Genetic Engineering of *Lactobacillus casei* for Surface Displaying the Green Fluorescent Protein: an Effort towards Monitoring the Survival and Fate of Probiotic Bacteria in the Gastrointestinal Tract Environment

COLIN CHAN

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
For the degree of

Master of Science in
Department of Biochemistry, Microbiology, and Immunology

University of Ottawa,
Ottawa Ontario, Canada

Abstract

With the introduction of antibiotics in animal feed becoming less popular, the agricultural industry has begun a shift towards the use of probiotics in animal feed. Since there is no current method to evaluate the risks of using genetically modified probiotics in animal feed. The goal of this project was to create a genetically modified model organism for risk assessment. The genetic marker for that was chosen was GFP that was to be expressed on the surface of the cell. The fluorescent properties allow for visualisation of the genetically modified bacteria and the surface expression would allow for the easy capture and recovery of the bacteria for culturing and cell counts. Genome wide screens were performed using the CW PRED algorithm to locate proteins with LPXTG motif for cell wall anchoring. 16 hypothetical proteins were detected and 6 were selected as candidates for possible surface display of GFP. Of these candidates, the novel *L. casei* protein LSEI_2320 was found to be expressed at the mRNA during early growth by RT PCR and at then protein level during stationary phase with western blot. This LPXTG protein was found at the surface of *L. casei* ATCC334 during stationary phase and late stationary phase with immunofluorescence microscopy. A genetically modified *L. casei* ATCC334 was constructed using the surface protein LSEI_2320 locus as a region for recombination with the pRV300 suicide plasmid. Genetic modification of the locus by the insertion of a GFP reporter region just before the predicted signal peptide site resulted in the abrogation of the expression of LSEI_2320 from the cell surface at the late stationary phase. It appears that this particular gene is not necessary to cell survival even though it is abundantly expressed on the cell surface and can be used as a location for genetic modification in *L. casei* ATCC334.

Acknowledgements

I would like to first and foremost thank my supervisor, Dr. Min Lin, for his guidance and encouragement throughout the entirety of my graduate studies. Completion of thesis would not have been possible without his persistent motivation and patience. I feel especially grateful to have had the opportunity to work in his lab.

As well, I would like to thank my thesis advisory committee members Dr Sean Li, Dr. Jiewen Guan and Dr. Susan Nadin-Davies for contributing valuable insight and advice towards my research project.

I would also like to give special thanks to all the members, past and present, in the lab, especially Han Hong Dan, Cathy Zhang and Laura Neil. They have continually provided me with their constant assistance and support that made my graduate studies truly memorable.

And most importantly, I would like to thank my family, particularly my parents, for their never-ending support, patience, and love. They have been my compass and source of strength during every hurdle of my studies.

Table of Contents

List of Abbreviations and Terms

LTA	Lipoteichoic acid
PGSA	Poly-γ-glutamate synthetase A
OmpA	Outer membrane protein A
FliC	Flagellin subunit
HR	Hypervariable region
UPEC	Uropathogenic Escherichia coli
FMDV	Foot-and-mouth disease virus
OspA	Outer surface protein A
RBS	Ribosome-binding site
EA1	Extracellular antigen 1
TTC	Tetanus toxin fragment C
LAB	Lactic acid bacteria
IgA	Immunoglobulin A
IL	Interleukin
cnb	Collagen binding protein
APF	Aggregation promoting factor
SlpA	S-layer subunits
FACS	Fluorescence-activated cell sorting
ldh	Lactose dehydrogenase
MCS	Multiple cloning site
ETEC	Enterotoxigenic *E. coli*
HCE	High constitutive expression
SARS	Severe acute respiratory syndrome
MIC	Minimum inhibitory concentration
GFP	Green fluorescent protein
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase PCR
ORF	Open reading frame
cDNA	Complementary DNA
eGFP	Enhanced GFP
PMSF	Phenylmethanesulfonyl fluoride
IPTG	Isopropyl β-D-1-thiogalactopyranoside
EM	Electron microscopy
ELISA	Enzyme-linked immunoabsorbent assay
CFIA	Canada Food Inspection Agency
GM	Genetically-modified
FISH	Fluorescent in situ hybridization
DAPI	4',6-diamidino-2-phenylindole
LPXTG motif	Lysine, Proline, any amino acid, Threonine, Glycine
pLIC plasmid	Ligation independent cloning

List of Figures

List of Tables

Chapter 1: Introduction

1.1 Bacterial cell surface

There are a few fundamental differences between Gram-positive (**Figure 1.1**) and Gram-negative bacteria (**Figure 1.2**). Firstly, Gram-positive bacteria are comprised of only a single cell membrane but have a rather thick peptidoglycan cell wall, whereas Gram-negative bacteria only have a thin layer of peptidoglycan (Hessle et al. 2000). The cell wall itself is a rigid structure that provides a degree of mechanical protection to the cell by preventing cellular bursting with exposure to a hypotonic environment (Rogers and Forsberg 1971). Gram-positive bacteria, consisting of a single cell membrane, allows protein export to the cell surface to be more efficient compared to Gram-negative organisms. Since Gram-negative bacteria have both an inner membrane which consists of phospholipids and lipopolysaccharides in the outer leaflet of the outer membrane and phospholipids in the inner membrane (Silhavy et al. 2010), the proteins that are cell wall associated in Gram-negative bacteria are not surface exposed (Lee & Schneewind. 2001). The phospholipid membranes act as a semi permeable barrier that allows for the selective uptake and diffusion of solutes to and from the environment (Roy 2009). Gram-positive bacteria also have lipoteichoic acids (LTAs), which are anchored to the cell membrane and extend out from the cell. The functional role of LTAs have been implicated in cell division via the inhibition of autolysins (Holtje and Tomasz. 1975) and in the functions of the glycocalyx. Teichoic acid (TA) is linked to the peptidoglycan, extending outward from the cell. TAs along with LTAs and peptidoglycan form a network of negative charges that can attract cations to the cell wall, such as sodium and magnesium (Neuhaus and Baddiley 2003) and play a role in cell wall rigidity.

