectively).

3.25 Destructions of cells using glass beads

Glass beads of pre-determined amount and size are used to disrupt cells when shaken vigorously. This causes the glass beads to collide with the cells in the sample, finally disrupting the cells and releasing the interior biomolecules. This method makes it possible to determine intracellular protein production in bacteria (e.g. *L. plantarum*).

Materials:

FastPrep tubes

Glass beads (106 microns and finer)

PBS

Procedure:

1. *L. plantarum* cultures harboring the appropriate plasmids were harvested and washed as described in section 3.24, step 1–6.

2. The cell pellet was diluted in 1 ml PBS and transferred to a cooled FastPrep tube containing 1.5 g glass beads.
3. The FastPrep tube was run in the FastPrep® FP120 Cell Disrupter, keeping the speed 6.5 m/s for 45 sec.
4. The tube was centrifuged at 13000 rpm for 30 sec, and the supernatant aspired carefully, before transferred to a new Eppendorf tube.
5. Step 4 was repeated.
6. The cell-free protein extract was stored at –20°C.

3.26 Prepare cells for freeze drying

Freeze drying uses vacuum to dry at approximately –40°C (OPS DIAGNOSTICS 2001), which causes distress on cells and proteins. Therefore a freeze drying medium is added to help protect cells and proteins. The combination resulting in the best protection varies with protein type and bacterial strain (Leslie et al. 1995). In this study, bacterial cell pellets harvested through centrifugation were diluted in freeze dry medium, containing skimmed milk and fructose (Øverland 2013) to help preserve the cultured cells.

Material:

PBS

Freeze dry medium:

11% (w/v) skim milk

12 g/L Fructose

Procedure:

1. Bacterial cells charged with LysM-containing proteins were prepared as in section 3.21, step 1–8. The cell pellets were further washed 3x using PBS, before the supernatant was discarded.
2. The sample was centrifuged at 5000 x g, at 4°C for 10 min, before the supernatant was discarded and the pellet re-suspended in 1 ml PBS.

3. The sample was centrifuged at 5000 x g, at 4°C for 3 min, before the supernatant was discarded and the pellet re-suspended in 1 ml PBS.
4. Step 3 was repeated two times.
5. The cell pellet was added 1 ml freeze dry medium and freeze dried overnight.
6. The freeze dried cells were stored in darkness at room temperature.
7. To hydrate the cells, 1 ml sterile dH_2O was added.

3.27 Lysozyme treatment

Proteins that are embedded in the cell wall structure could be difficult to obtain by primary antibodies, which results in undetected proteins when analyzed by flow cytometry or microscopy. Therefore cells with potentially embedded proteins were treated with lysozyme, which disrupts the peptidoglycan layer, possibly revealing the buried proteins beneath.

Materials:

Lysozyme 100 mg/ml

PBS

Procedure:

1. *L. plantarum* cultures harboring appropriate plasmids were harvested and washed according to section 3.24, Step 1 –6.
2. After dissolving the pellets in 300 µl PBS and 200 µl lysozyme, they were incubated at 37°C for 30 min. Negative samples were included by adding 500 µl PBS only, before incubating at 37°C for 30 min.
3. The cells were washed 3x with PBS and centrifuged at 5000 x g for 2 min at 4°C, in between.
4. The cells were then pre-stained for flow cytometry (see section 3.23).

4. RESULTS

4.1 Intracellular production of 3014-TB and 2162-TB in *Escherichia coli*

3014-TB and 2162-TB are proteins combining the promising antigens Ag85B and ESAT6 with the LysM anchoring domains; 3014 (contains a single LysM domain) and 2162 (contains two LysM domains). Ag85B and ESAT6 were selected due to their promotion of high immune responses against tuberculosis (TB) in mice, guinea pig and non-human primate models (Langermans et al. 2005; Olsen et al. 2001; Olsen et al. 2004). The lysM domains were retrieved from the N-terminal of the gene fragment encoding Lp_3014 and Lp_2162 (Fig. 1.3), the signal sequence (pSIP) located at the N-terminal of the LysM domains were replaced with a 7 histidine, His-tag. This was done to ensure the intracellular production in *E. coli* and the possibility to purify the protein. The LysM domains were fused to the N-terminal of the antigens (Ag85B-ESAT6), and a DC-targeting peptide was added to the C-terminus of these fusion proteins to promote targeting of dendritic cells (Tyler et al. 2013).

4.1.1 Construction of 3014-TB

Constructs from previous studies, *pLp_AG85B_ESAT6-DC* (Øverland 2013) and *pLp_3014Gag24p* (Urdal 2013) (The plasmids are listed in Table 2.4) were used as templates to generate the fragments needed to construct the plasmid *p3014_Ag85B-ESAT6-DC* for production of *3014*-TB (Figure 4.1). The template *pLp_3014Gag24p* were used to generate the fragment *3014*_LysM, using primers 3014_F and 3014_R, while *pLp_AG85B_ESAT6-DC* were used to generate the fragment *Ag85B_ESAT6-DC*, using primers AG85Fus3014_F and AGDCHisR. The two fragments were fused together by a PCR reaction to create the final fragment, *3014-Ag85B-ESAT6-DC* (Figure 4.1)

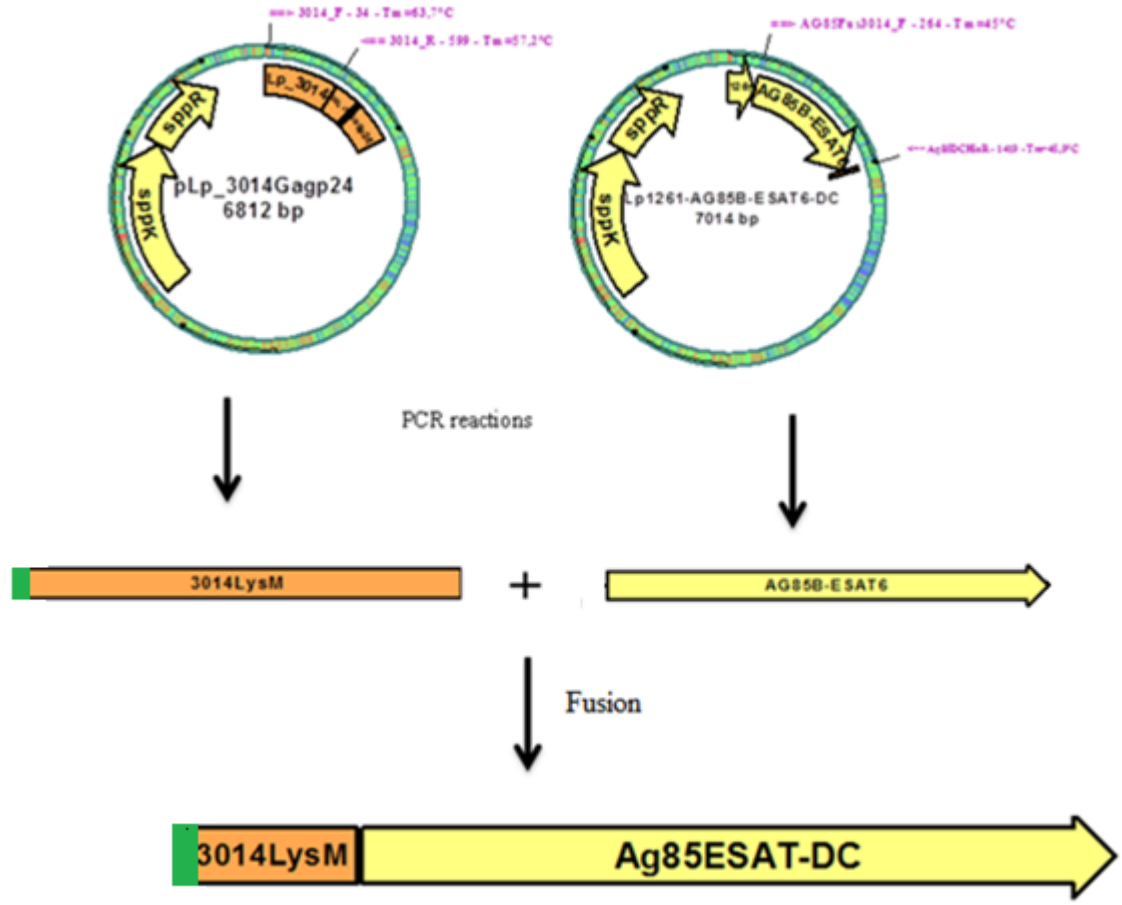

Figure 4.1: Strategy for generating the fragments *3014*_LysM and *Ag85B-ESAT6*. The PCR reaction with *pLp_3014Gagp24* as template and 3014_F and 3014_R as primers resulted in the *3014*_LysM fragment. Another PCR reaction with *Lp1262-AG85B-ESAT6-DC* as template and primers AG84Fus3014_F and AGDCHisR resulted in the *Ag85B-ESAT6* fragment. The two PCR-fragments have overlapping ends and were fused together with a PCR reaction with primers Ag85Fus3014F and Ag85DCHisR, resulting in the final fragment, *3014-Ag85B-ESAT6-DC*. The His-tag is located at the 3'-end in the final fragment *3014-Ag85B-ESAT6-DC* and is visualized in green.

The gene fragment *3014-Ag85B-ESAT6-DC* was ligated into a BglII and EcoRI digested pBAD, yielding the final construct called *p3014-Ag85B-ESAT6-DC* (Figure 4.2). Purified pBAD plasmids were digested using BglII and EcoRI, resulting in two fragments with the sizes 50 bp and 3964 bp. The fragments were separated according to size on agarose gel, leaving the larger fragment (3964 bp) free to be extracted and purified (Figure 4.2).

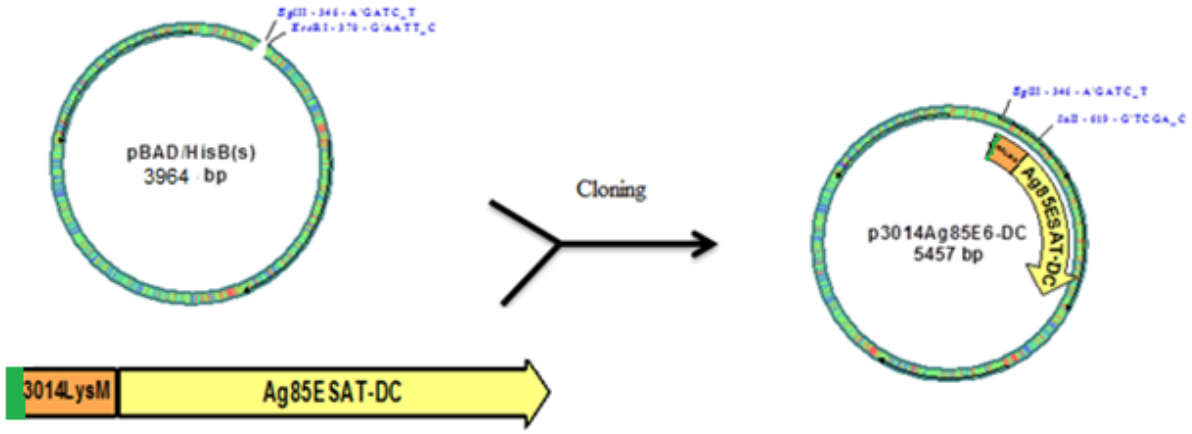

Figure 4.2: The cloning strategy for *3014*-TB. The BglII and EcoRI digested and purified pBAD fragment (3964 bp) were, by using In Fusion cloning reaction, cloned with the gene fragment *3014-Ag85B-ESAT6-DC*, yielding the final construct *3014*-TB.

4.1.2 Construction of 2162-TB

A second construct, 2162-TB differs from 3014-TB only in the N-terminal anchor. The gene fragment *2162*_SVUSP used in the second construct contains two LysM domains, separated by a PST linker region (Figure 1.3). C-terminally of the second LysM domain is a short PST linker sequence of 17 amino acid residues, which was included to create a proper distance between the LysM anchors and antigens. Hopefully, by elongating the distance between the LysM domains and the Ag85B-ESAT6-DC (from Lp_3014 to Lp_2162, this distance increases 1.5 times); this would yield the proper distance for the optimal binding-affinity of LysM domain and for the N-terminus His-tag.

3014-TB was used as the starting point to construct 2162-TB. 3014-TB was digested with SalI and BglII (Figure 4.2), and the resulting 5600 bp fragment was isolated from the gel. The genomic DNA of *L. plantarum* was used as template to yield the gene fragment *2162*_SVUSP, using the primers 2162_USP_F and 2162_SVUSP_R (Figure 4.3). The BglII and SalI digested 3014-TB, yielding the 5600 bp pBAD fragment were, using In Fusion reaction, cloned with the gene fragment *2162*_SVUSP, producing the final construct *2162*-TB (Figure 4.3).

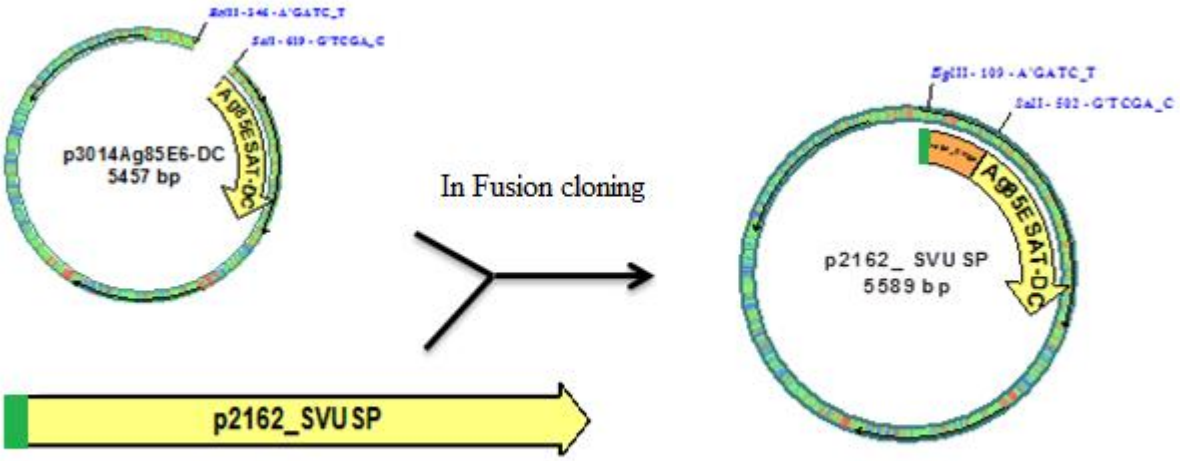

Figure 4.3: The cloning strategy for *2162*-TB. The genomic DNA of *L. plantarum* was used as a template to extract *2162*_SVUSP with the primers 2162_USP_F and 2162_SVUSP_R. The digested *3014*-TB with the restriction enzymes BglII and SalI, was in Fusion cloned with the PCR-retrieved fragment *2162*_SVUSP, to result in the complete plasmid called *2162*-TB. The N-terminus His-tag is visualized in green.

The plasmids, *3104*-TB and *2162*-TB, were transformed into chemically competent *E. coli* TOP10 cells, transformants were selected using ampicillin resistance, to locate the cells harboring the pBAD-derivatives. Transformants containing the correct plasmids (as assessed by colony PCR) were cultured for long-term storage as glycerol stocks at –80°C.

4.2 Protein expression

The use of *Escherichia coli* strains to produce proteins in large quantities before purification is a commonly used method (Eckert et al. 2006; Ohnuma et al. 2007; Olsen et al. 2001; Tyler et al. 2013), and was therefore chosen in this study to produce 3014-TB and 2162-TB. *E. coli* cells harboring the plasmid, *3014*-TB, were used to optimize the intracellular production of 3014-TB in *E. coli*.

4.2.1 Intracellular expression of 3014-TB in *Escherichia coli*

Protein production in *E. coli* was run though series of optimizations, due to the production of 3014-TB as inclusion bodies (see section 3.19 and Fig. 4.5). By varying the growth temperature (from 18^0C–37^0C), the arabinose concentration (0%–20%) used to induce the expression of 3014-TB, and the hours incubated (4–24 hours) after induced, hopefully, 3014-TB would avoid resulting in inclusion bodies.

E. coli cells were grown in 500 ml (BHI medium) cultures, in the temperatures 37^0C, 30^0C and 18^0C. *E. coli* has an optimal growth temperature of 37^0C; however, by using lower growth temperatures, the growth rate was reduced. The reduced growth rate of the *E. coli* cells increased the time for the proteins (as 3014-TB) to fold properly, and avoided the issue of 3014-TB being produced as inclusion bodies. The expression of the gene fragment, *3014*-TB, in *E. coli* cells was controlled by an L-arabinose promoter system, araBAD (Greenfield et al. 1978), which uses arabinose as the effective inducer. *E. coli* cells were initially induced with arabinose concentrations ranging from 0–20% (Figure 4.4 and 4.5). The time given to produce 3014-TB in *E. coli* cells, after induced with arabinose, was ranging from 4–24 hours (Figure 4.4 and 4.5).

Firstly, *E. coli* cells were grown in 10 ml cultures at 37^0C and 30^0C, where each culture was induced using the arabinose concentrations ranging from 0–20% (0%, 0.002%, 0.02%, 0.2%, 2%, 20%). After inducing the *E. coli* cells, each culture was divided into two tubes, each containing 5 ml of the original *E. coli* culture. One of the 5 ml *E. coli* cultures was incubated for 4 hours after induced, while the other one was incubated for 24 hours. The culture that was incubated for 24 hours, in contrast to the culture incubated for 4 hours, revealed a higher total protein yield, and was therefore the incubating time used for incubation in the following work.

After the incubation of cells for 24 hours, the *E. coli* cells were disrupted using sonication. After 5 min of sonication, the cultures were divided into supernatants and cell-pellet using centrifugation; both were run on SDS-PAGE (Figure 4.4 and 4.5). A negative control was included to all experiments conducted in this work; *E. coli* TOP10 cells harboring a pBAD-derivative not expressing the antigens Ag85B-ESAT6-DC.

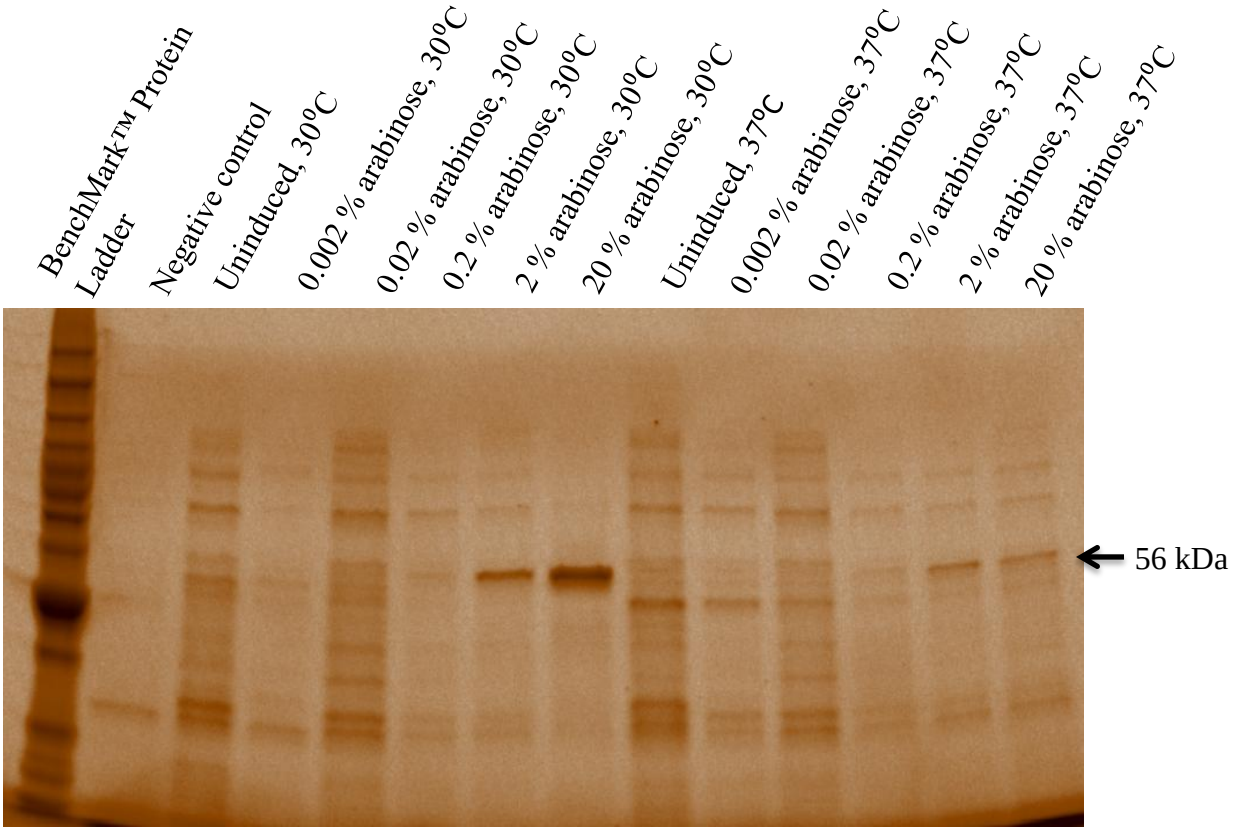

Figure 4.4: SDS-gels of cell extracts from cells harboring *3014*-TB harvested 24 hours after induced with the indicated amounts of arabinose and grown at the indicated temperatures. The expected size of the overexpressed protein, 3014-TB is 56 kDa, and the band corresponding to this size is indeed observed in some of the samples. Negative control; were grown at 30^0C and induced with 20% arabinose.

Figure 4.4 depict that soluble 3014-TB was obtained in some of the cultures. Cultures grown at 30^0C had the highest amount of soluble protein, indicating that the reduced growth rate at this temperature increased the amount of properly folded proteins. Analysis of the pellets obtained during the cell disruption procedure, showed that several of the samples contain large amounts of insoluble 3014-TB (Figure 4.5). Thus, inclusion bodies seem to be formed.

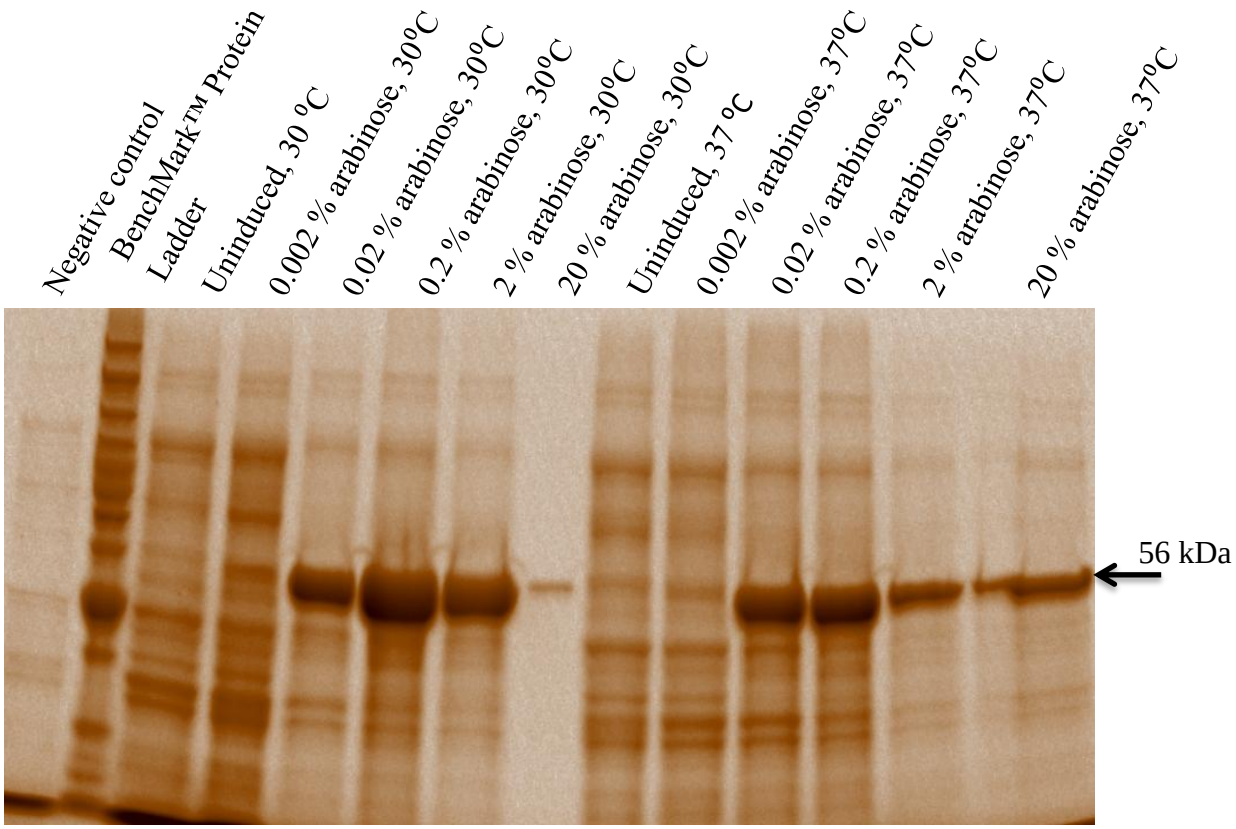

Figure 4.5: SDS-gel of cell pellets from cells harboring *3014*-TB harvested 24 hours after induced with the indicated amounts of arabinose and grown at the indicated temperatures. The expected size of the overexpressed protein, 3014-TB is 56 kDa, and the band corresponding to this size is indeed observed in some of the samples, as inclusion bodies. Negative control; were grown at 30^0C and induced with 20% arabinose.

Samples revealing the lowest production of inclusion bodies were the cultured grown at 30^0C and induced with low arabinose concentrations, as 0% and 0.002% (Figure 4.5). Figure 4.4 reveals that the cells grown at 30^0C, induced with the highest arabinose concentrations (2% and 20%), produces soluble proteins at 56 kDa, surprisingly the band at 56 kDa were not detected using western blotting (analyzed using the antibodies ESAT6 Mouse mcAb (ab26246) and Penta-His™ Antibody, BSA free, hereafter named anti-ESAT6 and anti-His, respectively).

Further optimization were therefore tried to hopefully achieve soluble 3014-TB. Finally, by decreasing the growth temperature to 18^0C, induced using arabinose concentrations ranging from

0% –0.4% (0%, 0.02%, 0.2%, 0.3 % and 0.4%), soluble 3014-TB was detected on SDS-PAGE (Figure 4.6).

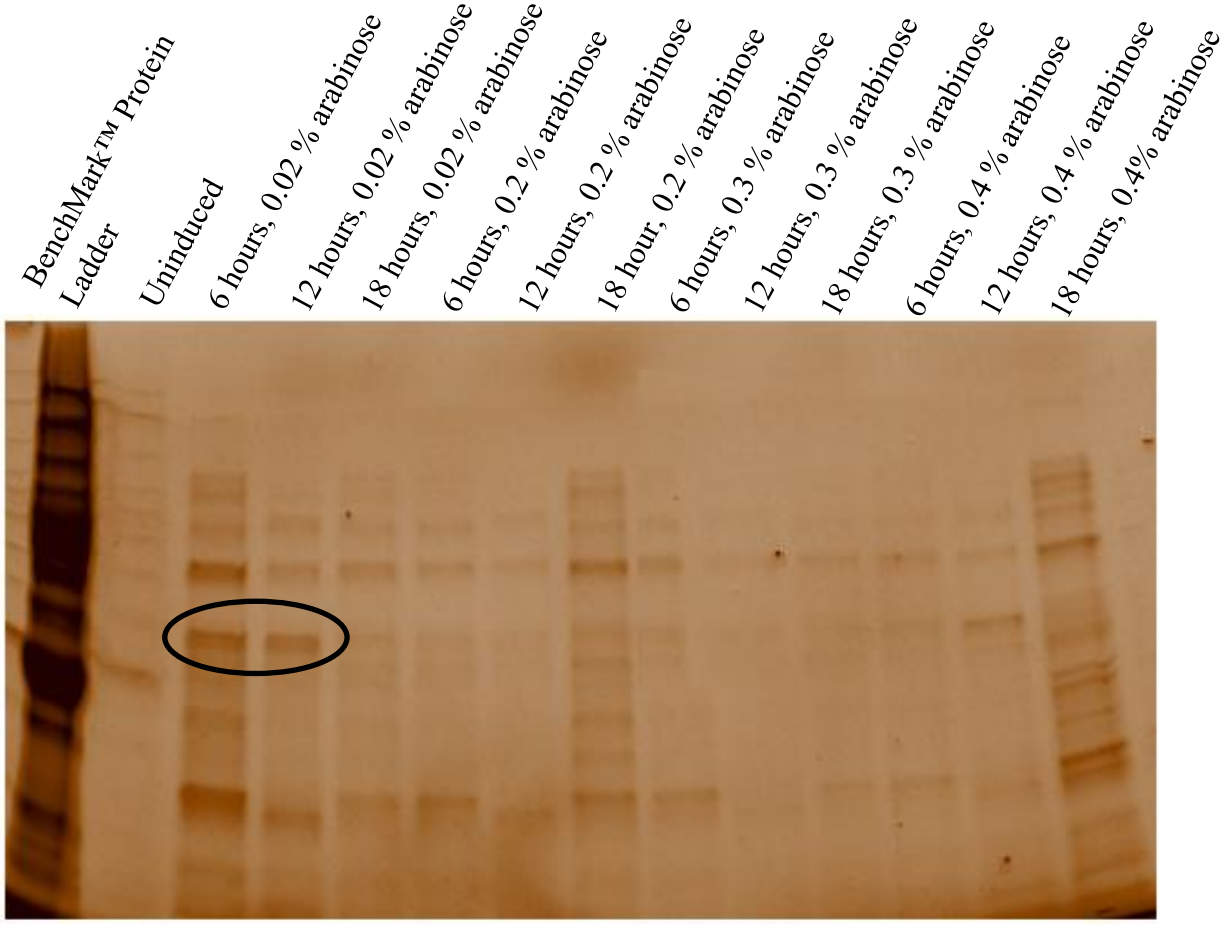

Figure 4.6: SDS-gels of cell extracts from cells harboring *3014*-TB grown at 18°C, induced with the indicated amounts of arabinose and harvested after the indicated time of incubation. The expected size of the overexpressed protein, 3014-TB is 56 kDa, and the band corresponding to this size is indeed observed in some of the samples. The highest yield of 3014-TB is marked; these are induced with 0.02 % arabinose and harvested after 6 and 12 hours. Negative control; were grown at 18^0C, induced with 0.02% arabinose and harvested after 12 hours.

The samples revealing the highest production of soluble 3014-TB were detected in the samples containing *E. coli* cells grown at 18^0C, induced with the final arabinose concentration 0.02 % and harvested after incubating for 6 and 12 hours (Fig. 4.6). Western blots confirmed that the

band at ca. 56 kDa observed in these samples, depicted in Figure 4.6, indeed is 3014-TB (Appendix, Fig. A1).

The following conditions were used to produce soluble 3014-TB in *E. coli* cells harboring the *3014*-TB; the cells were grown in 18^0C, induced with 0.02% arabinose followed by incubating for 12 hours (cells were incubated for 12 hours after induced for reasons concerning convenience during the work period). These optimal conditions were used in all further attempts to produce 3014-TB in *E. coli*.

4.2.2 Intracellular expression of 2162-TB in *Escherichia coli*

Producing soluble 2162-TB in *E. coli* TOP10 cells, followed the optimized conditions discovered for the production of soluble 3014-TB, as described in section 4.2.1. Soluble 2162-TB was indeed detected using western blotting, as depicted in Fig. 4.7, when grown at 18^0C, induced with 0.02% arabinose followed by 12 hours incubation.

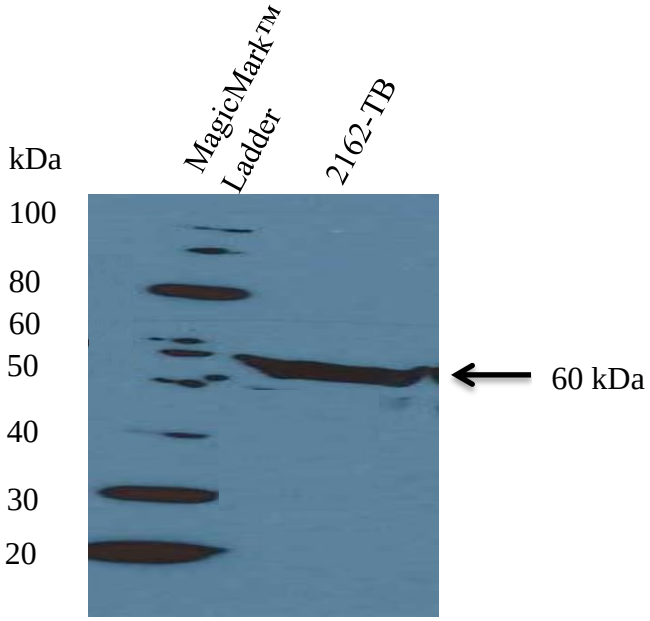

Figure 4.7: Western blot, using anti-ESAT6, showing cell extracts from cells harboring *2162*-TB grown at 18^oC, induced with 0.02 % arabinose, followed by incubation for 12 hours. The expected size of the overexpressed protein 2162-TB is 60 kDa, and the band corresponding to this size is indeed observed in the sample.

The following conditions were used to produce soluble 2162-TB in *E. coli* cells harboring the *2162*-TB; the cells were grown at 18^0C, induced with 0.02% arabinose followed by incubating for 12 hours. These optimal conditions were used in all further attempts to produce 2162-TB in *E. coli.*

4.3 Purification of proteins from cell extracts of *E. coli*

3014-TB and 2162-TB were purified from cell-free protein extracts of *E. coli* cells, using Ni-NTA columns, as described in section 3.15.

4.3.1 Purification of 3014-TB

Protein extracts from *E. coli* cells grown at 18^0C, induced with 0.02% arabinose and harvested after 12 hours incubation, were used as a starting point for purification of 3014-TB, using Ni-NTA column. Purifications of *E. coli* protein extract containing soluble 3014-TB, revealed, using Western blotting (data not shown), no detectible soluble 3014-TB in the eluate. Therefore were several variations in the protocol tried. Buffer A would usually contain low concentrations of imidazole (100 mM) to prevent unspecific interactions. We attempted to reduce the starting imidazole concentration to 0 mM in order to prevent competition for 3014-TB to interact with the Ni-NTA beads. To release bound 3014-TB, the highest imidazole concentration (500 mM) was used in Buffer B (Fig. 4.8).

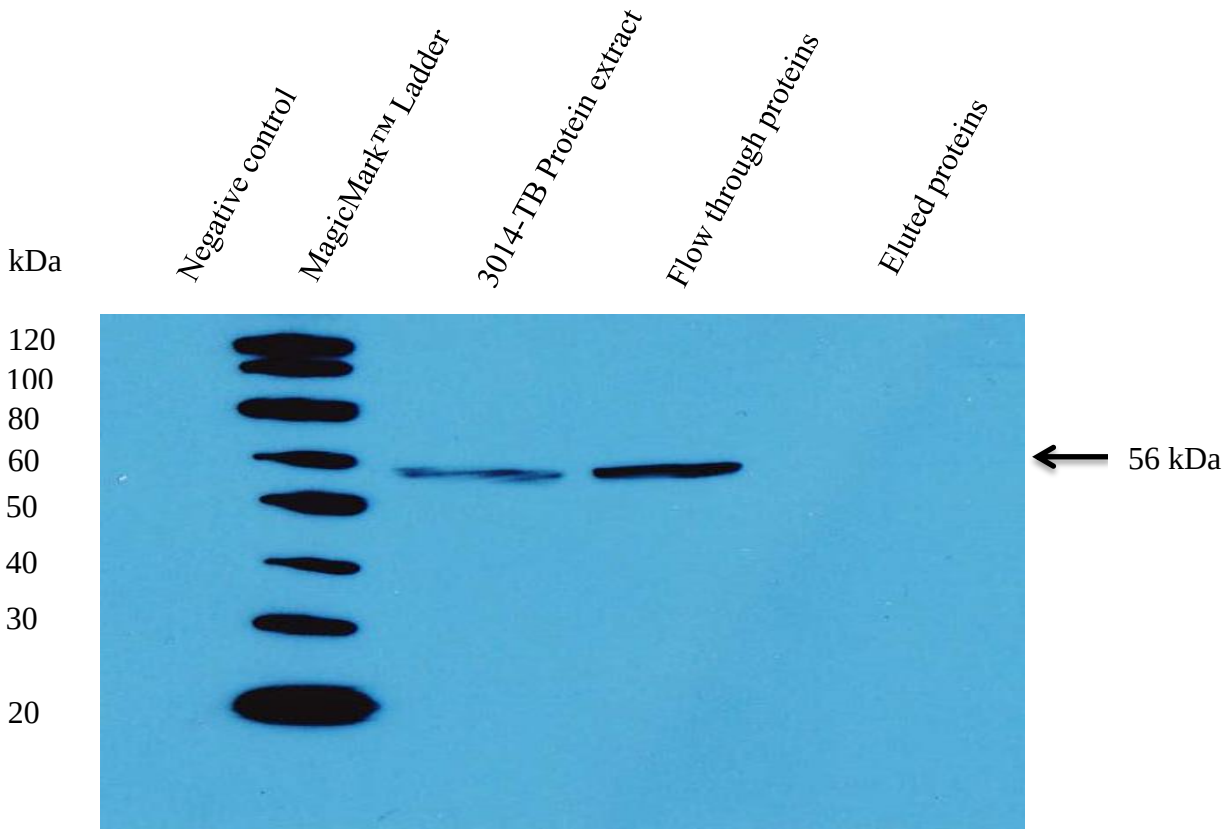

Figure 4.8: Western blot, using anti-His antibodies, showing the result of purification of 3014-TB from cell extracts from *E. coli.* The sample type is indicated above the lanes, and the sample size was 10 µl for all. The 3014-TB protein appears in a full-length form (56 kDa) in the flow through. Note that no bands were detected in the eluate.