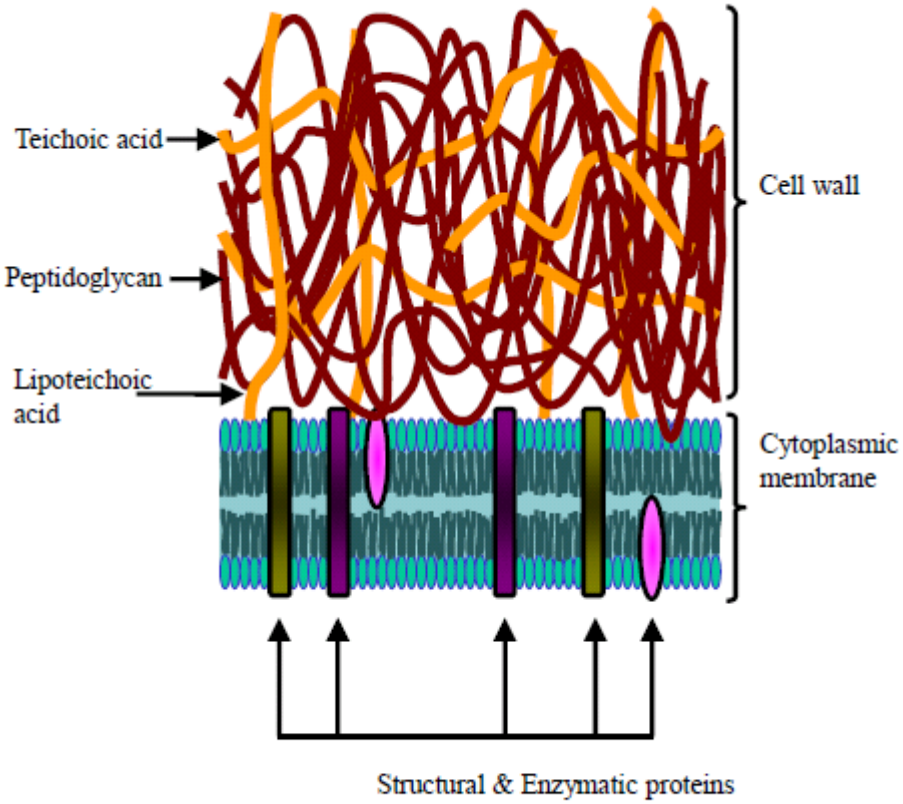


Figure 1.1 General model of the Gram-positive bacterial surface

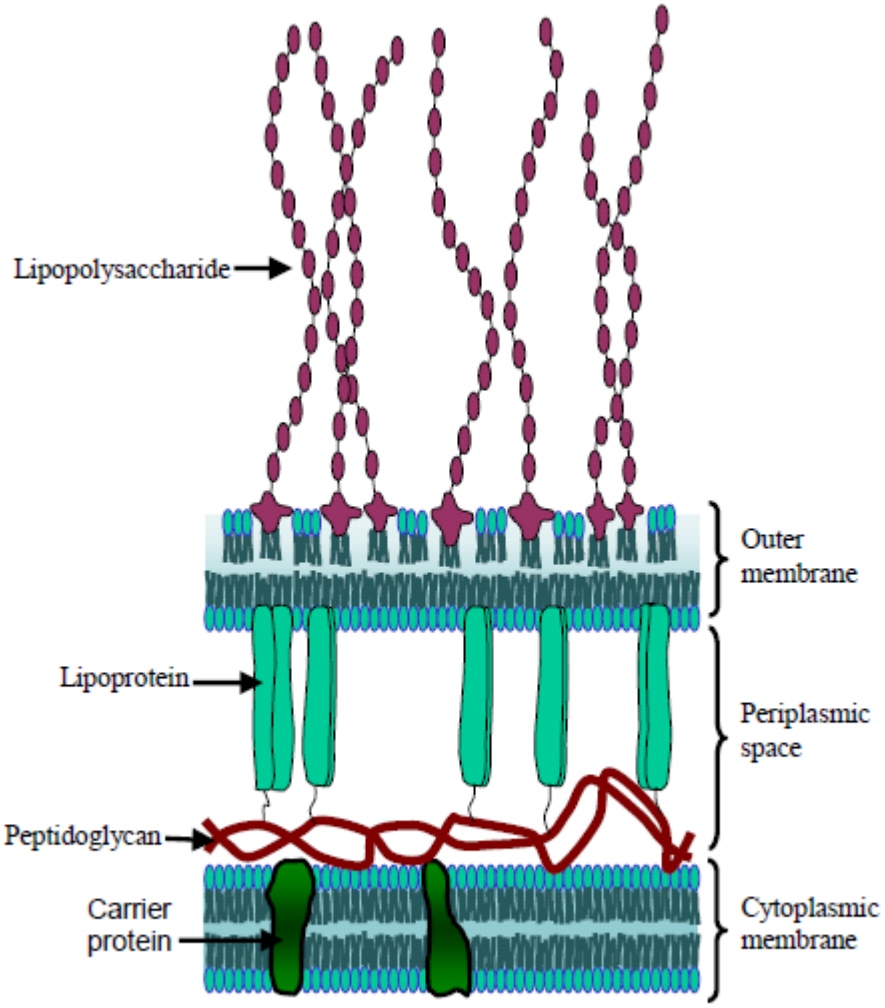


Figure 1.2 General model of the Gram-negative bacterial surface

Thus, the negatively charged compenets of the Gram-positive cell surface play important roles in the uptake of nutrients, minerals, and antibiotics, and can act as anchoring molecule for enzymes, including autolysins and adhesins.