Also, with this buffer system (buffer A contains 1 M Tris HCl pH 7.4 and buffer B contains 1 M Tris HCl pH 7.4 and 500 mM imidazole), all 3014-TB was found in the flow through fraction, when analyzed by Western blotting using both anti-ESAT6 (data not shown) and anti-His (Fig. 4.8), meaning that 3014-TB still did not bind to the Ni-NTA. This observation strengthened the idea that the His-tag in folded 3014-TB, somehow is embedded.

In subsequent experiments, attempts were made to release the potential embedded His-tag by a change in pH. Histidine as a positively charged amino acid, were tried released from the folded protein structure with a pH change in the buffers (A and B) from 7.4 to 8 (Fig. 4.9).

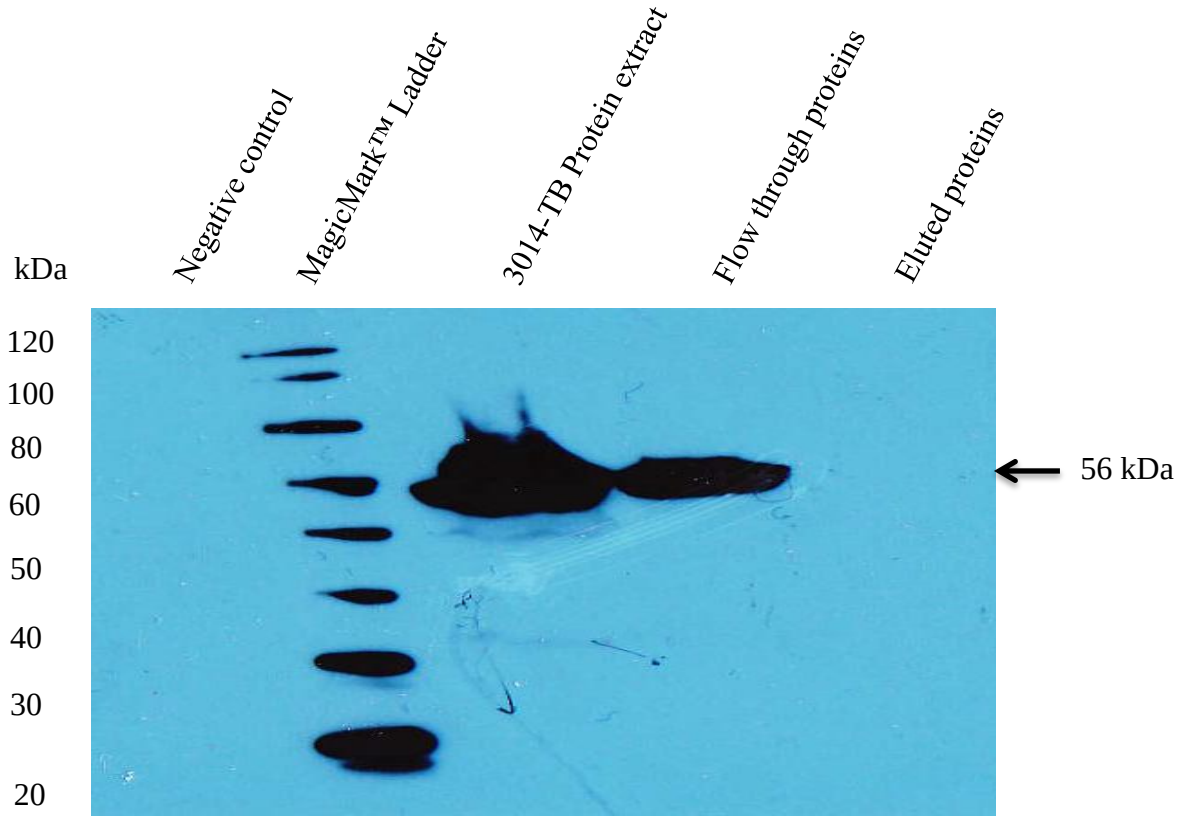

Figure 4.9: Western blots, using anti-His, showing the result of purification of 3014-TB from *E. coli* cell extract, purified in pH 8. The sample type is indicated above the lanes and the sample size was 10 µl for all. The 3014-TB protein appears in a full-length form (56 kDa) in the flow through. Note that no bands were detected in the eluate.

The variation of pH in the buffer solution did not lead to improved purification of 3014-TB (Fig. 4.8). Further elevation of pH values were not tested, since pH > 8 could affect the complete protein structure and cause denaturation.

Previous studies revealed that LysM domains in plant proteins bind to chitin (Onaga & Taira 2008). Although the same has never been observed for bacterial proteins containing LysM domains, an attempt was made to purify 3014-TB using chitin beads. Chitin beads were mixed with protein extract in Eppendorf tubes, and incubated at room temperature for 10 min. After centrifugation, the supernatant was collected, representing the flow through. Buffer solution containing 40 mM Tris Hcl pH 7.4 and 4 M ammonium sulphate was then added to the chitin beads and after incubating for 10 min, the new supernatant was collected by centrifugation. SDS-

gels showed that also in this case, 3014-TB appeared in the flow through (data not shown), suggesting that the LysM used in this study do not bind to chitin.

In an alternative approach attempts were made to solubilize 3014-TB from inclusion bodies, followed by purification under denaturing conditions. This would hopefully increase the possibility to purify the proteins provided that it would be possible to subsequently regain their structure under refolding conditions. For this purpose pellets harvested from *E .coli* cultures grown at 30^0C, induced with 0.2 % arabinose, and incubated for 26 hours (Fig. 4.5), were denatured for purification. Several denaturing conditions were tested to dissolve 3014-TB (Bævre Heggset 2005) (see section 3.19 for details). Incubation with 8 M urea gave the highest yield of resolved 3014-TB (data not shown) and protein solubilized in this manner was subjected to purification (Fig. 4.10).

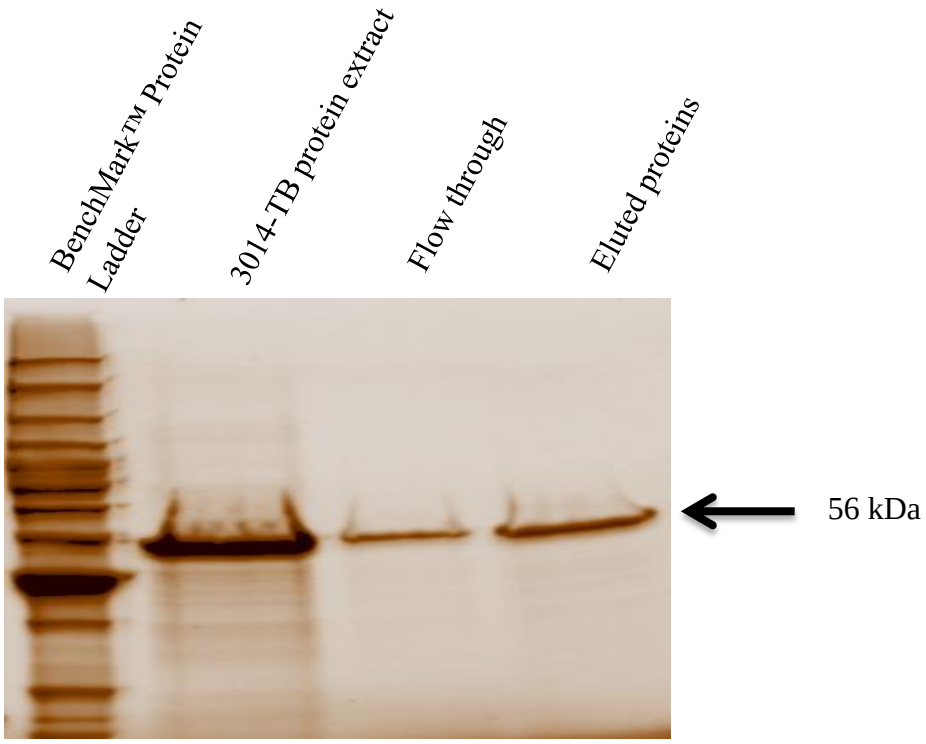

Figure 4.10: SDS-gels showing the result of the purification of *E.* coli protein extract containing solubilized 3014-TB, using 8 M urea. The 3014-TB protein was kept in a solubilized state during the purification, by using buffers containing 8 M urea. 3014-TB appears as a full-length protein (56 kDa) in both the flow through and the eluate, though a higher percentage of solubilized 3014-TB is found in the eluate.

Soluble 3014-TB was almost completely purified using 8 M urea as the denaturant during purification (Fig. 4.10), resulting in the His-tag no longer being embedded in the protein structure, and, is therefore free to interact with the Ni-NTA beaded column. Two refolding strategies were tested in the attempt to obtain a soluble 3014-TB in the absence of denaturants. Refolding by dilution in "Refolding solution" (Bævre Heggset 2005), led to protein precipitation, while refolding using dialysis resulted in soluble 3014-TB (data not shown). Though soluble, 3014-TB was no longer detectable at the cell surface of *L. plantarum* (data not shown). The final purpose of soluble 3014-TB was to bind to gram positive bacteria, such as *L. plantarum* (see section 1.5.2); therefore, 3014-TB was given as *E. coli* total protein extracts in the following binding-studies (see section 4.4).

4.3.2 Purification of 2162-TB

Considering the problems met when working with 3014-TB, two candidates of 2162-TB were made; these were named 2162-TB_L and 2162-TB_S. The difference between the two *2162* constructs is the length of the PST linker region between the last LysM domain and the Ag85B-ESAT6-DC antigen (Fig. 1.3, Lp_2162; the area after the last LysM domain). A longer linker region could potentially avoid the embedded His-tag that potentially occurred in 3014-TB (section 4.3.1). The last PST linker in 2162-TB_L, separating the last LysM domain from the antigen, is 240 amino acid residues long, while the last PST linker region in 2162-TB_S only contains 17 amino acid residues. The two variants of 2162-TB were produced intracellularly in *E. coli* according to the optimal conditions described in section 4.2.2 (i.e. grown in 18^0C, induced with 0.02 % arabinose followed by incubating for 12 hours.). The protein extracts were applied onto Ni-NTA columns using the optimal buffer combination; buffer A containing 1 M Tris HCl pH 7.4, and buffer B containing 500 mM imidazole and 1 M Tris HCl pH 7.4 (see section 4.3.1). Following the purification of 2162-TB, the samples were analyzed by SDS-PAGE (Appendix, Fig. A2), and the presence of 2162-TB was verified by western blotting, using antibody anti-His (Fig. 4.11).

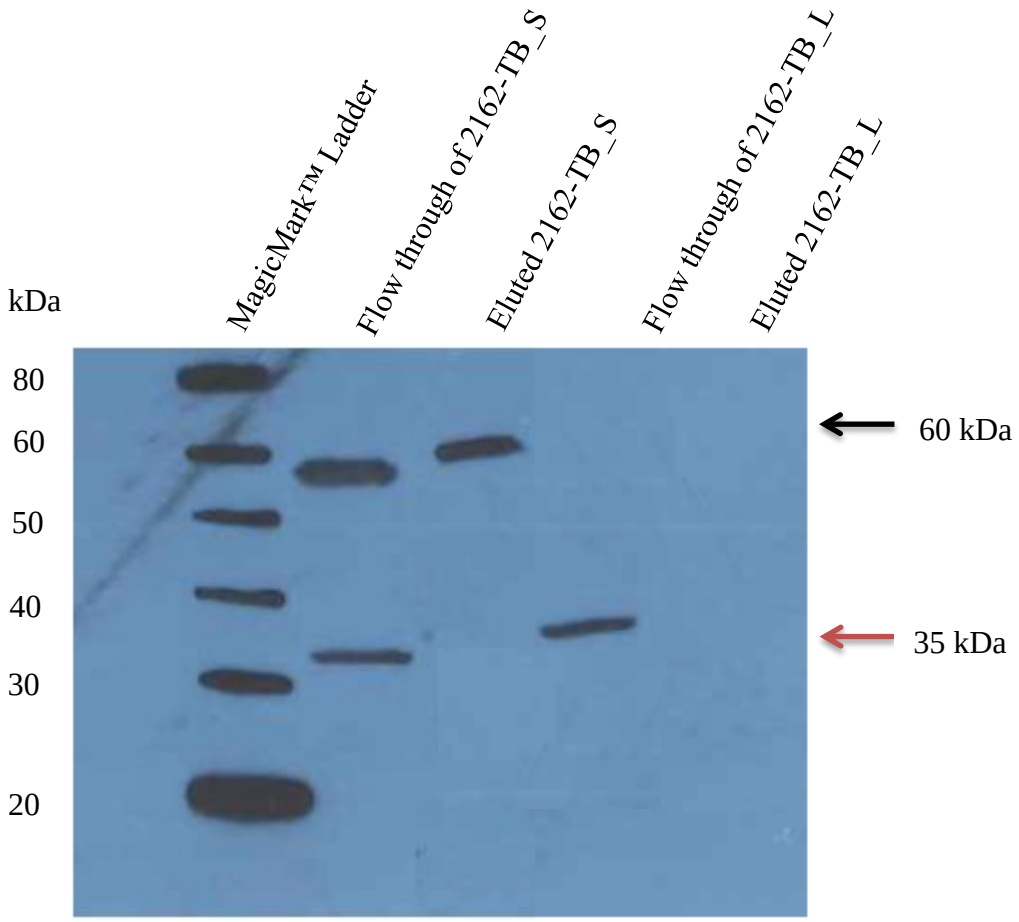

Figure 4.11: Western blot showing results of the purification of the two variants of 2162-TB from *E. coli* cell extract, using anti-His. The sample type is indicated above the lanes and the sample size was 10 µl for all. The 2162-TB_S protein appears both in a full-length form (60 kDa) and a punctuated form (35 kDa), while 2162-TB_L protein only appears in the punctuated form (35k Da). Note that 2162-TB_S protein is detected in the eluate as a full-length protein (60 kDa).

The protein 2162-TB_S was depicted in a punctuated form (35 kDa) in the flow through, and in a full-length form (60 kDa) in both the flow through and the eluate (Fig. 4.11). Western blotting, using anti-ESAT6 to verify 2162-TB_S, only reveals the full-length protein of 60 kDa in the eluate, which implies that only the full-length protein of 2162-TB_S was purified.

The N-terminus of the 2162-TB_L protein was the only fragment of 2162-TB_L depicted (35 kDa) in Figure 4.11, using anti-His, which indicates that 2162-TB_L was either degradation/cleaved off or not fully expressed. Western blotting using anti-ESAT6, detected no

bands in either flow through or eluate, indicating that the longer PST linker region in 2162-TB_L is exposed for cleavage. With this in mind, the 2162-TB_L was discarded and the 2162-TB_S were used further (referred to as 2162-TB hereafter).

After purification, purified 2162-TB was mixed with living *L. plantarum* cells, to potentially bind the cell surfaces. The protein concentration of purified 2162-TB was measured to be 1.67 mg/ml, which were too low to result in detectable amounts of 2162-TB at the cell surfaces of living *L. plantarum* cells (data not shown). Therefore, the total protein extract of *E. coli* containing soluble 2162-TB were given in the following binding-studies (see section 4.4).

4.4 Anchoring of 3014-TB and 2162-TB to the cell surface of living bacteria

Due to the problems faced during purification, both 3014-TB (Fig. 4.6) and 2162-TB (Appendix, Fig. A2) were given as total protein extracts when mixed with living bacteria. Protein extracts from *E. coli* containing either 3014-TB or 2162-TB were stored in 4°C, or used immediately to bind to gram positive bacteria. Living *L. plantarum* cells (Table 2.3) were used as delivery vehicles, due to the peptidoglycan layer. Total protein extracts of *E. coli* containing either 3014-TB or 2162-TB was mixed with *L. plantarum* cells, to determine if 3014-TB and 2162-TB was capable to bind the cell surfaces of living *L. plantarum.* Different protocols were tested to determine the potential optimal binding conditions.

Cells harvested from overnight cultures of *L. plantarum* were washed with PBS, and the cell pellets were used in the bindings-studies. 1 ml of total protein extracts containing either 3014-TB or 2162-TB, with a total protein concentration varying from 8.8 mg/ml to 11.7 mg/ml, was mixed with the washed cells from 1 ml of overnight culture, followed by incubation at room temperature for 30–60 minutes (see section 3.21 for more details).

4.4.1 Incubating *L. plantarum* with 3014-TB proteins

After co-incubating protein extracts from *E. coli* cells containing 3014-TB with *L. plantarum* cells for 30 min at room temperature (see section 3.21, for more details), the cells were harvested through centrifugation. The resulting cell pellet was washed 3 times by adding 1 ml PBS, before vortexing and centrifugation; the supernatant after each centrifugal run was saved, resulting in 4

supernatants (called supernatant 1–4 hereafter). The same protocol was followed for the co-incubation of total protein extracts containing 3014-TB and *L. plantarum* cells for 40, 50 and 60 min. The goal was to potentially find the conditions of incubation that yielded the highest amount of detectable 3014-TB at the cell surface of *L. plantarum*, therefore, *L. plantarum* harvested in both stationary phase and exponential phase was used to co-incubate with 3014-TB. After incubation, the final cell-pellets incubated in 30–60 min were analyzed by western blotting, using anti-ESAT6 (Figure 4.12).