Gram-positive bacteria may also contain capsules that are comprised of S-layer proteins, polysaccharides, or proteoglycans. The S-layer is attached to the secondary cell wall components such as polysaccharides associated with the cell wall in the case of *Lactobacillus* (Sara and Sleytr 2000). The main role of the capsule is to protect the bacterium from environmental hazards, including the phagocytosis by immune cells, the complement system and also from attack by antimicrobial peptides (Llobet et al. 2008). Furthermore, they also play a role in evading the immune cells of the host and are also involved in the formation of biofilms (Anderson et al. 2010). Other notable features on the cell surface include flagellum that span the entire cell membrane and cell wall for the purpose of cellular motility, which uses a molecular motor to power the filament that causes a twisting motion (Mora et al. 2009) in contrast to the eukaryotic motility involving whipping or back and forth motion (Lin et al. 2012). Adhesins are located on the cell wall and membranes. These pili proteins are used for attachment to surfaces and other cells and can allow bacteria to exhibit different tissue tropism during infection (Kline et al. 2009).

Various cell surface interactions can occur on bacterial surfaces, including adherence and uptake of genetic material (Wang and Taylor 1990) and nutrients. One important nutrient for bacteria in the environment is iron, which usually requires the use of siderophores to capture the ions and transporter proteins, such as an ATP binding

cassette transporter, to internalize the complex for processing (Miethke and Marahiel 2007). Secretion of proteins and polysaccharides, which makes up the capsule, is another important process on the bacterial cell surface (O'Riordan and Lee 2004).

1.2 Cell membrane translocation signals and motifs

There are generally two ways with which proteins can become surface localized: 1) a sec dependent pathway (**Figure 1.3**) and 2) a twin arginine/TAT pathway. In the first case, protein destined for surface localization must pass through the cell membrane before they can reach the surface. The Sec translocon is able to facilitate this method of transportation to nascent peptides (**Figure 1.3**), but it requires the presence of an N-terminal signal peptide that is recognized by the signal recognition particle (SRP) (Lycklama and Driessen 2012). The signal peptide itself contains a short stretch of positive amino acids at the N-terminal region, a central hydrophobic region, and a polar C-terminal region that contains the cleavage site (Von Heijne 1990). For Gram-positive bacteria, this can be about 29-31 residues long (Von Heijne and Abrahmstn 1989). The protein is subsequently cleaved by signal peptidase I to produce a mature protein (Auclair et al. 2012) (**Figure 1.4**).

The twin arginine translocon (TAT) also follows a very similar process that involves a signal peptide and a signal peptidase. The difference in the two translocation pathways being that the Sec translocon can only transport unfolded protein while the TAT system can translocate a fully folded protein (Robinson et al. 2011). The TAT signal sequence is distinct from the sec signal sequence in that it has a longer sequence of 37 residues due to a longer N-terminal region and is less hydrophobic. The defining feature

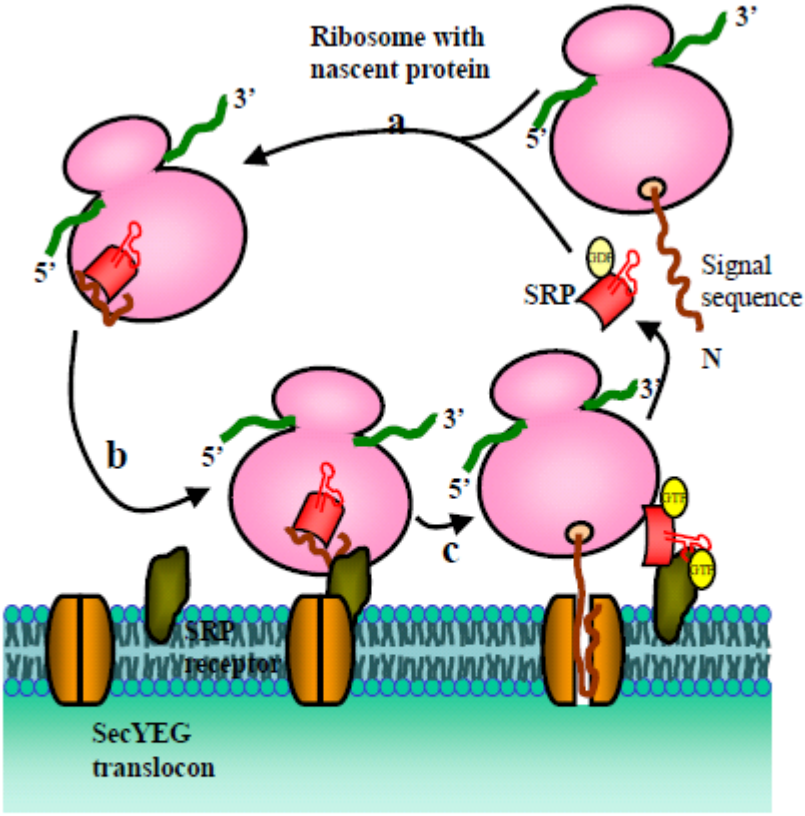


Figure 1.3 Important steps in the secA mediated protein secretion. a. The signal recognition particle (SRP) recognises the Signal peptide and binds to the ribosome nascent protein complex. b. The SRP directs the ribosome nascent protein complex to dock at the SRP receptor. c. Translation resumes with the nascent protein being threaded through the SeYEG translocon.

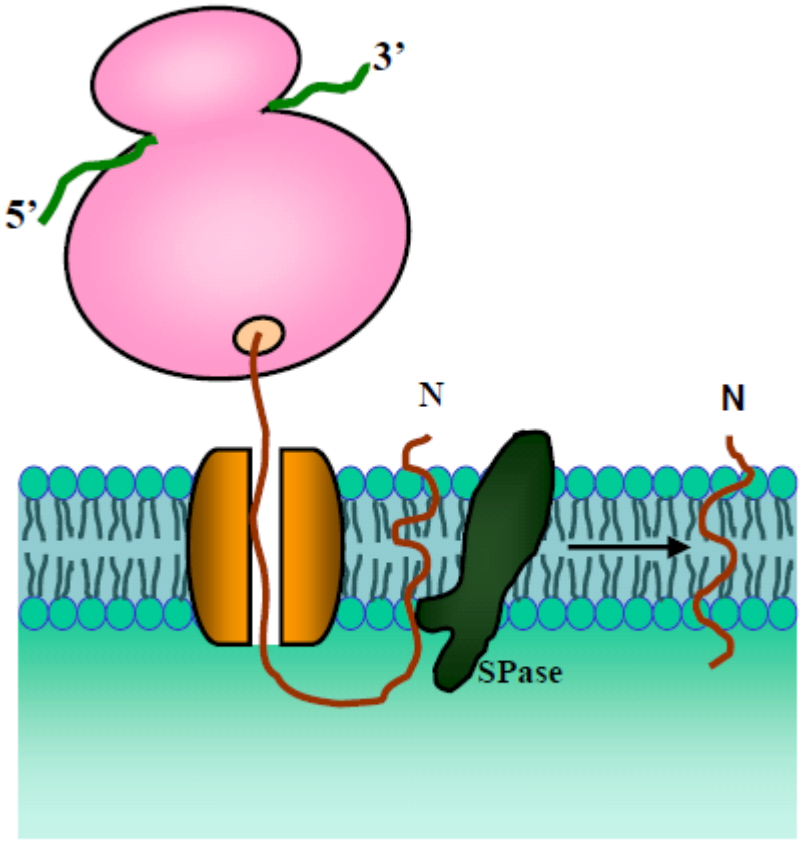


Figure 1.4 Cleavage of the signal peptide via the signal peptidase to remove the signal peptide

is a motif between the N-terminal region and the hydrophobic center, (S/T)RRxFLK (Bendtsen et al 2005).

1.3 Motifs for protein anchoring to the cell surface

After the protein leaves the cell membrane, there are various means with which they can attach to the cell surface, including covalent or non-covalent attachment (**Figure 1.5**). For example, proteins containing LPXTG motifs (Marraffini et al. 2006) and many lipoproteins attach covalently (Sutcliffe and Harrington 2002) via their lipobox motif, while LysM domains (Buist et al. 2008) and GW repeats attach non-covalently (Renier et al. 2012).

With attachments to the cell wall, proteins can be covalently linked to the peptidoglycan via enzymes called sortases. Sortases can recognize LPXTG motifs in the C-terminal of nascent proteins and attach them to the peptidoglycan by cleaving the threonine and glycine residues (Maresso and Schneewind 2008). In addition, adhesins and pili generally follow the same sort of anchoring process as LPXTG proteins and are covalently linked to the cell wall (Oh et al. 2008). In the case of lipoproteins, these can be anchored to the cell membrane via their N-terminal lipobox domains. Proteins that attach to lipids require a motif called a lipobox, ending in a cysteine and follow a similar format to the signal peptide. The enzyme prolipoprotein diacylglycerol transferase covalently attaches the protein to diglyceride before sorting and subsequent cleavage is facilitated by signal peptidase II (Kovacs-Simon et al. 2011).