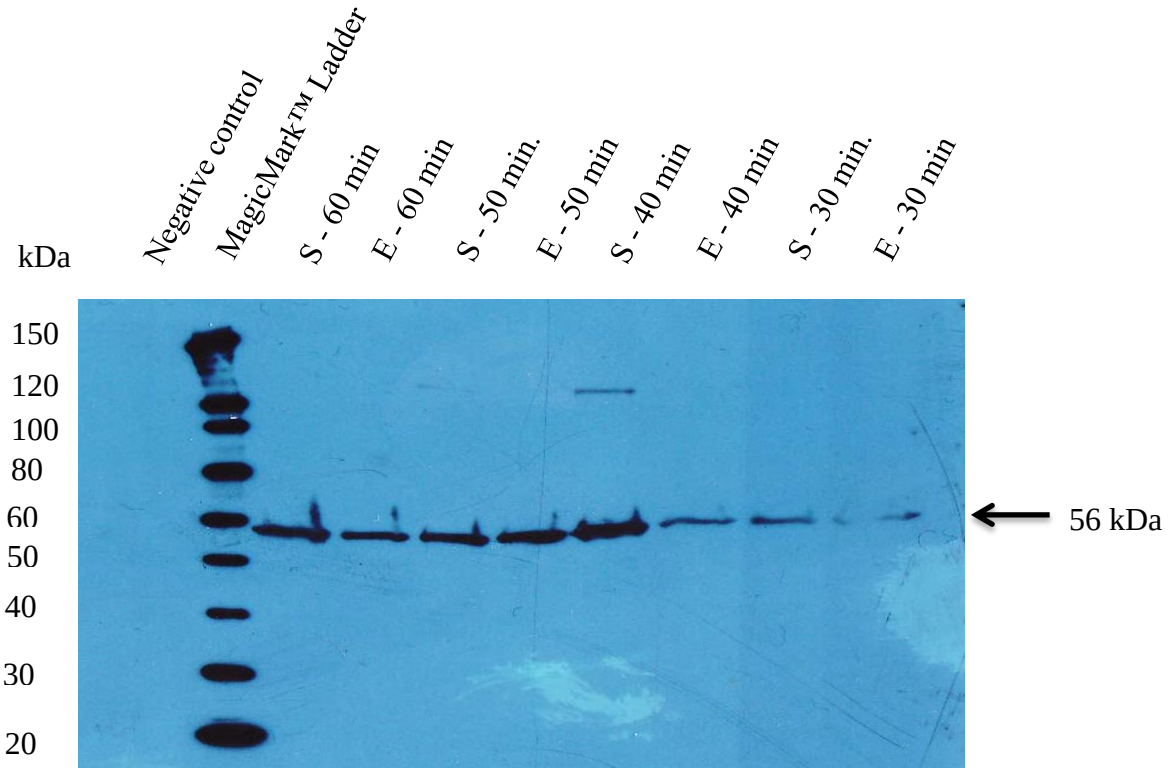

Figure 4.12: Western blot, using anti-ESAT6, showing results following the co-incubation of protein extracts containing 3014-TB from *E. coli* with living *L. plantarum* harvested in the stationary (S) or exponential (E) phase. 3014-TB was incubated with *L. plantarum* for 30 to 60 min at room temperature. All cell samples reveal 3014-TB (56 kDa) displayed at the cell surface of *L. plantarum*. Negative control; *L. plantarum* harvested in exponential phase, incubated for 60 min with protein extracts from *E. coli* containing a pBAD-derivative, not expressing the Ag85B-ESAT6-DC antigen.

3014-TB was depicted to bind to the cell surface of *L. plantarum* cells, when analyzed by western blotting, using anti-ESAT6 (Fig. 4.12) and anti-His (data not shown). 3014-TB binds to living *L. plantarum* after 30 min, and by increasing the incubation period thus increases the amount of the protein 3014-TB displayed on the cell surface of *L. plantarum* (Fig. 4.12). The goal was to bind all soluble 3014-TB in the protein extract from *E. coli* to the cell surfaces of *L. plantarum* cells, therefore, after 1 hour of incubation the supernatants 1–4 were analyzed by western blotting, using anti-ESAT6 (Appendix, Fig. A3); unbound 3014-TB would be detected in supernatant 1, while poorly bound 3014-TB would be detected in any of the three supernatants 2–4. 3014-TB was not depicted in the supernatants 1–4 (Appendix, Fig. A3), and therefore, 60 min of incubation were used in the following bindings-studies.

Protein extracts containing 3014-TB incubated for 60 min with *L. plantarum* cells, were using the total protein concentration of 11.7 mg/ml. However, by gradually increasing the total protein concentration to 44 mg/ml, thus resulted in the gradual increase of the amount of 3014-TB displayed at the cell surface, analyzed by western blotting (data not shown). The detectable amounts of 3014-TB at the cell surface were not depicted to increase when the total protein concentrations used were exceeding 44 mg/ml (data not shown). For simplicity, 11.7 mg/ml of the total protein concentrations of protein extracts containing 3014-TB were used in the following work.

4.4.2 Unspecific interactions to the cell surface of *Lactobacillus plantarum*

The use of protein extracts containing 3014-TB or 2162-TB rather than the purified 3014-TB or 2162-TB could result in potential background signals; by interpreting the potential bound additional substances of the protein extract (e.g. proteins not containing TB-antigens) to the cell surfaces of *L. plantarum* cells, as positive signals. Negative controls were treated equally as the sample containing 3014-TB and 2162-TB (described in section 4.4.1); 1 ml of protein extracts were incubated for 1 hour with living *L. plantarum* cells harvested in exponential phase. In this manner, the potential unspecific interaction to the cell surfaces of *L. plantarum* interpreted as positive signals would be detected. Four negative controls were tested in this work, analyzed by flow cytometry; *L. plantarum* WCFS1 not mixed with protein extracts (called *L. plantarum* only) and *L. plantarum* WCFS1 mixed with protein extracts from;

- *E. coli* TOP10 harboring a pBAD-derivative containing a gene fragment not expressing 3014-TB (Table 2.4; Section 4.2.2, negative control), called *E. coli* TOP10.
- *E. coli* TOP10 harboring an empty pBAD plasmids (Table 2.3, unpublished by G. Mathiesen).
- *E. coli* TOP10 harboring a pBAD-derivative containing the Ag85B-ESAT6 antigens, lacking the LysM domain (Table 2.3) (called ESAT6 only).

After co-incubating protein extracts with *L. plantarum* cells, *L. plantarum* only were incubated with 1 ml PBS, the samples were analyzed by flow cytometry, using anti-ESAT6 and secondary FITC labeled antibody HRP-Rabbit Anti-Mouse IgG (referred to as FITC-labeled antibody hereafter) (Fig.4.13).

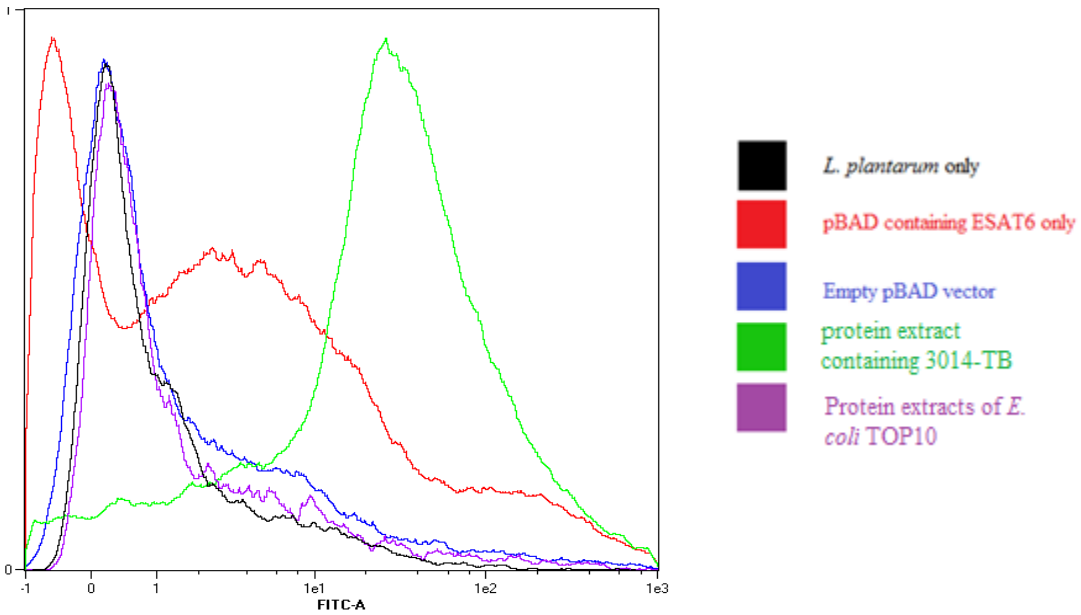

Figure 4.13: Flow images using anti-ESAT6, displaying the potential unspecific interactions to the cell surface of *L. plantarum*. The color scheme is shown to the right of the histograms, explaining the individual protein extracts bound to living *L. plantarum* cells, which are represented by each colored histogram. The positive control (green) is the protein extract containing 3014-TB bound to the cell surfaces of *L plantarum*. Note that *L. plantarum* cells charged with ESAT-6 only is the negative sample displaying an unspecific reaction.

L. plantarum charged with 3014-TB has a clear shift towards the right (Fig. 4.13), indicating the high amount of FITC-labeled secondary antibodies at the cell surface. The primary antibody

anti-ESAT6 recognizes the specific ESAT6-epitopes in 3014-TB, followed by FITC-labeled secondary antibody recognizing and binding anti-ESAT6. When cells are analyzed using a laser beam, more detected FITC-labeled antibody causes the line representing that sample to shift towards the right (after normalizing the number of cells and FITC-detection in the cell sample without the added protein extract, the black histogram in Fig. 4.13) (Section 3.23). The only negative control that revealed an unspecific interaction with the *L. plantarum* cells was the protein extract containing ESAT6-only (red histogram, Fig. 4.13).

Flow cytometry is a very sensitive method; therefore, the sample containing ESAT6 only and *L. plantarum* cells (red histogram, Fig. 4.13) was analyzed by western blotting using anti-ESAT6, to confirm the unspecific interaction (data not shown). The western blot depicted no unspecific interaction, implying that the previously observed unspecific binding (Fig. 4.13) was insignificant or too low to be recognized using western blotting. On the other hand, the western blot confirmed strong binding of 3014-TB (Appendix, Fig. A1).

4.4.3 3014-TB binds to a broader range of bacteria strains

3014-TB with the N-terminal Lysine Motif (LysM) domain would theoretically have the ability to anchor all bacteria strains containing peptidoglycan. Protein extracts containing 3014-TB were, therefore, co-incubated for 1 hour at room temperature with 14 different bacterial strains (excluding the already tested *L. plantarum* WCFS1) harvested in the exponential phase (see section 4.4.1 for more details; the bacteria strains are listed in Table 4.1). In this manner, detected amounts of 3014-TB displayed on the cell surface of each bacteria strain, analyzed by western blotting, could be visually compared using the high intensity bands representing each bacterial strain. This would give an indication of the potential alteration of 3014-TB's ability to anchor the different gram positive bacteria (Fig. 4.14).

Table 4.1. Bacterial strains used to test binding of 3014-TB

Lactobacillus spp	**Other strains**
L. gasseri	*Streptococcus faecalis*
L. brevis	*Pediococcous acidolactic*
L. sakei	*Enterococcus faecium*
L.coryneformis	*Pediococcus pentisaceus*
L. lactis subspecies lactis (lactis subsp. lactis)	*Carnobacterium pisicola*
L. rhamnosus GG.	*Bacillus subtilis*
L. acidophilus	*E. coli*

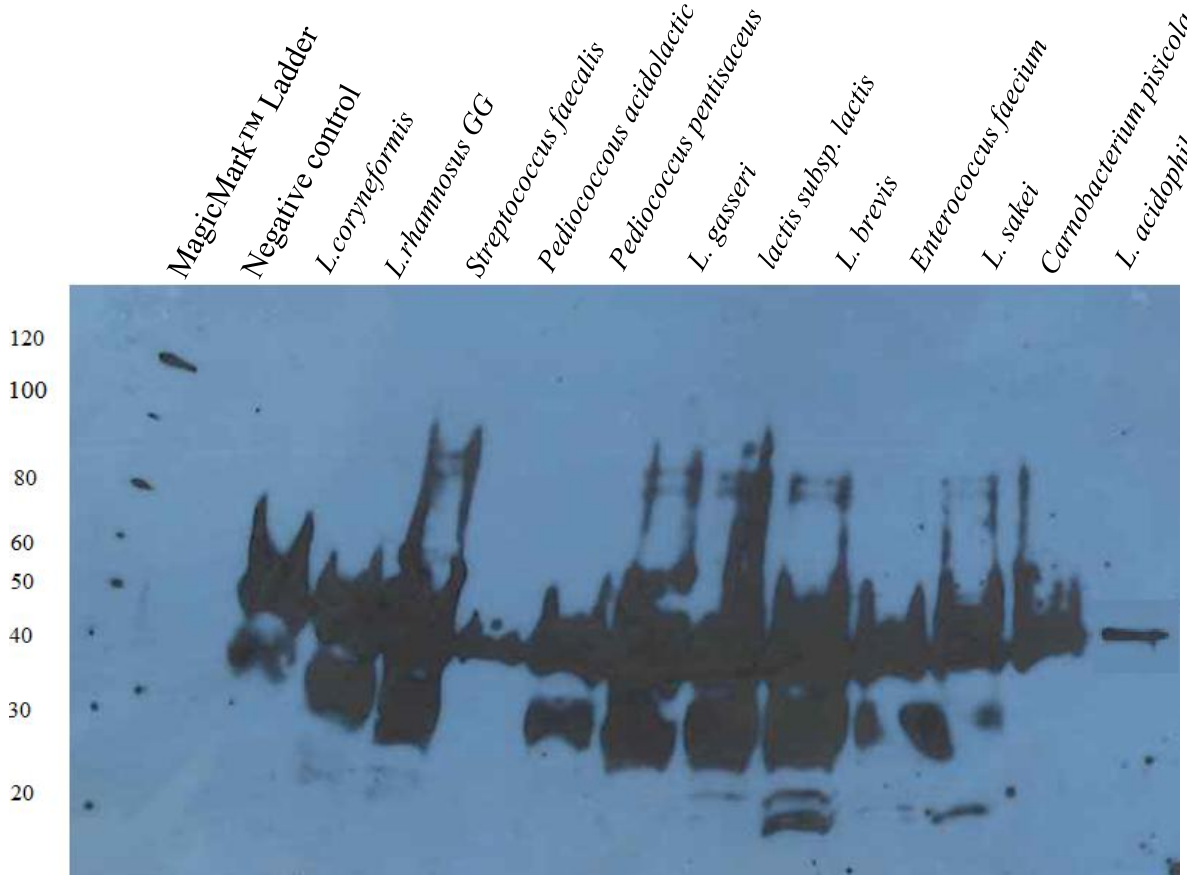

Figure 4.14: Western blotting, using anti-His, showing the results after co-incubating protein extract containing 3014-TB from *E. coli* with the 12 bacterial strains indicated in each lane. The protein 3014-TB appears in a full-length form (56 kDa) at all cell surfaces, excluding the negative control. 3014-TB was also detected as truncated forms (approximately 20, 30 and 40 kDa) and elongated forms (approximately 80 kDa) in some of the samples. Negative control: *L. rhamnosus GG* cells co-incubated with protein extracts from *E. coli* TOP10 cells harboring a pBAD-derivative, not expressing the antigen, Ag85B-ESAT6-DC.

3014-TB was displayed on the cell surfaces of all 12 strains of *Lactobacillus* that were tested, both as a full-length protein and truncated/elongated forms, using both anti-ESAT6 (Fig. 4.14, excludes *L. plantarum*) and anti-His (data not shown). The negative control is depicted in Figure 4.14, and as expected, reveals no positive signals for proteins containing His-tags. *E. coli* is gram-negative and lacks the peptidoglycan outer layer, and was therefore considered to function as an additional negative control (Appendix, Fig. A2). 3014-TB was displayed in high amounts at the cell surfaces of *E. coli*, indicated by the high intensity band (Appendix, Figure A2), while *B. subtilis* (as gram-positive) displayed low amount of 3014-TB on the cell surfaces, indicated by the low intensity band (Appendix, Fig. A2).

Fig. 4.14 shows that the amount and degree of fragmentation of anchored 3014-TB varied. A few strains were elected for further work, namely *B. subtilis*, *L. gasseri*, *L. rhamnosus GG* and *L. plantarum* WCFS1. *L. plantarum* was selected because it was the standard strain used in this and previous (Øverland 2013) studies; *L rhamnosus GG* was selected because it is part of the Idoform® capsules given to stabilize the gut's microflora, and were proven to be the *Lactobacillus* strain most stable in bile, in a study by Kõll et al. (2008). *L. gasseri* was selected because it is known to elicit immune activities in humans (Selle & Klaenhammer 2013) and modulate dendritic cells *in vitro* (Luongo et al. 2013). *B. subtilis* was selected due to the promising results used as a delivery vehicle, discovered in a study by Amaguni and Tzipori (2012) (described in section 1.3.2).

The growth phase of the bacteria (stationary or exponential phase) anchoring most 3014-TB to the cell surface needed to be determined. Samples from each of the four bacteria strains in both stationary and exponential phase were collected and added 11 mg 3014-TB, before incubated for 1 hour. The amount of 3014-TB on each surface was determined by western blots, using ant-ESAT6 (Fig. 4.15).

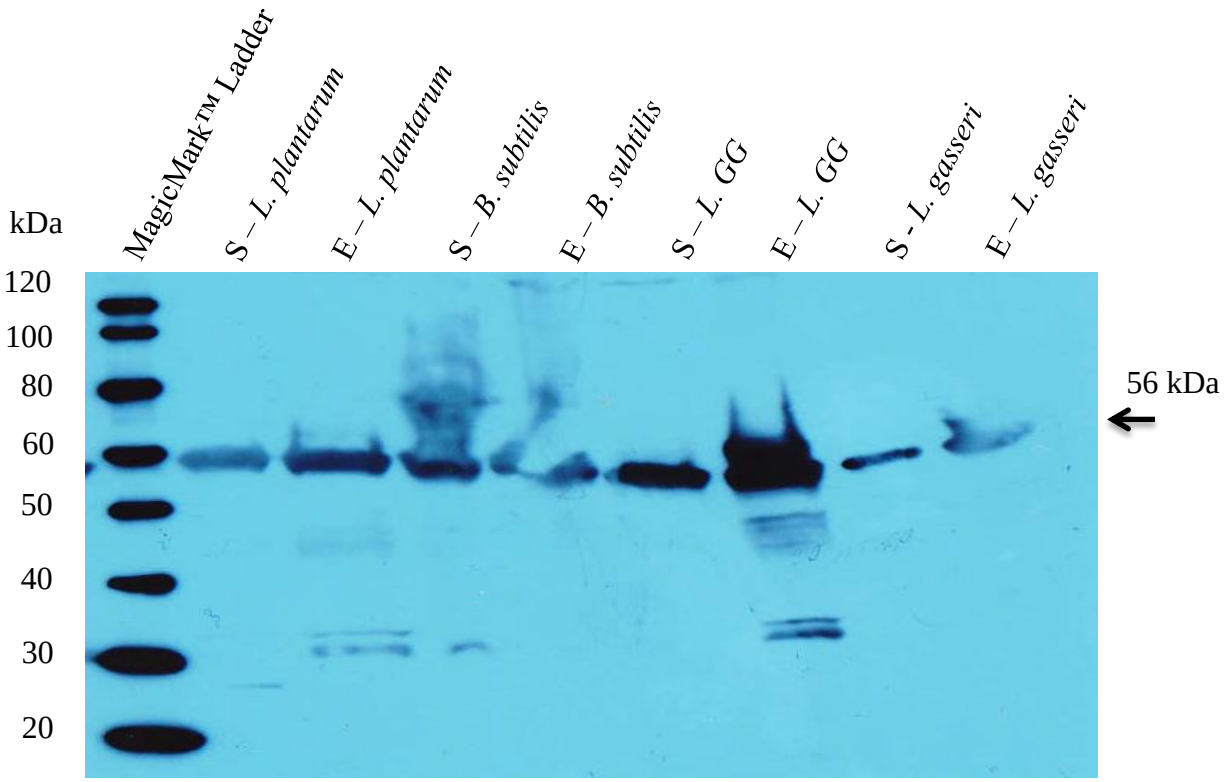

Figure 4.15: Western blot, using anti-ESAT6, showing the results after co-incubation of protein extract containing 3014-TB with *L. plantarum, L. gasseri, L. rhanmosus GG* and *B. subtilis* harvested in the exponential (E) phase and stationary (S) phase. 3014-TB is displayed as a full-length protein (56 kda) and as truncated forms (30 and 45 kDa) at the cell surfaces of the bacterial strains tested, harvested in both stationary- and exponential phase. Note that 3014-TB is displayed in higher amounts when incubated using bacterial strains harvested in the exponential phase, indicated by the higher intensity band.

The four bacterial strains tested, harvested in the exponential phase, depicted higher amounts of 3014-TB displayed at their cell surfaces (Fig. 4.15), which is indicated by the higher intensity bands. The same was depicted by western blotting using anti-His (data not shown). Flow cytometry were used to further confirm that the bacterial strain harvested in their exponential phase were optimal when incubated with 3014-TB (Fig. 4.16).

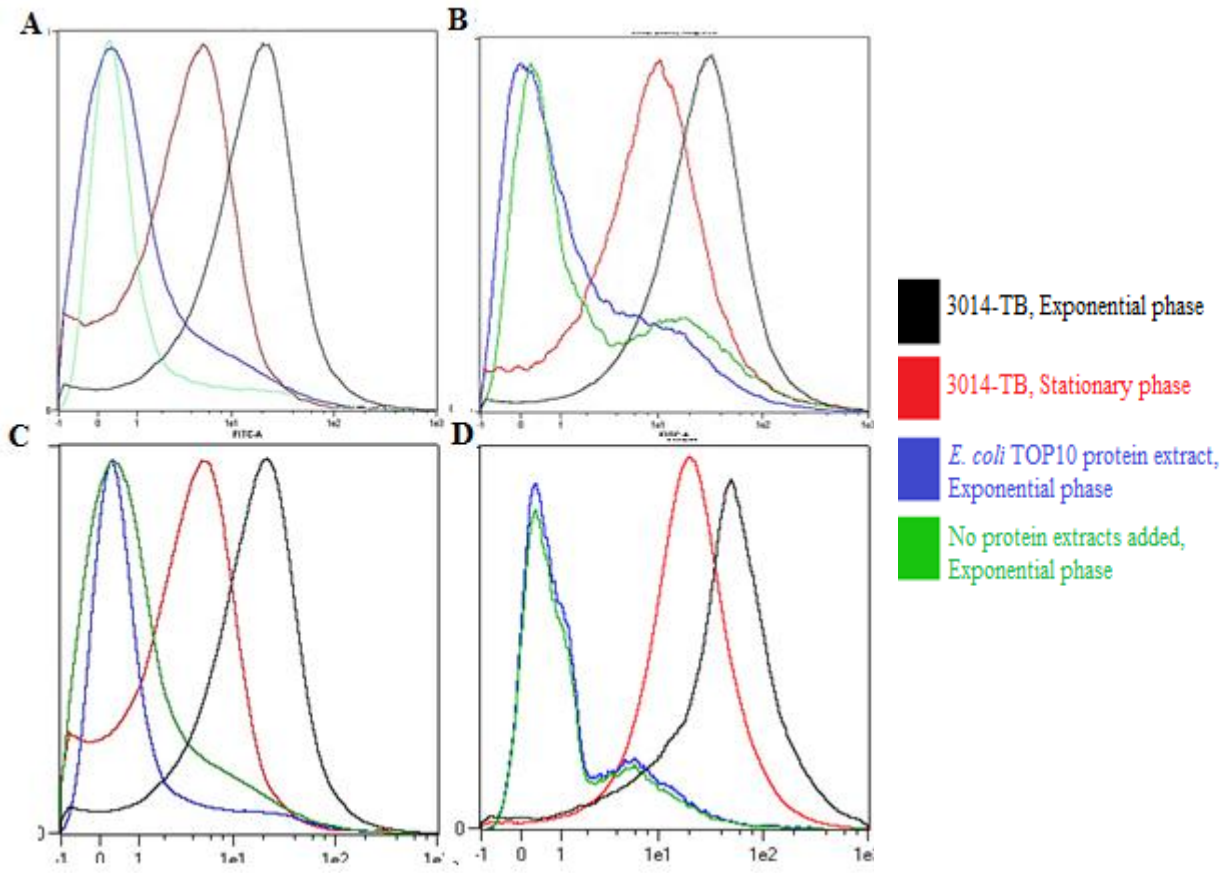

Figure 4.16: Flow images of *B. subtilis* (A), *L. plantarum* (B), *L. GG* (C), *L. gasseri* (D) in both stationary and exponential phases, displaying 3014-TB at the cell surfaces. The color scheme is shown to the right of the histograms, explaining the individual protein extracts added and the cells' phase when harvested, represented by each colored histogram.

Generally, the line shifted furthest to the right was 3014-TB bound to the cells that were harvested in the exponential phase (Fig. 4.16, black histogram), which confirms the results depicted by western blot (Fig. 4.15); the highest amounts of 3014-TB was observed when cells harvested in the exponential phase were used.

4.4.4. 2162-TB anchored to the cell surface of *Bacillus subtilis* and *Lactobacillus spp.*

Following the discoveries of 3014-TB attached to the bacterial strains used (see section 4.4.1 and 4.4.3), protein extracts containing 2162-TB from *E. coli* were co-incubated with *L. plantarum, L. gasseri, L. rhamnosus GG* and *B. subtilis*, harvested in exponential- and stationary phase. The protein extracts containing 2162-TB were incubated for 1 hour at room temperature, with bacterial strains, before analyzed by western blotting, using anti-His (Fig. 4.17).

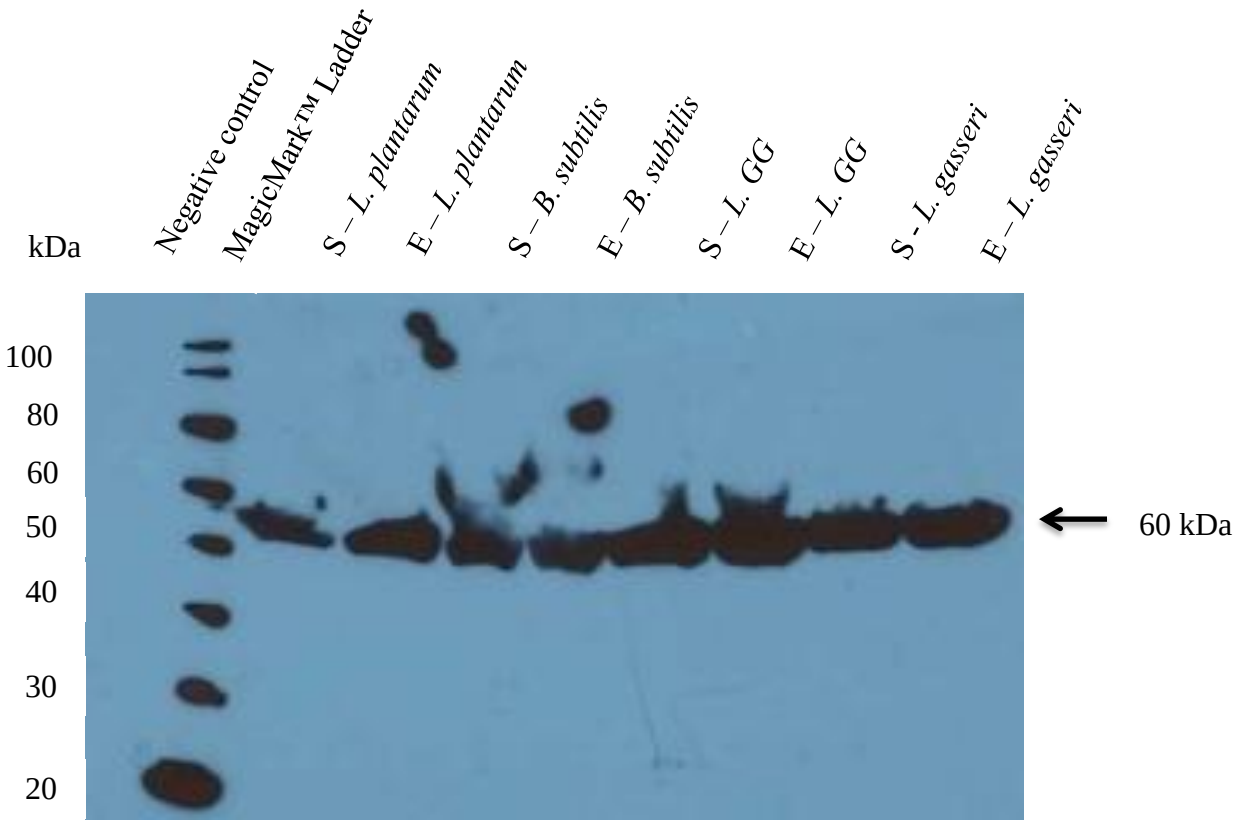

Figure 4.17: Western blot, using anti-His, showing the results of co-incubated protein extracts containing 2162-TB with the indicated bacterial strains, harvested in the stationary- (S) and exponential (E) phase. The bacterial strains are indicated above the lanes. 2162-TB is displayed as a full-length protein of 60 kDa, in almost equally amounts on the cell surfaces of the bacterial cells harvested in both stationary and exponential phase.

2162-TB displayed on the cell surfaces of the four strains were depicted in Fig. 4.17, the same degree of anchored 2162-TB was observed for cells harvested in both stationary- and exponential phase. After analyzing the samples by flow cytometry, using anti-ESAT6 (data not shown), the

protein 2162-TB was observed to anchor *B. subtilis* cells, harvested in the stationary- and exponential phase, in the same degree. For *L. plantarum*, *L. GG* and *L. gasseri* the highest degree of anchored 2162-TB was observed when cells were harvested in the stationary phase (data not shown).

In all further work, 2162-TB and 3014-TB were anchored to cells harvested in the exponential phase to simplify the comparison of the results obtained for both proteins.

4.5 Effect of low pH and bile on 3014-TB and 2162-TB

Proteins displayed on delivery vehicles, which are orally administrated, need to resist degradation in low pH values and high bile concentrations (Marteau et al. 1997). In addition, 3014-TB and 2162-TB anchored to cell surfaces of bacteria must resist longer storing times, so the vaccine can be manufactured and transported between countries all over the world without losing effect.

4.5.1 3014-TB and 2162-TB after freeze drying and storage

The protein extracts containing 2162-TB or 3014-TB were stored at 4°C. After 3 weeks of storage, neither 2162-TB nor 3014-TB was observed at the cell surfaces following co-incubation (data not shown). To elongate the stability of 3014-TB and 2162-TB during storage, the proteins were tried stored while attached to the bacterial cell surfaces, using freeze drying (see section 3.26). Due to the distress caused by freeze drying on cells and proteins, a freeze dry medium (composed of skimmed milk and fructose) were added before bacteria cells charged with 2162-TB or 3014-TB were freeze dried overnight. Following the freeze drying, the samples were stored in the dark, at room temperature for 1 week. The freeze dried bacteria cells displaying the 3014-TB or 2162-TB were kept stable during 1 week of storage (Fig. 4.18).

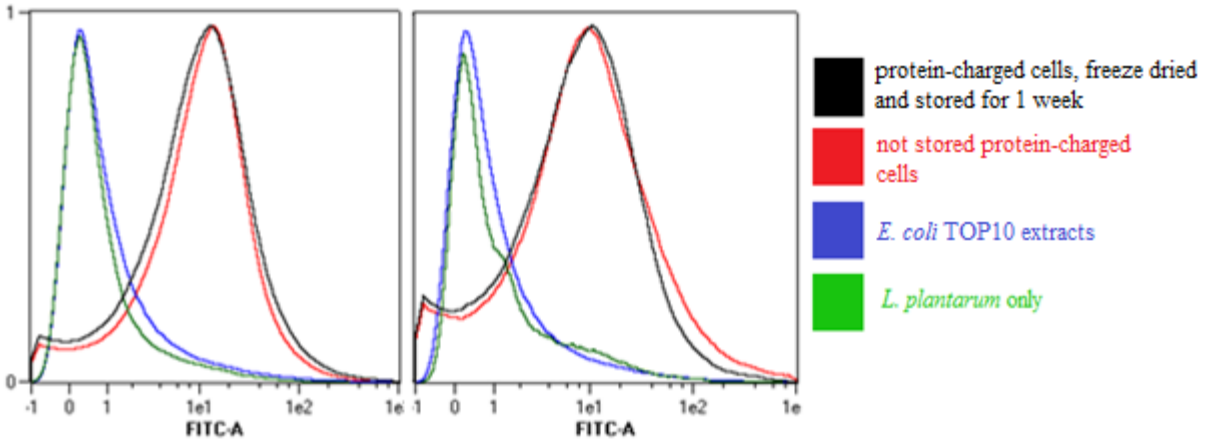

Figure 4.18: Flow cytometry to analyze the effect of freeze-drying and storage on *L. plantarum* displaying 3014-TB (left) and 2162-TB (right). The color scheme is shown to the right of the histograms. The control samples (green and blue) were analyzed after freeze drying and 1 week of storage.

The histogram representing *L. plantarum* displaying 3014-TB or 2162-TB that were stored for 1 week (Fig. 4.18, black histogram), depicts the same shift as for *L. plantarum* displaying 3014-TB or 2162-TB without storage (Fig. 4.18, red histogram). This indicated that the charged *L. plantarum* was stable after 1 week of storage. *L. plantarum* charged with 3014-TB were additionally stored for 3 months after freeze drying; to observe the increased storing time's effect on the stability of *L. plantarum* displaying 3014-TB (Fig. 4.19).

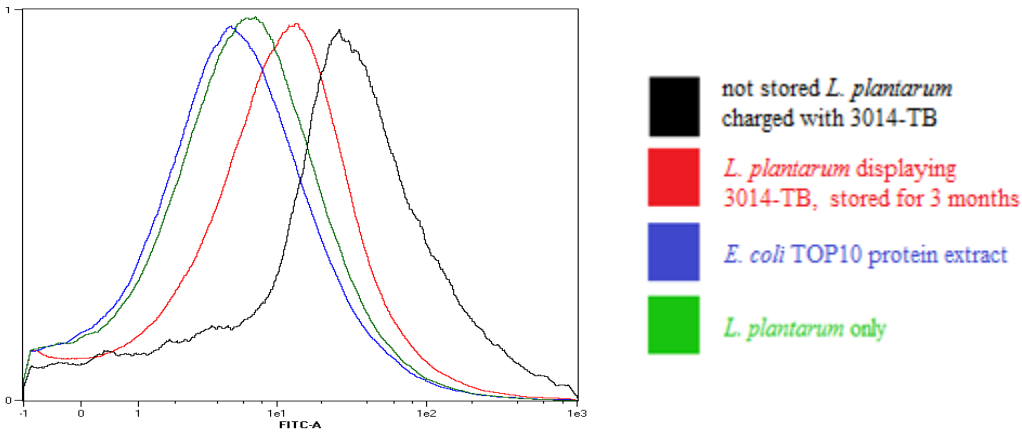

Figure 4.19: Flow images of *L. plantarum* displaying 3014-TB after freeze drying and 3 months of storage. The color scheme is shown to the right of the histograms. The control samples (green and blue) were analyzed after freeze drying and 3 months of storage.

The negative samples stored for 3 months (green and blue histogram, Fig. 4.19), not displaying ESAT6-epitopes, were almost shifted equally towards the right as the *L. plantarum* cells charged with 3014-TB (red and black histograms, Fig. 4.19). This tendency was not observed for the analyzed negative samples stored for 1 week (green and blue histogram, Fig.4.18), which indicates that the freeze drying medium, when stored with samples for longer time periods, probably results in an unspecific interaction with the primary antibody anti-ESAT6. The data indicate that *L. plantarum* cells displaying 3014-TB have the ability to be stored using freeze drying, but, for now, only in shorter time spans.

4.5.2 *L. plantarum* cells displaying 3014-TB or 2162-TB, incubated in low pH values

Before the vaccine reaches the mucosal surfaces in the intestine, the vaccine has to resist the low pH values found in the stomach, while the proteins displayed at the cell surfaces need to remain intact (Marteau et al. 1997).

The time needed to resist low pH depends on the food ingested. For example, the pH composition in a human stomach after ingesting a yoghurt starts at pH = 5 (20 min), then lowered to pH = 4 (40 min), before pH = 2 is kept for 60 minutes. Finally the pH value 1.8 is kept for more than 80 min (Marteau et al. 1997). According to this, the *L. plantarum* cells displaying 3014-TB or 2162-TB were incubated in pH values ranging from 1.5–6.5 for 2 hours at 37°C. After incubation, the samples were washed 3x, using 1 ml of PBS (as described in section 3.21, step 5–7) and stained following the pre-stained protocol (section 3.23) for analyzing by flow cytometry, using anti-ESAT6 (Fig. 4.20).

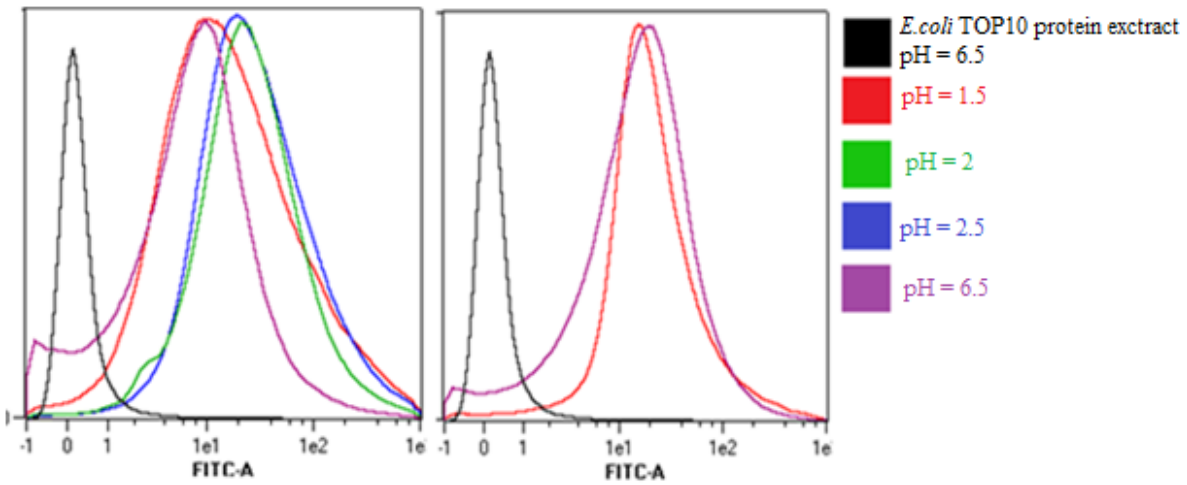

Figure 4.20: Flow images of *L. plantarum* displaying 3014-TB (left) or 2162-TB (right), incubated for 2 hours in a broad range of pH values. The color scheme is shown to the right of the histograms. *L. plantarum* displaying 3014-TB/2162-TB was incubated in pH regulated (indicated by the color scheme) MRS medium. The negative control (black) is *L. plantarum* charged with the protein extract of *E. coli* TOP10 (harboring a pBAD-derivative, not expressing the antigen Ag85B-ESAT6-DC, incubated in MRS medium, not pH-regulated.