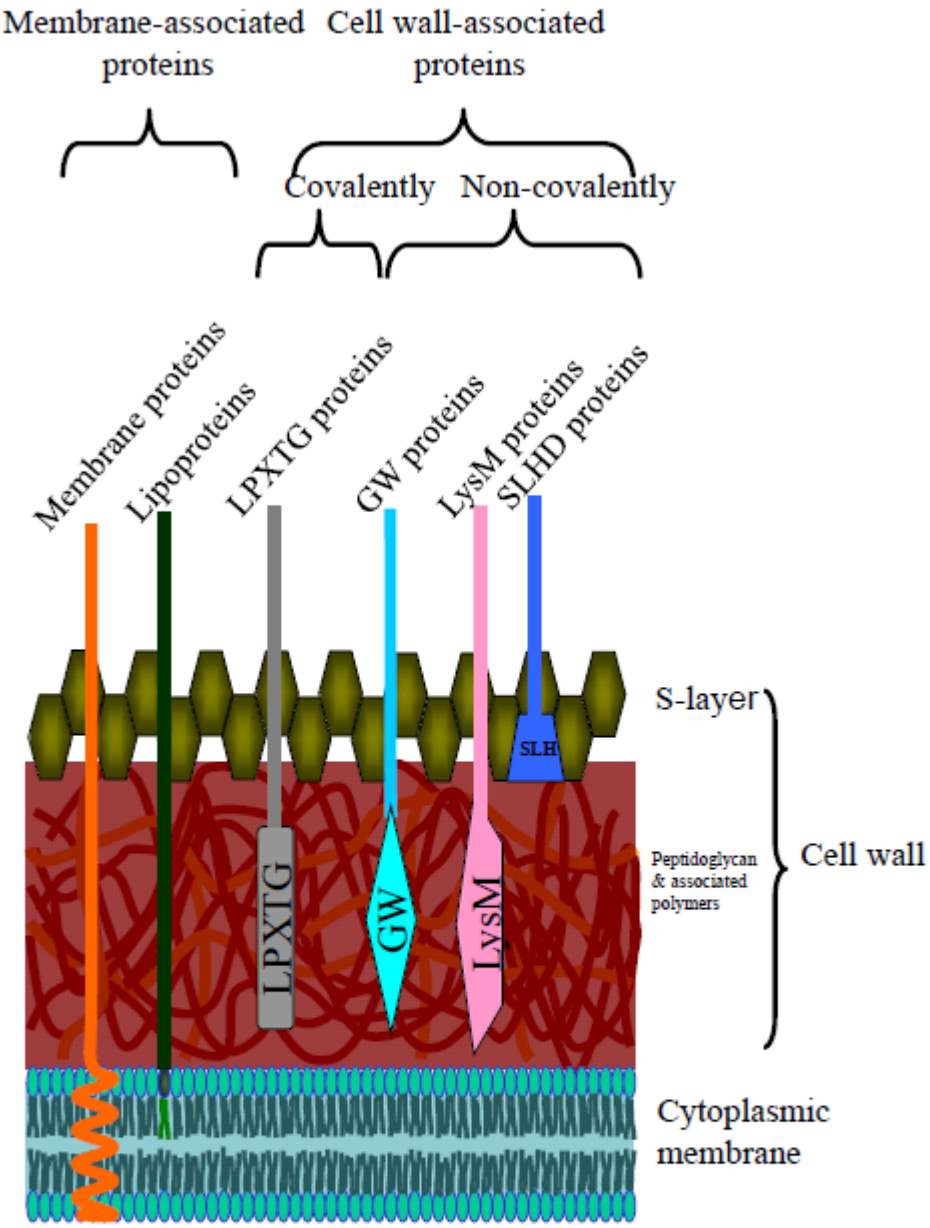


Figure 1.5 Various Surface protein anchors for Gram-positive bacteria. Membrane proteins can either be permanently associated with the membrane (Integral membrane proteins) or indirectly associated with the plasma membrane (Peripheral membrane protein) . Lipoproteins are covalently linked to the phospholipids. LPXTG proteins are covalently linked to the cell wall peptidoglycan. Non-covalent cell wall proteins have cell wall binding domains like the GW modules and the LysM domains. S Layer Homology domains (SLHD) bind to secondary cell wall polymers such as teichoic acids.

Transmembrane proteins follow the same general pattern of transport via the Sec A system; however; the complex may require the aid of a membrane insertion protein called YidC to facilitate their insertion within the membrane (Welte et al. 2011). The primary difference between transport of type I and type II membrane proteins is that the signal anchor is not cleaved in type II membrane proteins whereas the type I membrane proteins are cleaved. Furthermore, the N-terminal for Type I proteins is extracellular while the C-terminal for type II proteins is extracellular. For predicting the orientation of the protein, the positive inside rule (where more positively charged residues tend to stay on the cytoplasmic side) is applied and the transmembrane segments are hydrophobic (Xie and . Dalbey 2008).

For noncovalent binding to the LTA, there are C-terminal GW domains that can associate with the cell surface component These mostly consist of repeats of 80 residues containing a glcin-tryptophan dipeptide (Ebbes et al. 2011). LysM domains consist of approximately 44-65 amino acids long. They are found in a variety of proteins involved in bacterial wall degradation and can also offer binding to the peptidoglycan cell wall. LysM domains can be found in either the N or the C terminal of the protein (Buist et al. 2008). In addition, some species of bacteria also have choline associated with their LTA and thus can result in the attachment of choline binding proteins. Choline binding proteins are very similar to the GW repeats but are usually shorter at 20 amino acids, may contain proline rich domains, and usually found at the C-terminal of the protein (Desvaux et al. 2006).

S-layer proteins are also exported from the cell cytosol. However, they are not directly attached to the cell as they are usually attached through secondary polymers. They generally possess a signal sequence for export, repeats of the S-layer homologous motifs of 50-60 residues, and a crystallization domain for use during self assembly (Sara and Sleytr 2000).

Flagellum proteins are totally distinct subset of proteins since their signals for export are different from the others in that they are not cleaved. These proteins use a type III secretion system to aid in the assembly of the flagellum (Blocker et al. 2003). The variable region of FliC can be modified in such a way that up to 187 residues can be deleted or that it can accomidate the insertion of up to 36 residues (Tanskanen et al. 2000).

Another type of popular surface display protein has been the poly-γ-glutamate synthetase A (PGSA) protein from *Bacillus subtilis*, which is a membrane protein that is particularly used as a C-terminal co-display. This is because only the C-terminal region is exposed to the extracellular environment (Bloois et al. 2011). One extensively studied anchoring protein is the *Streptococcus pyogenes* M6 protein, which is a LPXTG protein that is capable of expressing many peptides on the cell surface of different bacteria with its localization signal (Pozzi et al. 1992).

1.4 LPXTG motif

The LPXTG motif is located in the C-terminal region of nascent peptides. Similar to other proteins destined for the cell surface, it must first pass through the SecYEG

translocon through the action of the SecA ATPase, which facilitates this process by containing a signal peptide recognized by the signal peptidase I enzyme at the N-terminal of the protein. Upon recognition, the signal peptide will be cleaved and the protein will be secreted. The LPXTG motif acts as a type of retention signal that slows down the secretion of the nascent peptide so that it can be recognized by sortase A. This enzyme facilitates proteolytic cleavage after the threonine residue in the LPXTG motif (**Figure 1.6**), allowing for covalent crosslinking of the peptide to the bacterial peptidoglycan cell wall. Deletion of sortase A in *L. salivarus* was capable of inhibiting the expression of 3 predicted surface proteins and also reduced the adherence of the mutant bacteria to human epithelial cells (van Pijkeren et al. 2006). The most recent work showed a *L.acidophilus* displaying FliC of *Salmonella* using the mub anchor that contained the LPXTG motif (Kajikawa et al. 2011). The motif also appears to be well characterized and is used extensively in phage display. Literature has shown that it is possible to anchor biotin to phage capsids using the LPXTG motif and thus, it plays an important role in labelling (Hess et al. 2012).

The type of anchoring system can vary when applied to surface display. This only depends on the original protein as far as surface display is concerned.