L. plantarum displaying either 2162-TB or 3014-TB was stable after incubation in pH-regulated (from 1.5–6.5) MRS medium (Fig. 4.20). The four bacteria strain charged with 2162-TB or 3014-TB, after freeze dried and stored for 1 week, were incubated in pH-regulated MRS medium (data not shown), revealing the same stability as for the samples observed in Fig. 4. 20.

4.5.3 *L. plantarum* cells displaying 3014-TB or 2162-TB, incubated in bile

After resisting the low pH values in the stomach (see section 4.5.2), the vaccine has to resist bile concentrations reaching 4% in the small intestine. The bile concentrations vary from 0.8–4% after ingesting a meal (Marteau et al. 1997), just as the pH values vary. Therefore, *L. plantarum* charged with 3014-TB/2162-TB, were incubated for 1 hour in bile concentrations of 2% at 37°C, before analysis by flow cytometry (Fig. 4.21).

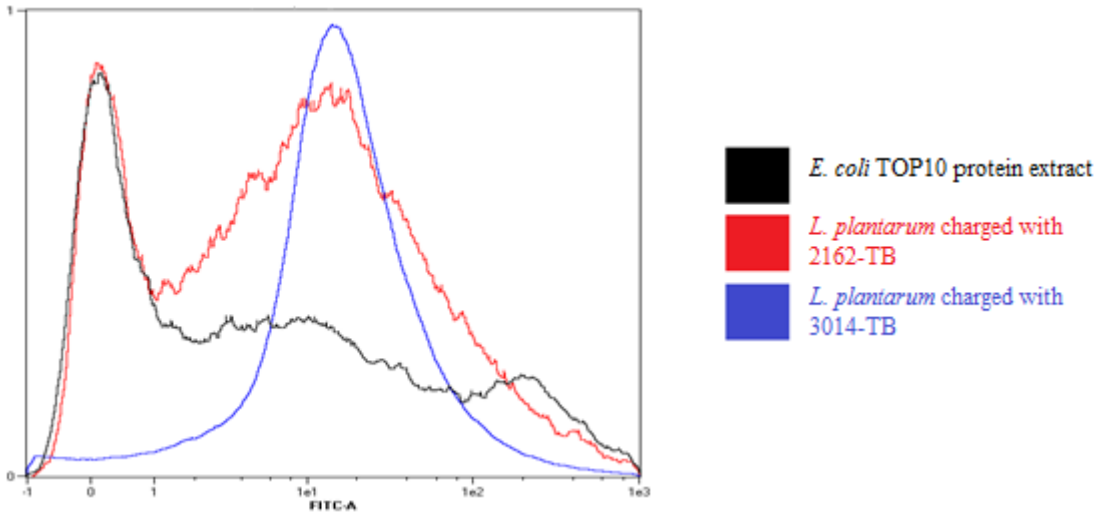

Figure 4.21: Flow images of *L. plantarum* displaying either 3014-TB or 2162-TB, incubated in 2% bile for 1 hour, at 37°C. The color scheme is shown to the right of the histograms. *L. plantarum* displaying 3014-TB/2162-TB was incubated in MRS medium containing 2% bile as the final concentration. The negative control (black) is *L. plantarum* charged with protein extract of *E. coli* TOP10 (harboring a pBAD-derivative, not expressing the antigen Ag85B-ESAT6-DC), incubated in MRS medium containing 2% bile as the final concentration.

The results indicate that the *L. plantarum* charged with 3014-TB (Fig. 4.21, blue histogram) resists incubation in 2% bile better than *L. plantarum* charged with 2162-TB (Fig. 4.21, red histogram), but there are uncertainties since the negative control reveals an unspecific interaction with the anti-ESAT6. Pellets treated with bile become strongly colored, which potentially are misinterpreted as positive results, analyzed by flow cytometry. This was also the case analyzing with western blots (data not shown).

After incubating the negative control in MRS medium containing 4% bile as the final concentration, the unspecific interaction with anti-ESAT6 increased, causing the histogram representing the negative control to shift equally towards the right as histograms representing the samples including 3014-TB and 2162-TB. The anchored 3014-TB and 2162-TB to *L. plantarum* cells' ability to resist incubation in bile must be analyzed further using another system, not affected by the color given after bile incubation.

4.6 Expression of Lp_3014-TB and Lp_2162-TB in *Lactobacillus plantarum*

In studies done by Tjåland (2011) and Øverland (2013), the proteins containing tuberculosis (TB) antigen were expressed in *L. plantarum,* and displayed on the cells' own surface (self-expressing *L. plantarum*). The approach in the present study has taken a different angle, since heterologously produced proteins were anchored to non-GMO *L. plantarum*, which does not express these proteins. Both studies depend on using *L. plantarum* as a delivery vehicle for displaying proteins containing TB-antigens, and were therefore compared; potentially determining the better approach for anchoring proteins to delivery vehicles.

4.6.1 The construction of Lp_*2162*-TB and Lp_*3014*-TB

The gene fragments of *2162*-TB and *3014*-TB were altered to include the original signal sequence placed at the N-terminus of the proteins. The new fragments were ligated into NdeI and EcoRI digested pSIP plasmids, producing the final fragments *Lp_2162*-TB and *Lp_3014*-TB (Fig.4.22), which uses the vector system of *L. plantarum* (Sørvig et al. 2005) to make self-expression possible.

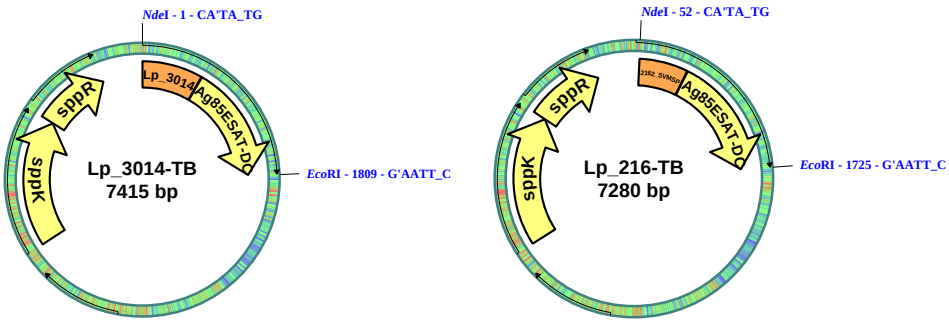

Figure 4.22: Schematic overviwe of the final constructs of Lp_*3014*-TB and Lp_*2162*-TB respectively, cloned into the pSIP system. In these constructs, the signal sequence (leading the expressed proteins out of the cell) is located at the N-terminus. The restriction sites used to digest the pSIP-plasmids are visualized.

The final constructs (Fig. 4.22) were transformed into *E. coli* TOP10 competent cells, the plasmids were isolated and purified before transformation to electrocompetent *L. plantarum*.

4.6.2 Lp_3014-TB and Lp_2162-TB displayed at *Lactobacillus plantarum* surfaces

The production of Lp_3014-TB and Lp_2162-TB in *L. plantarum* cultures was detected using western blot analysis of cell extracts obtained after cell-disruption with glass beads (data not shown), using cells harvested 2 hours after induction with 25 ng inducing peptide SppIP (see section 3.24 and 3.25). *L. plantarum* cultures were induced before they reached OD_{600}= 0.33, and after induced, the growth of *L. plantarum* cultures were monitored in the following 5 hours (a total of 8 hours; Appendix, Fig. A5). *L. plantarum* expressing the proteins, Lp_2162-TB or Lp_3014-TB, were revealed to have a slower growth rate than the *L. plantarum* cells not harboring a pSIP-derivative.

The extracellular attached proteins on *L. plantarum*'s own surface were verified with flow cytometry; 2 and 5 hours after induced and treated with lysozyme. Lp_2162-TB was embedded in the structure after 2 and 5 hours, where lysozyme treatment was used to visualized the proteins at the cell surface (Appendix, Fig. A6). The highest amount of detected proteins of Lp_2162-TB on the cell surface was after 5 hours incubation and lysozyme treatment. Lp_3014-TB was partly displayed at the cell surface after incubated for 2 and 5 hours, and, Lp_3014-TB was completely revealed after lysozyme treatment after incubated for 2 hours (Appendix, Fig. A7). However, lysozyme treatment after incubated for 5 hours (Appendix, Fig. A7), revealed a histogram with two separated tops; suggesting that the already displayed Lp_3014-TB were degraded after lysozyme treatment, while the previously embedded proteins of Lp_3014-TB became exposed. The degree of displayed proteins of Lp_3014-TB on the cell surface was almost similar for 2 and 5 hours, but even when split in two, the top depicting the displaying Lp_3014-TB proteins after 5 hours incubation was shifted furthest to the right.

The amount of proteins detected on cell surfaces of self-expressing *L. plantarum*, never exceeded the amount of proteins detected on *L. plantarum* displaying 3014-TB or 2162-TB (Fig. 4.23).

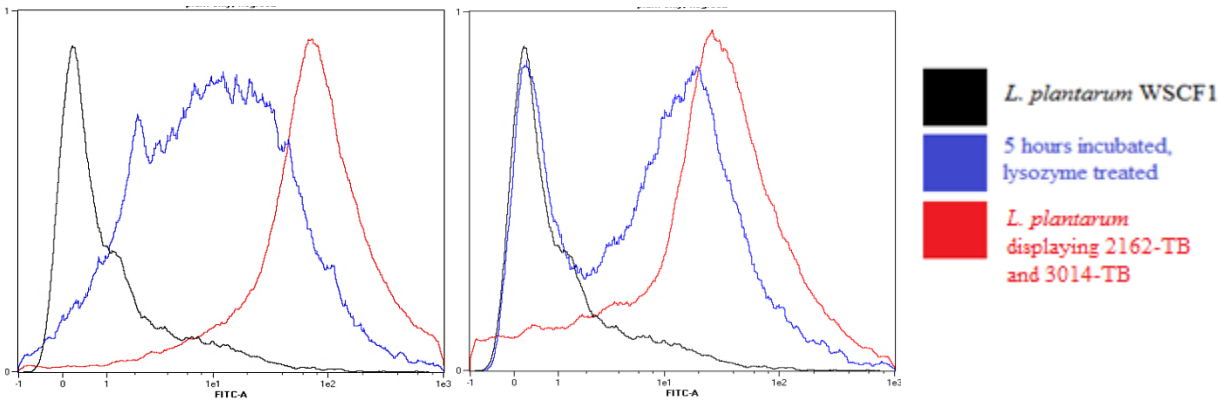

Figure 4.23: Flow images comparing *L. plantarum* expressing and displaying Lp_2162-TB (right) or Lp_3014-TB (left), to *L. plantarum* charged with 2162-TB or 3014-TB, respectively. The color scheme is shown to the right of the histograms, explaining the individual samples represented by each colored histogram. The negative sample was *L. plantarum* cells not harboring a pSIP-derivative, induced with SppSIP and incubated for 5 hours. 3014-TB and 2162-TB were incubated for 1 hour at room temperature with *L. plantarum* cells harvested in exponential phase.

5. DISCUSSION

5.1 Intracellular production of 3014-TB and 2162-TB in *Escherichia coli*

The display of proteins on lactic acid bacteria (LAB) leading to a vaccine is a well-known approach (Daniel et al. 2011; Wells 2010; Wells & Mercenier 2008), depending on the use of GMO bacteria. The novelty of the present study lies in developing methods to non-covalently anchor heterologous proteins to LAB, to create a non-genetically modified organism (non-GMO) displaying a vaccine (Bosma et al. 2006). For a heterologous protein to anchor gram positive bacteria, the proteins need to be produced in large quantities through easy cloning and bacterial culturing. *E. coli* is well adapted for this purpose and was therefore chosen for this study. The two antigens from *Mycobacterium tuberculosis* (*Mtb*) fused together to the Ag85B-ESAT6-DC fusion protein, have been explored in previous studies based on using GMO-LAB, and have shown promising results (Tjåland 2011; Øverland 2013).

The proteins were intracellularly expressed through the inducible pBAD-vector system, which uses the araBAD promoter to enhance expression of the protein of interest. After optimizing the expression-conditions, 3014-TB and 2162-TB were successfully produced intracellularly in *E. coli* cultures. The optimal conditions were grown at 18°C, induction with 0.02% arabinose and incubation for 12 hours after induction. *E. coli*, which is a typical mesophilic bacterial strain (Broweze et al. 1978) grows in temperatures from 15–40°C, and both growth rate and the rate of translation vary within this temperature interval. The lowest growth temperature for *E. coli* is proven to be 7.8°C (Shaw et al. 1971), and at such low temperatures protein synthesis will be reduced to a minimum, or completely stop (Jones et al. 1987). A temperature of 18°C, which was used in this study, causes a slow growth rate for *E. coli*; which was considered as an advantage, because the proteins, 3014-TB and 2162-TB, would be given time to fold properly, into potentially soluble proteins. *E. coli* did still express proteins at this temperature, and did indeed produce soluble 3014-TB and 2162-TB. *E. coli* cultures grown under these conditions produced full-length 3014-TB (56 kDa) and 2162-TB (60 kDa), as confirmed with western blots.

5.2 Purifying 3014-TB and 2162-TB from *Escherichia coli* protein extract

To obtain optimal samples for subsequent anchoring to LAB, attempts were made to purify His-tagged 3014-TB and 2162-TB from cell extracts of *E. coli* strains. The use of a His-tag (7 Histidines), attached either at the N- or C-terminus of the protein have been explored prior to this work, as an affinity tag to purify particular proteins (Lee et al. 2014; Ma et al. 2014).

His-tagged proteins were released from the *E. coli* interior using sonication, which breaks the cells using intervallic sound waves, resulting in protein extracts. The challenge of continuously discover the protein 3014-TB in the flow through were met in the attempt to purify the protein from the resulting protein extracts using a Ni-NTA column. This challenge was assumed to arise from an embedded His-tag in 3014-TB, since the problems remained even after optimizing the Buffer composition; buffer A were optimize to contain 1 M Tris HCl pH 7.4, excluding imidazole completely, preventing competition for 3014-TB to bind the Ni-NTA column. Buffer B contained 1 M Tris HCl pH 7.4 and 500 mM imidazole, causing any potential proteins bound to the Ni-NTA column to be released into the eluate.

By denaturing the protein 3014-TB in 8 M urea before purified, using the buffer compositions described (included the denaturant, 8 M urea); 3014-TB was depicted in the eluate in a higher detectable amount than in the flow through. Following the purification of 3014-TB, the protein was tried refolded by the removal of denaturants, either gradually using refolding solution, or fast, using dialysis. The gradual removal of denaturants caused aggregation of proteins, while the fast removal resulted in a soluble 3014-TB which unfortunately did not have the ability to anchor the cell wall of *L. plantarum*.

Hopefully, by elongating the distance between the His-tag and the antigens (Ag85B-ESAT6-DC), the His-tag would avoid being embedded in the protein structure. The two constructs included in this study, 3014-TB and 2162-TB, differed in the numbers of LysM domains in their anchoring domains, where 3014 contained one LysM domain while 2162 contained two LysM domains (Fig. 1.3 and 5.1). Expanding the anchoring domain from one domain (3014) to include two domains (2162) increased the length of the protein fragment between the His-tag and the antigens by 1.5-fold. To avoid the embedded His-tag, two 2162-TB candidates were made varying in the last PST linker region (Figure 5.1).

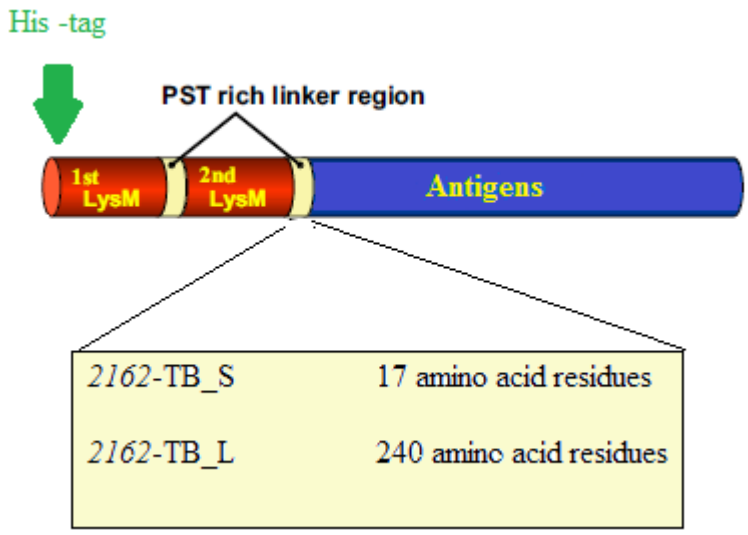

Figure 5.1: Illustration of a protein containing 2 LysM domains, separated by PST linker regions. This protein is built similarly to 2162-TB, with the two LysM domains separated by a PST linker region. In contrast, 3014-TB only contains one LysM domain, followed by a PST linker region, separating the LysM domain from the antigens. Following the 2nd LysM domain in 2162-TB, is an additional PST linker region, separating the LysM domain and the antigens; the number of residues included in the two 2162-TB candidates is given at the bottom of the figure. This figure is an alteration of (Ohnuma et al. 2007).

The natural Lp_*2162* gene encodes a double LysM domain (Fig. 1.3 and 5.1), with a long PST linker region following the second LysM domain. The two constructs were made by ending the PST linker region at two natural points, resulting in a longer fragment (*2162*_L) and a shorter fragment (*2162*_S) (Fig. 5.1). The PST linker region is increased by 223 amino acid residues, from 2162-TB_S to 2162-TB_L (Fig. 5.1), which also equally increases the length between the His-tag and the antigens.

2162-TB_S and 2162-TB_L were tried purified in the optimal buffer compositions described, revealing 2162-TB_S in a full-length form (60 kDa) and a punctuated form (35 kDa), while 2162-TB_L were only depicted in a punctuated form (35 kDa). The punctuated form of 2162-TB_L implies that the longer PST linker region was exposed to cleavage by proteases. In addition to being cleaved, 2162-TB_L was not depicted in the eluate, leaving 2162-TB_S as the only candidate of 2162-TB to be depicted in the eluate, and was therefore the candidate of 2162-TB included in the further experiments (referred to as 2162-TB hereafter).

While 3014-TB and 2162-TB eventually could be produced as soluble proteins in *E. coli*, 3014-TB could unfortunately not be purified. Whereas 2162-TB could be purified, the resulting purified proteins were not detected at the surfaces of *L. plantarum* cells, which could be explained by the low yield of purified 2162-TB (1.67 mg/ml). Therefore, 3014-TB and 2162-TB were administrated as total protein extracts to anchor the surfaces of gram positive bacteria. There are disadvantages following the administration of protein extracts rather than purified proteins, one being the protein extracts' additional substances, which potentially could bind to the cell surfaces yielding, unknown effects by altering the cell surface. The use of protein extracts containing 3014-TB and 2162-TB, rather than the purified proteins, also has the disadvantage that administrated antigen concentration is unknown and hard to control. If the elicited immune response depends on the concentration of antigens administrated, and there is a threshold needed to elicit an immune response (Wells & Mercenier 2008), the protein concentration being unknown and uncontrollable may cause problems.