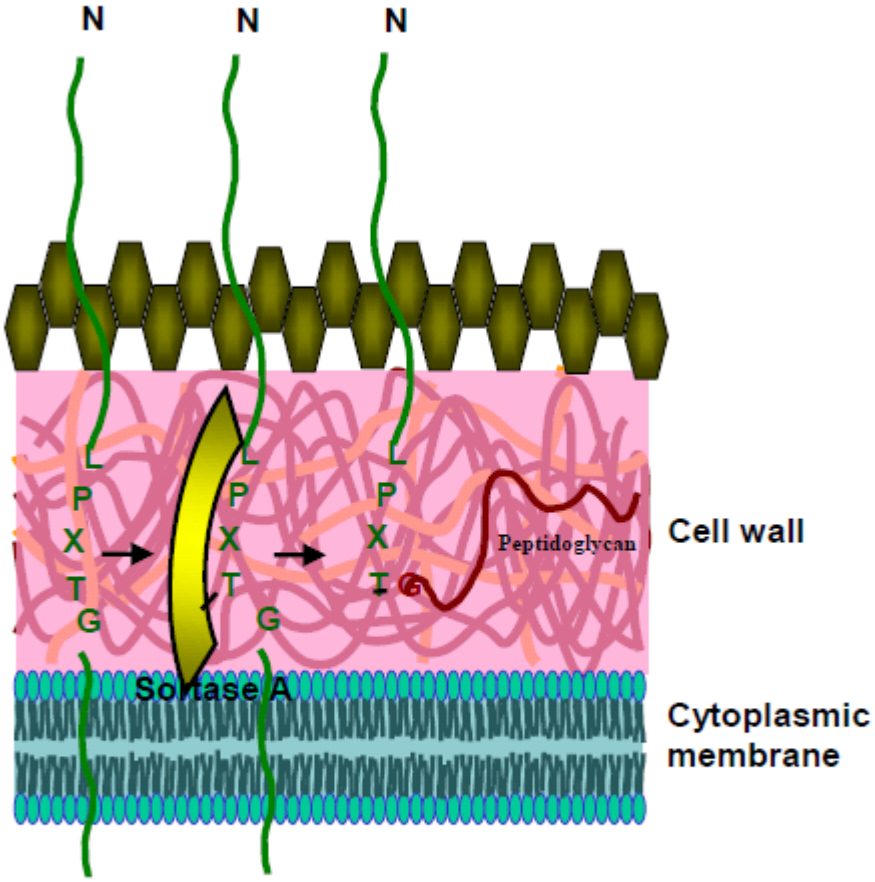


Figure 1.6 Sortase mediated anchoring to the Gram-positive cell wall. Sortase recognises the LPXTG motif on the nascent protein and causes a cleavage between the threonine and glycine residues. The sortase then attaches the cleaved peptide to the peptidoglycan.

1.5 Surface Display of proteins in Bacteria

Surface display is a protein engineering technique that uses parts of bacterial proteins that are known to be associated with the cell surface (ie. anchor/scaffold) to express other proteins (ie. target) on the cell surface. A typical surface display system will usually have the displayed protein fused to the cell surface-associated domain of an anchoring protein. In order to do this, there should be extensive knowledge on how anchoring proteins become cell surface localized in order to take advantage of their localization signals. For example, localization of anchoring proteins can be due to covalent linkage to the peptidoglycan of the cell wall via sortases in conjunction with signal peptidase I, or via attachment to the phospholipid membrane via apolipoprotein N-acyl-transferase (see lipobox/lipoproteins) in conjunction with signal peptidase II. In addition, surface display systems have also taken advantage of pili and flagella as well.

Non-covalent linkages to the cell surface consist of transmembrane proteins and S-layer proteins, while other proteins can interact with parts of the cell wall, such as techoic acid or LTA via electrostatic attraction. Early on, Gram-negative surface display systems, such as *Escherichia coli*, were used since they were the most well studied bacteria at the time. However, ongoing research has also increased our knowledge of other bacteria such as Gram-positives as well. With Gram-positive bacteria, surface display can be easier to achieve since proteins only need to pass through one membrane bilayer before surface localization is achieved compared to Gram-negatives that requires passing through two membrane bilayers. For example, the outer membrane protein A (OmpA) that anchors to the peptidoglycan of Gram-negative bacterial outer membrane

was used to display small peptides (such as FLAG, myc) at regions where the loops of the protein are exposed to the extracellular environment (Verhoeven et al. 2009). However, the exposed loops were a major limitation in size for the target protein. For Gram-negative bacteria, the protein must be translocated to the periplasmic space before it can reach the outer membrane, which can take place via both sec dependent and sec independent methods.

Various Gram-negative bacterial surface proteins have been used for surface display, such as lipoproteins, flagellin subunit, fimbrial proteins, and S-layer proteins. For instance, the plasmid encoding TraT lipoprotein was used to display the C3 epitope of the Vp1 protein (residues 93-103) of the polio virus via inserts at residues 125, 180, or 200 (Taylor et al. 1990). This type of configuration requires the protein to be anchored via its N-terminal to the phospholipids. Flagellin subunit (FliC) has been used in *E. coli* for surface display of YadA from *Yersinia enterocolitica* (302 residues), inserted into the central region of FliC, which replaced 58 residues (Westerlund-Wikström et al. 1997). The advantage of this system is that a single flagellum will contain many thousands of flagellin subunits that can provide high expression, where the maximum amount allowed for replacement in this system was about 187 amino acids from the central region of FliC. Another Gram-negative feature that has been used for surface display is the fimbrial proteins. The P fimbriae F11 major subunit hypervariable regions (HRs), HR1 and HR4, from uropathogenic *E. coli* (UPEC) were used to display epitopes for foot-and-mouth disease virus (FMDV) (9 residues) and *Plasmodium falciparium* (14 residues) (Van Die et al. 1988). Furthermore, S-layer proteins have also been reported to be scaffolds for

Gram-negative display systems. The RsaA protein of *Caulobacter crescentus* was able to display a 12 residue long portion of *Pseudomonas aeruginosa* strain K pilin (Bingle et al. 1997).