5.3 Anchoring 3014-TB and 2162-TB to the cell surface

The use of LysM domains to non-covalently anchor heterologous proteins to the cell wall of gram positive bacteria is an increasingly popular field in the development of vaccines (Bosma et al. 2006; van Roosmalen et al. 2006; Xu et al. 2011), because LysM domains are known to bind the varying and complex structure of peptidoglycan, chitin (Ghuysen 1986; Wong et al. 2013) and potentially teichoic acid (Navarre & Schneewind 1999). Due to the LysM domains abilities to anchor this cell wall structures, 3014-TB and 2162-TB were expected to bind to the cell surfaces of a broad range of gram positive bacteria.

After co-incubating gram positive bacterial cells with protein extracts for 1 hour, the western blots revealed that the vaccine-constructs, 3014-TB and 2162-TB, were bound to all the 14 gram-positives tested in this study. 3014-TB and 2162-TB were also observed at the gram-negative *E. coli* (the strains are listed in Table 4.1). Surprisingly, 3014-TB and 2162-TB were found to anchor less efficiently to *B. subtilis* (Appendix, Fig. A3). Only a small amount of 3014-TB was observed at the surfaces of *B. subtilis* compared to similar *Lactobacillus spp* used in this, and previous studies (Steen et al. 2003). So far, there is no explanation for why LysM-containing proteins anchor less efficiently to the cell wall of *B. subtilis*, but LysM-containing proteins have

been discovered to anchor *B. subtilis* at specific locations, in a ring-like manner, surrounded by areas rich in lipoteichoic acid, yielding visually lower densities of displayed LysM-contained proteins in contrast to *Lactobacillus spp* (Steen et al. 2003). LysM domains are thought to bind the NAG monomers of the peptidoglycan structure, which interestingly is the only similarity discovered between peptidoglycan and chitin (Visweswaran et al. 2012). If the structure that LysM domain recognizes could be determined, this information could be used to potentially specify the LysM domains' binding affinity towards the type of bacterial strain used.

3014-TB and 2162-TB, due to the LysM anchor, were detected to anchor a broad range of gram positive bacteria, including the gram negative bacteria, *E. coli*, using western blotting and flow cytometry. Both western blotting and flow cytometry uses immunostaining, which exploits the antigen-antibody-interaction to detect (e.g.) a protein of interest, and is considered as reliable methods to verify the presence of a particular protein (Lea 2006). If the confocal microscopy were to be included as a method to further investigate 3014-TB and 2162-TB on the cell surfaces, the distribution of the proteins on the cell surfaces could be determined as well. Therefore, confocal microscopy should have been included in this work.

Western blotting used to detect particular proteins is considered to be semi-quantitative, because the amount of proteins in the sample is indicated by the band intensities. Flow cytometry, on the other hand, discriminates between the individual cells in the analyzed sample, yielding quantitative results of the sampled cells (Lea 2006). Though quantitative, the concentrations of 3014-TB and 2162-TB displayed at the cell surfaces should be thoroughly measured using e.g. Bradford method. The concentration of 3014-TB and 2162-TB were not determined in this study, mainly due to the use of total protein extracts when co-incubating with bacteria. Determining the protein concentration of the particular protein and avoid the interfering protein, are challenging (Rosenberg 2005), and therefore, 3014-TB and 2162-TB should be purified using another affinity tag, before determining the concentration of the purified protein.

5.4 Comparing the potential of 3014-TB and 2162-TB to bind to cell surfaces of *L. plantarum*

An orally administrated vaccine has to resist harsh environments in the gastrointestinal tract (GIT). Antigens displayed at the cells surface are thought to be more immunogenic compared to cytoplasmic antigens (Wells & Mercenier 2008). However, displayed antigens are more exposed for degradation during their passage through the digestive tract, and therefore, is the choice of lactic acid bacteria as delivery vehicle considered to be important, due to their resistance against these harsh GIT environments (Wells & Mercenier 2008). *L. gasseri* is known to survive low pH values (2.5), *L. plantarum* is more stable at these pH values, while *L. rhamnosus GG* is more tolerant against bile compared to *L. gasseri* and *L. plantarum* (Kõll et al. 2008). For the delivery vehicles charged with 3014-TB or 2162-TB to elicit immune responses, the proteins 3014-TB and 2162-TB has to stay on the cell surfaces through the GIT passage; facing lower pH values, as 1.8, and bile concentrations ranging from 2–4% (Marteau et al. 1997).

In this study, *L. plantarum* cells charged with 3014-TB or 2162-TB were incubated in pH values ranging from 1.5–6.5, and bile concentrations ranging from 2–4 %. The amount of 3014-TB and 2162-TB displayed at the cell surfaces after pH-incubation were compared to the *L. plantarum* cells displaying 3014-TB or 2162-TB not incubated in pH, using flow cytometry. Flow cytometry gives a more qualified quantitative guess than western blotting. The histograms representing the *L. plantarum* cells displaying 3014-TB and 2162-TB incubated in low pH values revealed almost the same shift compared to the histogram representing the untreated protein-displaying cells, which indicates that 3014-TB and 2162-TB hardly were affected by the low pH values. The effect following 2% bile administration indicates that 3014-TB resists bile incubation better than 2162-TB. However, the negative control (cells not displaying 3014-TB or 2162-TB) represented by a histogram revealed, after incubated in bile, a shift towards the right which implied an unspecific interaction. After increasing the bile concentration from 2% to 4 %, the histogram representing the negative control were observed to shift equally to the right as the histogram representing the samples containing 3014-TB and 2162-TB. The cell pellet following bile administration is brightly colored, which probably caused this shift in the histogram representing the negative sample. Other methods (not affected by the colored cell pellet) should therefore be included before the final conclusions are drawn.

L. plantarum cells charged with 3014-TB and 2162-TB, analyzed by flow cytometry, revealed the potential to stay stable during storage for one week, after freeze drying. The histogram that represented cells displaying 3014-TB or 2162-TB were, after storage, observed to shift equally towards the right as the histograms representing the cells displaying 3014-TB and 2162-TB not stored before analyzed. Freeze drying causes distress on cells and proteins, and the cells and proteins are protected by adding a freeze dry medium to the sample. The optimal effect of the freeze dry medium on the particular bacterial strain or protein used, depends on the chosen combination of the freeze drying medium's components (Leslie et al. 1995). Because the *L. plantarum* cells displaying 3014-TB and 2162-TB were observed to be stable for only one week, the freeze dry medium targeting the particular combination of bacterial strains and proteins used in this work, should be further optimized.

Finally, data obtained from this study reveals that both 3014-TB and 2162-TB anchors to the cell surfaces of gram positive bacteria. Additionally, the cells displaying 3014-TB or 2162-TB have the ability to resist degradation during incubation at low pH and possibly bile, and can be stored in a freeze-dried form for at least 1 week. Future work should account for the challenges facing the limiting time of storage, by changing the component of the freeze dry medium to result in the optimal combination, yielding the best protection for *L. plantarum* cells displaying 3014-TB or 2162-TB. The protein-displaying cells' ability to resist degradation in bile needs to be determined, using a method that will not be affected by the colored cell-pellet. The 3014-TB or 2162-TB displayed *L. plantarum* cells should preferably be tested in environments including more than one factor at a time; e.g. expose the displayed cells to the low pH values and a protease-activity subsequent to the environments detected in the stomach.

5.5 Comparisons of the constructs 2162-TB and 3014-TB

Some examples in literature reveal the need for larger clusters of LysM domains, N- or C-terminally located in proteins, in order to obtain their optimal binding capacity (Bateman & Bycroft 2000; Petutschnig et al. 2010; Wong et al. 2013). Two LysM containing anchoring domains were therefor tested for the antigen-containing protein used in this study, which hopefully would help determining the better anchor: 2162 (two LysM domains) or 3014 (one lysM domain) (Figure 1.3).

Co-incubation of 3014-TB and *L. plantarum, L. rhamnosus GG, L. gasseri* or *B. subtilis*, revealed surface exposed proteins using flow cytometry and western blotting. The same was observed upon co-incubation with 2162-TB. The amount of displayed proteins was visually estimated by comparing the band intensities in western blots or the histograms representing the samples shifts towards the right in flow cytometri. Comparing the amount of displayed 3014-TB and 2162-TB at the cells surfaces revealed an inconsistency when comparing all the individual experiments conducted in this study (data not shown). This ambiguity could have been reduced by closely monitoring the concentration of the total protein extracts administrated before co-incubating the protein extracts with bacterial cells. The protein extracts containing 3014-TB and 2162-TB were usually stored in 4°C before incubated with bacterial cells; the ambiguity could have been reduced by continuously avoid storing the protein extracts before administrated.

Incubating cells displaying 3014-TB or 2162-TB in low pH values revealed an almost equal shift of the histograms representing each of the protein samples. In contrast, histograms representing cells displaying 3014-TB were usually shifted further to the right than the histograms representing 2162-TB charged cells when faced with bile. A possible explanation is that the smaller 3014-TB (56 kDa) is more protected from the surrounding environment, than the larger (60 kDa) and potentially more exposed 2162-TB. This could in an immunologically perspective, cause a disadvantage. Antigens protected in the folded protein structure could be hidden from interacting with immune cells to elicit an immune response.

So far, the data indicates that the cells displaying 3014-TB have the slighter advantage over cells displaying 2162-TB, regarding the stability when incubated in bile. In future aspects, the effect following bile incubation on the cells displaying either 3014-TB or 2162-TB should be determined using another method. The results for the protein amounts displayed at the cells surface was ambiguous, thus, no conclusions can be made. A final conclusion could be drawn after determining the potential elicited immune response.

5.6 Comparing *L. plantarum* cells charged with Lp_3014-TB or Lp_2162-TB to the cells charged with 3014-TB or 2162-TB

Prior to this study, *L. plantarum* had been used to express and expose the Ag85B-ESAT6 fusion antigen at its own surface, giving the disadvantage of resulting in a GMO vaccine (Tjåland 2011; Øverland 2013). The use of GMO is controversial (see section 1.6), and a non-GMO vaccine would be received better in many countries when introduced.

By altering 3014-TB and 2162-TB, the proteins were intracellularly produced in *L. plantarum* and displayed at the cell surface. To accomplish this, the signal peptide sequences present in Lp_2162 and Lp_3014 that were removed in the constructs discussed so far, were kept, in order for the fusion proteins to be secreted from the cells. The new Lp_*3014*-TB and Lp_*2162*-TB fragments were cloned into the *L. plantarum* pSIP inducible vector system. Images retrieved from flow cytometry of *L. plantarum* expressing Lp_2162-TB, suggested that the protein was displayed (Appendix, Fig. A6). The protein was only detected after lysozyme treatment, indicating that it was deeply embedded in the cell wall. Expressed Lp_3014-TB proteins, on the other hand, were suggested to be partly embedded in the cell wall of *L plantarum* (Appendix, Fig. A7), due to the split histogram representing *L. plantarum* cells expressing Lp_3014-TB after incubation for 5 hours and lysozyme treatment. Which implies deeply embedded Lp_3014-TB in the cell wall structure that was detected after lysozyme treatment, while the Lp_3014-TB that were displayed at the cell surface from the beginning, were degraded after lysozyme treatment (Fig. A7). Other embedded protein containing TB-antigens at the *L. plantarum* surfaces have elicited promising immune responses in mice (Øverland 2013), suggesting that embedded Lp_3014-TB and Lp_2162-TB on *L. plantarum* could potentially elicit immune responses as well.

Finally, expressed Lp_3014-TB or Lp_2162-TB were detected at the surfaces of *L. plantarum* cells after incubating for 5 hours and lysozyme treatment, followed by analyzing the samples by flow cytometry. The histogram representing *L. plantarum* cells displaying Lp_3014-TB or Lp_2162-TB were not shifted as far to the right as the histograms representing the *L. plantarum* cells displaying 3014-TB and 2162-TB, implying that an higher amount of antigens (Ag85B-ESAT6-DC) were presence at the cells displaying 3014-TB and 2162-TB. Hopefully, higher concentrations of displayed antigens could elicit stronger and longer-lasting immune responses.

5.7 Concluding remarks and future perspectives

Hopefully, results from this study will help future studies towards the development of a non-GMO vaccine against tuberculosis (TB). The promising antigens (Ag85B-ESAT6) have successfully been expressed intracellularly in *E. coli* and the heterologous proteins could be anchored to 14 different strains of gram-positive bacteria, through their peptidoglycan binding domain (LysM domain). The bacterial cells displaying the heterologous proteins provide an easy and low cost vaccine manufacturing method, and have shown resistance against stomach- and intestinal-like environments. The vaccine was resistant against low pH values (1.5–6.5) and possibly bile conditions (2%), and has the ability to be stored for at least 1 week. Future work should include determining the potential elicited immune responses, and address the main problems discovered during this work: the use of purified proteins as opposed to cell extracts and increasing storage stability.

Use of protein extracts rather than the purified protein may cause unknown side effects when administrated (see section 5.2). To avoid this, 3014-TB and 2162-TB should be purified, potentially using another small affinity tag. A small affinity tag has the advantage to fuse directly to the antigens in the proteins without altering the tertiary structures, and do not need to be removed after purification (Terpe 2003). If the small affinity tags do not result in purified proteins, the tag could be replaced within the protein, e.g. internally, or at the C- or N-terminus. The proteins could be purified using larger fused peptides, but this is a rather expensive and complicated protocol, therefore, small-affinity tags should be considered firstly. The choice of any tag (large or small) to be used to purify proteins depends on the protein-type used, the expression system of the protein and the proteins' application afterwards (Terpe 2003).

Current vaccines are usually manufactured in one country and then exported to the country where they are to be administrated. Marketed vaccines therefor need the ability to resist longer storing periods without degrading before being administrated. Degraded vaccines would be more vulnerable when facing the harsh environment found in the GIT, and possibly lose the potential to elicit an immune response, e.g. as a result of proteolytic attack in the stomach. Hopefully, by altering the freeze dry mediums' composition to result in an optimal protection towards the cells charged with 3014-TB and 2162-TB, the protein-charged cells' stability could exceed 1 week of storage and potentially after administrated.

Before the potential vaccines generated in this study can reach mouse trials, the ability for *L. plantarum* cells displaying 3014-TB and 2162-TB to elicit a potential immune response needs to be determined. Previous studies have revealed detectable immune responses against the antigens used (Ag85B-ESAT6) (Dietrich et al. 2006; Doherty et al. 2004) also when displayed on *L. plantarum* through self-expression (Øverland 2013). Thus, the potential to elicit an immune response is defiantly present. Furthermore, it has been shown that the 12-mer specific DC-targeting sequence attached C-terminally to the proteins used in this study stimulates the immunogenic effects of the antigen coupled to it (Mohamadzadeh et al. 2009; Øverland 2013). All in all, the cells charged with either 2162-TB or 3014-TB have the potential to elicit an immune response through the targeting of DC. Which, finally, would give the possibility to determine the better anchor-domain for the heterologous protein used in this study.

The future goal is to produce a vaccine against tuberculosis, which results in a better and longer-lasting immune response than the BCG-vaccine. Probably, both GMO and non-GMO routes will be developed. The present study provides a first step towards a non-GMO vaccine, which may provide a favorable choice in the future.

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APPENDIX

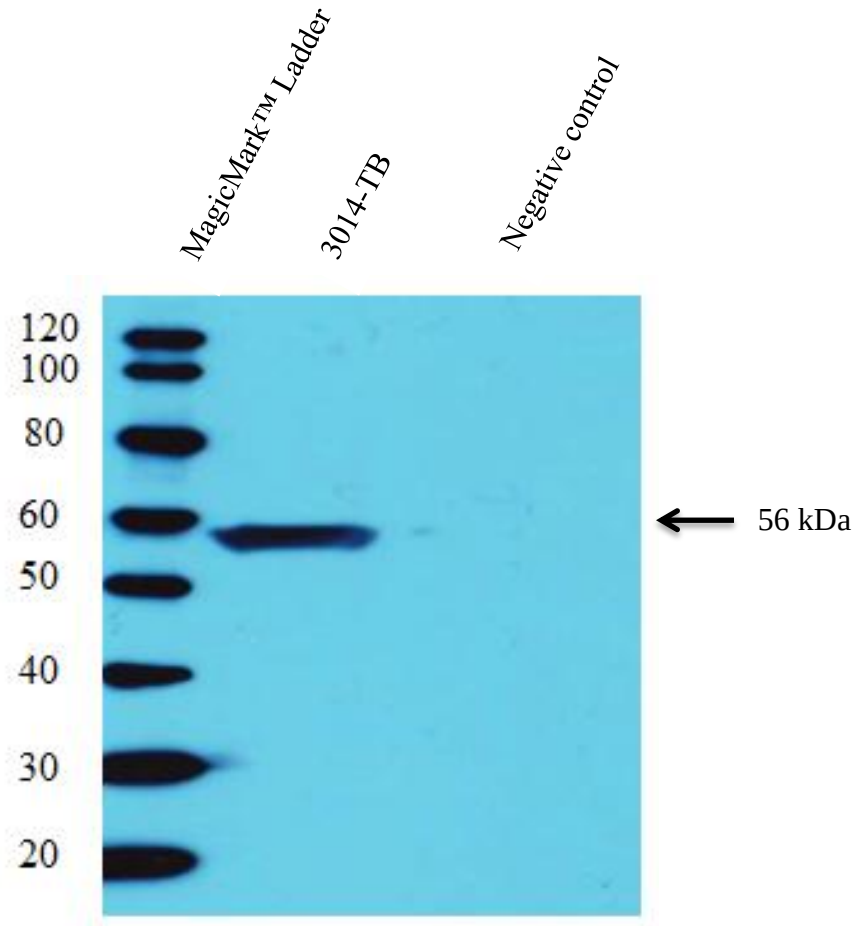

Figure A1: Western blot, using ESAT6, showing result of the optimal production of protein extracts containing 3014-TB, from *E coli.* 3014-TB was grown at 18°C, induced with 0.02% arabinose, followed by incubation for 12 hours. 3014-TB is shown as a full-length protein (56kDa). Negative control; protein extracts of *E. coli*, harboring a pBAD-derivative lacking the antigen (Ag85B-ESAT6-DC), grown in the same manner as the 3014-TB protein.