Other examples of surface display have involved protein A from *Staphylococcus aureus* as an anchor system for the display of the M3 malarial antigen (80 amino acids) and a albumin binding protein from *Staphylococcus carnosus* using *Staphylococcus carnosus* as the host (Samuelson et al. 1995). The system consists of utilizing the N-terminal signal peptide and promoter of the *Staphylococcus hyicus* lipase gene and the protein A C-terminal cell wall binding domain of protein A with the protein of interest fused between the two segments and subsequently displayed. Furthermore, a system was developed to express the outer surface protein A (OspA) of Gram-negative *Borrelia burgdorferi* (causative agent of lyme disease) as a membrane lipoprotein in a *Mycobacterium bovis* model. The anchoring protein used the promoter, secretion signal, ribosome-binding site (RBS), and first 6 peptides from the 19 kDa protein antigen of a *M. bovis* surface-associated lipoprotein (Stover et al. 1993). Cell surface-associated proteins have been another delivery vehicle in Gram-positive bacteria. For instance, an S-layer protein called extracellular antigen 1 (EA1) from *Bacillus antracis* has been used to anchor the tetanus toxin fragment C (TTC) from *Clostridium tetani* to the S-layer, which associates with the cell wall in a non-covalent manner. In addition to the above anchoring proteins, spore components from *Bacillus subtilis* have been used for surface display (Mésnage et al. 1999). This was the case when *B. subtilis* was able to express the TTC antigen from *C. tetani* on its own CotB spore protein (Ciabattini 2004).

1.6 Overview of *Lactobacillus*

Lactobacilli are rods or cocci, non-motile, Gram-positive members of the lactic acid bacteria (LAB) group that are catalase negative, and live in microaerophylic environments. They are also chemo-organotrophs and can live in a variety of environments that contain carbohydrates (Felis and Dellaglio 2007). They can live in the gastrointestinal tract (Walter 2008), female reproductive tract (Mosbah and Reda 2009). *Lactobacillus* also is used in the preservation of food and also used to make cheese, yogurt, salami, sauerkraut, pickles, olives and coffee. For animals, *Lactobacillus* can be used to make silage and has been used as probiotics for chickens, pigs and cattle (Bernardeau et al. 2006). Some species of Lactobacilli are part of the gut microbiota of animals, including *L. reuteri, L. mali, L delbrueckii* and *L.animalis* (Walter 2008), as well as probiotic organisms, such as *L. acidophilus*, *L. reuteri* and *L. casei* (Reid 1999). They are capable of restricting the growth of certain bacteria through their production of metabolites, such as lactic acid from simple sugars, which can subsequently lower the pH (Neal-McKinney et al. 2012). In addition, they can produce antibiotics, such as bacteriocins (Maqueda et al. 2008), and hydrogen peroxide (Mosbah and Reda 2009). Other effects are stimulating the productions of secretory immunoglobulin A (IgA) (Isolauri et al 2001), improving the tight junctions between intestinal epithelial cells (Ulluwishewa et al 2011) and improving immunotolerance (Johansson et al. 2011). Lactobacilli species in the gastrointestinal tract are important in the development of the immune system as well as playing a big role in the uptake of nutrients for the host organism. For instance, *L. paracasei* can modulate the immune system to produce more

interleukin (IL)-6 to reduce the damage caused during an inflammatory response. Furthermore, *L. acidophilus* was able to increase the intake of glucose by epithelial cells (Rooj et al 2010). *Lactobacillus* can also produce short chain fatty acids which can be taken up by enterocytes as nutrients (Naaber et al 2004). Their role is quite important since they can be taken to recolonize the gut after antibiotic therapy (Myllyluoma et al 2005).

1.7 Significance of *L. casei*

The non-motile *L. casei* mainly resides in the digestive tract and is capable of fermenting lactose to produce lactic acid. It is a Generally-recognized-as-safe (GRAS) organism and is known for having probiotic properties (Snydman 2008). This is in part due to its lactic acid production, which can discourage the growth of less desirable microbes. In the gut, it can stimulate the secretion of IgA antibodies. *L. casei* can also be used as a starter culture in the production of cheese, fermented foods, and other dairy products. In addition, it is also used in the fermentation of silage for animal feed. In addition, they have been used to surface display proteins for use in whole cell vaccines. The strain ATCC334 of *L. casei* in particular has putative genes annotated as well as a sequenced genome, which makes it a prime candidate for research as not much has been done in characterizing it aside from its carbohydrate metabolic pathways and cold stress proteins.

1.8 Surface display systems in *Lactobacillus*

Due to their lack of pathogenicity and reported probiotic properties in humans, lactobacilli are attractive candidates for nanobiotechnological applications, such as vaccine-delivery systems. Thus, there is increasing interest in research focusing on developing surface display systems in different species of *Lactobacillus*. Most of the surface display systems developed in *Lactobacillus* and other lactic acid bacterica (LAB) utilize the LPXTG motif on the C-terminus of cell wall anchoring proteins. Various other types of anchoring proteins, such as non-covalent transmembrane, cell surface-associated, S-layers, and covalent peptidoglycan-based anchoring proteins, have also been exploited for surface display.

Non-covalent Transmembrane proteins: Use of transmembrane proteins includes the use of the PGSA gene from Gram-positive *B. subtilus*, which is by far the most popular anchoring system to date. This consists of a type II integral membrane protein that can display the target protein via its C-terminal which is surface exposed (Blooise et al. 2011). This allows for a larger protein to be displayed compared with using the transmembrane loops, which can limit the size of the displayed protein to about 49.9 kDa (Narita et al 2006). Currently, the surface display systems for *L. casei* have been using the PGSA gene from *B. subtilis* as a scaffold. Its gene product is one part of the synthase complex and a 371 amino acid transmembrane protein with a molecular weight of 41 kDa (Ashiuchi et al. 2001). This protein consists of the N-terminus being required for anchoring (membrane association) with the C-terminus is situated extracellularly, where

foreign proteins are usually fused to the C-terminal of PGSA. This sort of anchoring is a non-covalent form since it is membrane associated.