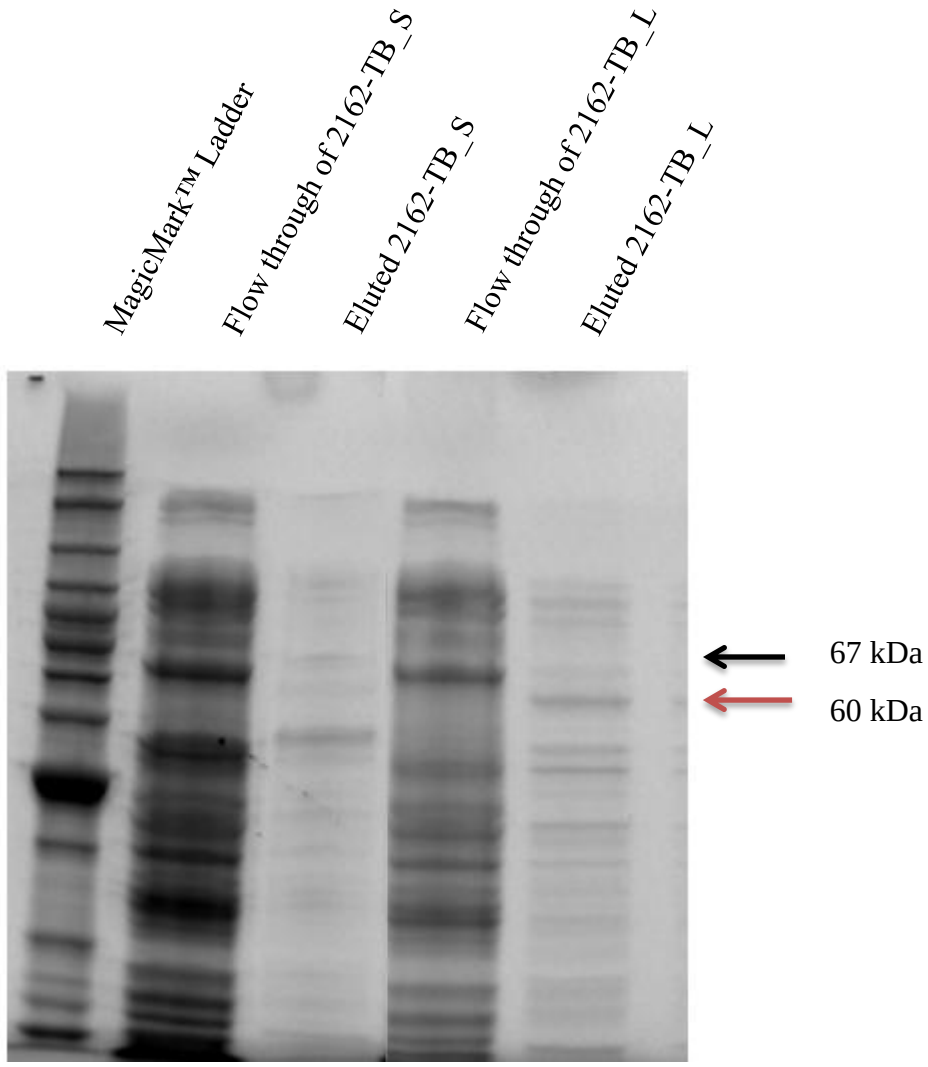

Figure A2: SDS-gels of cell extracts from cells harboring *2162*-TB_S and *2162*-TB_L, grown at 18°C, induced with 0.02% arabinose and harvested after 12 hours of incubation. The expected size of the overexpressed protein 2162-TB_S is 60 kDa, and the band corresponding to this size is indeed observed in the sample containing 2162-TB_S, while the expected size of the protein 2162-TB_L is 67 kDa, a band approximate to this size is observed in the flow through.

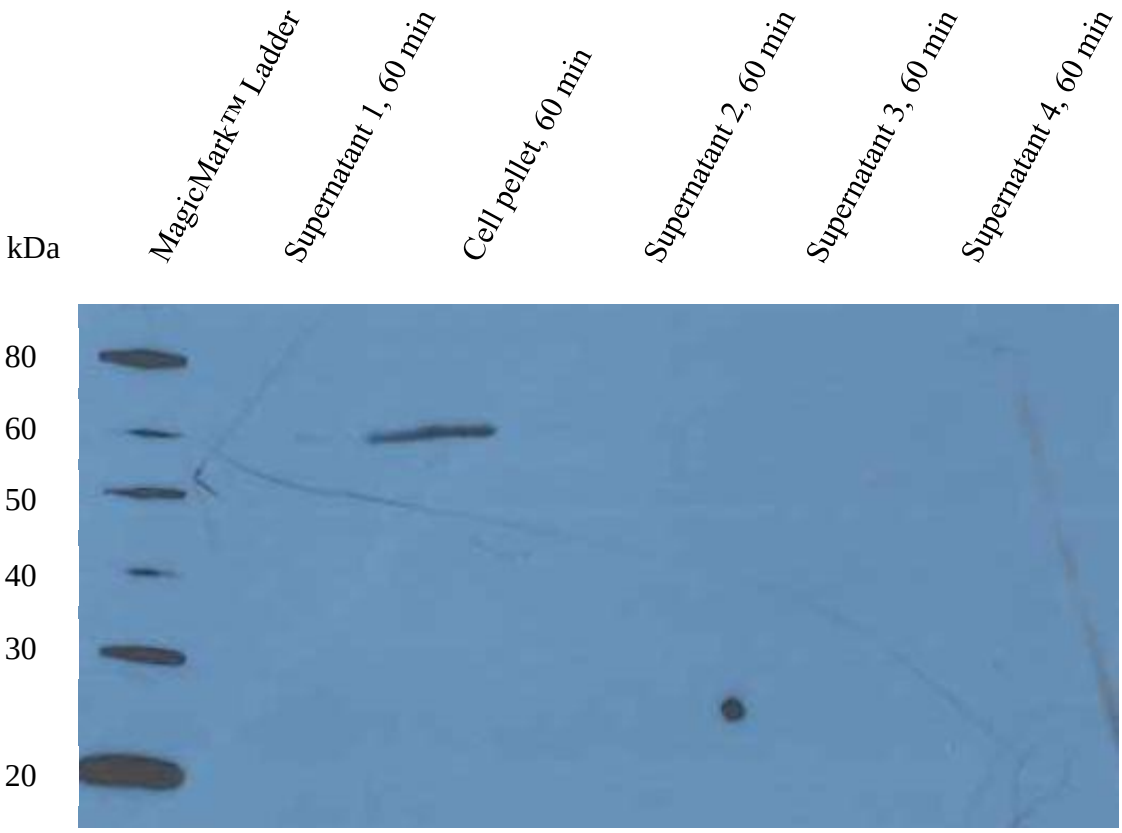

Figure A3: Western blot, using anti-His, depicting the resulting supernatant 1–4 and cell pellet after 60 min of co-incubation of protein extracts containing 3014-TB with *L. plantarum* cells. 3014-TB bound to living *L. plantarum* cells harvested in the exponential phase is shown, while 3014-TB is not observed in any of the four supernatants.

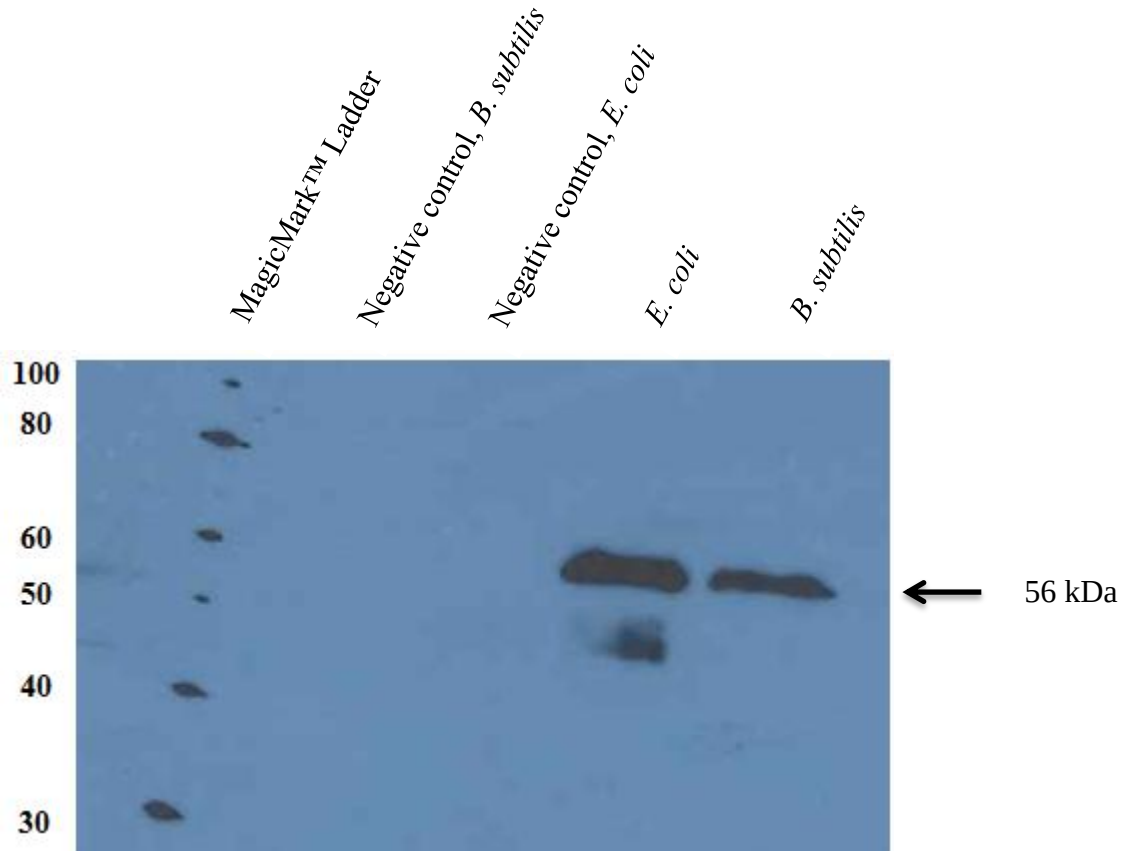

Figure A4: Western blot, using anti-His, showing the result following the co-incubation for 60 min, of *E. coli* and *B. subtilis* cells with protein extracts containing soluble 3014-TB. After 60 min co-incubation, 3014-TB was displayed at the cell surfaces of *E. coli* and the *B. subtilis*, both harvested in the exponential phase. The negative controls are protein extracts of *E. coli* harboring pBAD-derivative lacking the antigen Ag85B-ESAT6-DC, co-incubated with both *E. coli* and *B. subtilis* cells, harvested in their exponential phase.

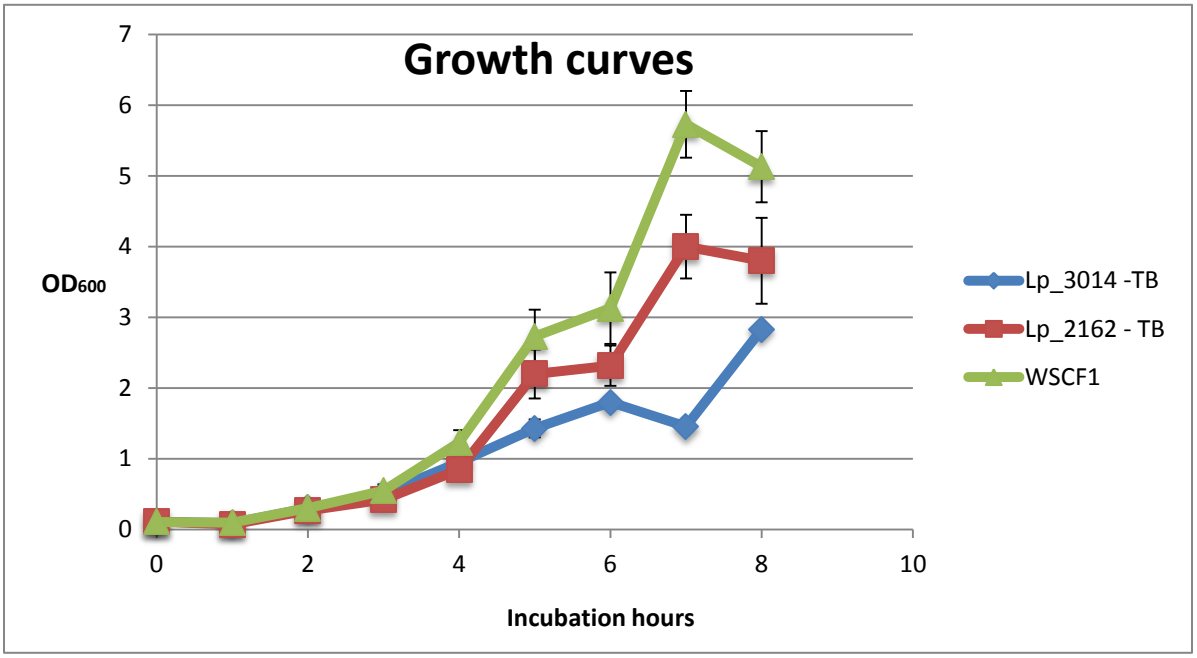

Figure A5: Schematic overview of the average growth for *L. plantarum* cells harboring different plasmids. The color scheme is listed to the right. All three were induced with inducer peptide 25 ng SppIP after approximately 3 hours. *L. plantarum* cells not harboring pSIP-derivative have a higher growth rate than the *L. plantarum* cells harboring the pSIP-derivatives, after induced with the inducer peptide. The standard deviation for each measured OD_{600} is given.

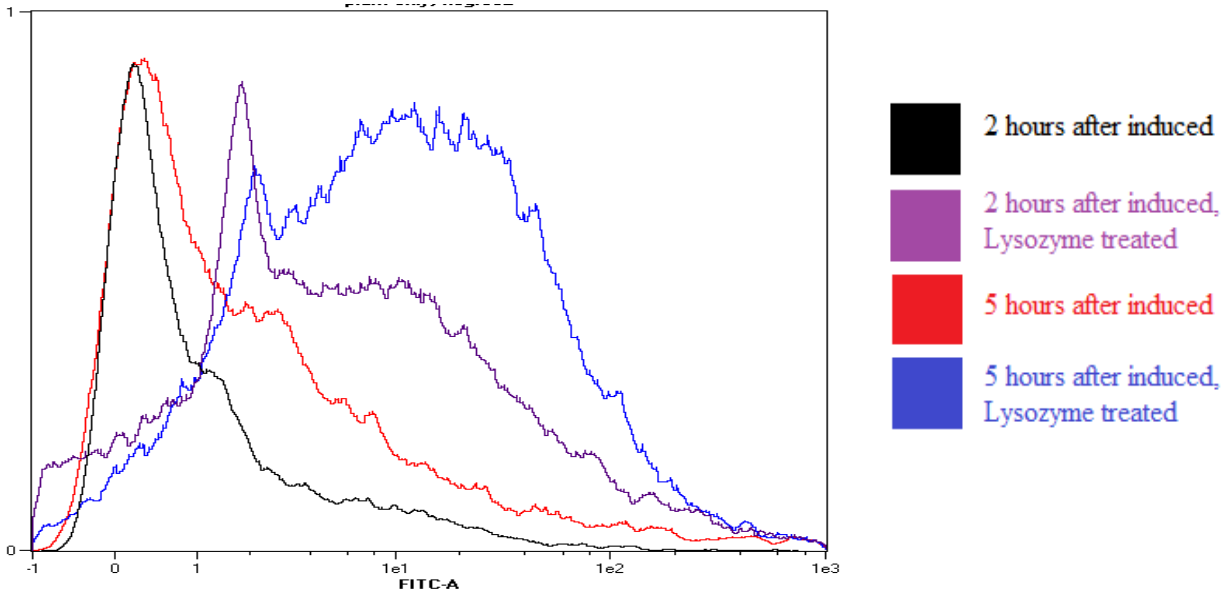

Figure A6: Flow images revealing Lp_2162-TB displayed at the surfaces of *L. plantarum* cells, after induced with the inducer peptide, SppSIP. The color scheme is listed to the right of the histograms, revealing each sample represented by each colored histogram. All samples were induced with the inducer peptide, SppSIP before the cells reached the OD_{600} = 0.33. *L. plantarum* WCSF1 (black histogram) do not harbor any pSIP-derivative, and were induced before reached OD_{600} = 0.33 and incubated for 5 hours, not treated with lysozyme.

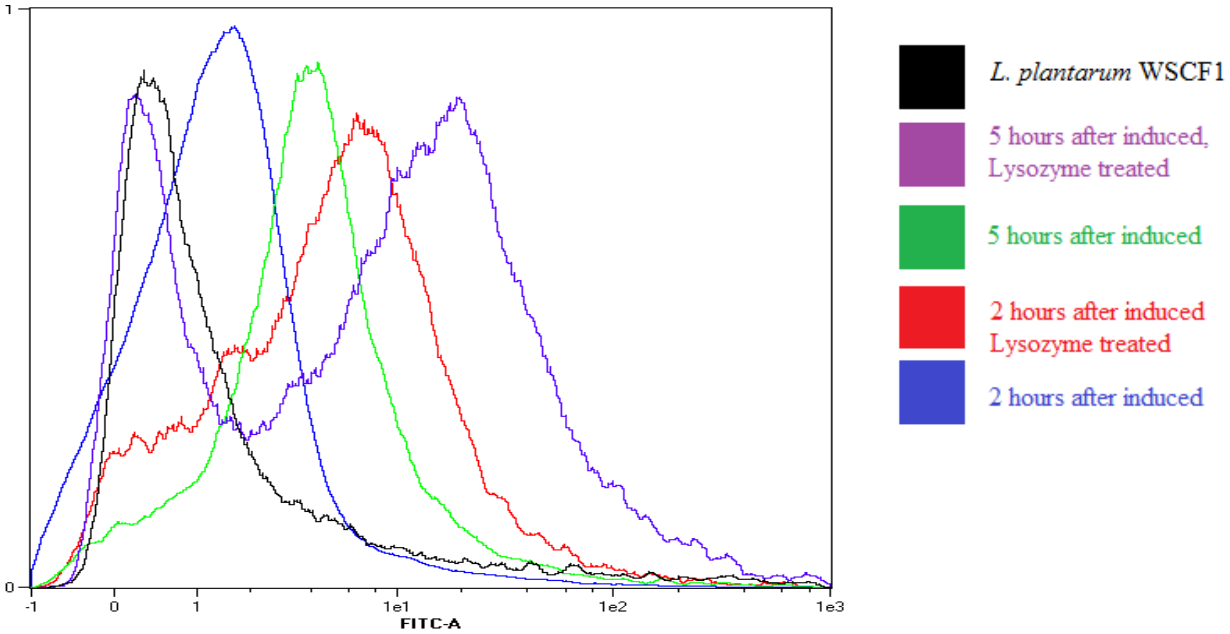

Figure A7: Flow images revealing Lp_3014-TB displayed at the surfaces of *L. plantarum* cells, induced with the inducer peptide, SppSIP. The color scheme is listed to the right of the histograms, revealing each sample represented by each colored histogram. All samples were induced with the inducer peptide, SppSIP before the cells reached the OD_{600} = 0.33. The *L. plantarum* WCSF1 (black histogram) do not harbor any pSIP-derivative, and were induced before reached OD_{600} = 0.33 and incubated for 5 hours, not treated with lysozyme.