Cell surface-associated proteins: *L. reuteri* is capable of using its own collagen binding protein (cnb) as an anchor for surface display. The target peptide was fused to the C-terminus of the full length cnb protein. For example, this system has been applied to the display of β-glucanase of *Fibrobacter succinogenes*, allowing the bacteria to digest β-glucan and aiding in nutrient uptake as a food additive (Huang et al. 2011). Its expression used a pNZ3004 vector and a LacA promoter of *L. lactis* for constitutive expression. The catalytic part of the glucanase (28 kDa) was fused to the C-terminal of the full length cnb gene. The cnb gene encodes a protein with a molecular weight of 29 kDa is cell surface associated and hypothesized to bind to teichoic acid via electrostatic interactions.

In *L. paracasei*, the heavy chain variable domain of llama antibodies raised against the SAI/II surface adhesion antigen of *Streptococcus mutans* was displayed using the C-terminal (last 78 residues) of aggregation promoting factor (APF) cell wall-associated protein of *L. crispatus* for cell wall binding (Martín et al. 2011). APF is cell surface bound via possible electrostatic interactions or non-covalent interactions via its conserved C-terminal domain (detected in a LiCl extraction). This surface display construct consists of a signal peptide (33 residues) for export and the N-terminal domain (75 residues) from APF (with a molecular weight of 24 kDa), flanking the inserted antibody between the two terminal domains. The gene successfully integrates into the genome using the vector pEM76 that contains an A2 phage integrase, used for site

specific integration at a 19bp attB in the leucine transfer RNA gene. The β-recombinase six sites present in the plasmid is used to remove unwanted sequences that occur during integration into the genome by deleting DNA flanked by two six sites. The approach used by this study makes surface display viable for longer term applications and also reduces the amount of foreign DNA present in the system. This was used to reduce infection from pathogens.

S-layer proteins: S-layer proteins have been also been reported for surface display applications. For instance, the S-layer subunits (SlpA) of *L. crispatus* are commonly used for display systems. In addition, they tend to be created as surface protein fusions in *E. coli* and subsequently mixed with the *Lactobacillus* to determine the occurrence of binding. The SlpA of *L. brevis* has also been utilized for surface display, such as the immunodominant VP1 epitope of the poliovirus (11 residues) and Myc tags (10 residues) (Åvall-Jääskeläinen et al. 2002). Development of these display constructs consists of insertions at 4 of the most hydrophilic parts (predicted to be surface exposed by an algorithm) of SlpA between Asp362 and Thr363, Lys249 and Ala250, Ala313 and Asn314 and Ala49 and Lys50. The vectors that have been used on this system were pVE6007 and pORI280, which allow for temperature sensitive integration into the genome via recombination involving RepA. The pORI280 requires RepA (encoded by pVE6007) in order to replicate at the permissive temperature. At the restrictive temperature, the RepA is absent and integration occurs between the regions of homology in which the pORI280 has inserted with the chromosomal encoded SlpA gene. The genes

were under the control of a nisin inducible promoter pnisA from *L. lactis* for the development of a vaccine delivery system.

Covalent Peptidoglycan-based anchoring: Proteins that have an LPXTG sorting signal, which covalently couple to peptidoglycan, require the use of a signal peptide at the N-terminus for export and a recognition sequence at the C-terminal for effective attachment to the cell wall. The surface display constructs developed with these LPXTG-containing encoding genes usually place inserts in between the signal peptide and C-terminal LPXTG motif.

For strong expression of surface displayed proteins by *Lactobacillus*, a good promoter has to be used to allow either high constitutive expression or high inducible expression without generating toxicity to the cell. For this reason, there are two systems for transforming cells for surface displayed protein expression: 1) a plasmid-based system and 2) a chromosome-based system.

Plasmid-based systems: Plasmid-based expression systems are much more suitable for shorter term studies that can take advantage of their higher copy number and hence higher protein expression levels. The advantages of using plasmid-based systems are that they are much more straightforward to make and do not require as much time. However, there are also drawbacks as the plasmids require selection to be maintained and the plasmids can become unstable. But as of yet, plasmid-based systems are by far the most popular system that is used in *L. casei* for a wide range of studies on protein

expression, surface display, and promoter activity. Furthermore, this system has been used to track the stability of transformed bacteria inside animal digestive tracts.

Chromosome based systems: Chromosomal expression is the preferred method of expression for longer term studies and usually relies on homologous recombination to replace an existing gene. However, they usually lack the higher levels of protein expression associated with plasmids, which can be mitigated by placing tandem copies of the surface display peptide into the chromosome. For effective integration to occur, shuttle vectors and suicide plasmids are normally used with the shuttle vectors being integrated into the chromosome at restrictive conditions of replication. These are mainly used to study the effects of knocking out genes in *L. casei*. One disadvantage of this system is that it requires more time to construct and screen compared to the plasmid approach.

1.9 Application of surface display

Applications of surface display are primarily for the development of vaccines that provide immunization of patients at the mucosal level by presenting immunogenic peptides at the cell surface that can be processed by dendritic cells and macrophages. The antigens that have been used for surface display are from various pathogens ranging from *E. coli* to human papilloma virus. Alternatively, biocatalysis is another application of surface display in which catalytic enzymes are displayed to allow interactions with substrates that the bacterium would be exposed to. Biocatalysts mainly consist of polysaccharide degrading enzymes, including β-glucanase and α-amylase. Furthermore,

surface display also has potential for environmental applications, such as bioremediation where expressed proteins (that can be in multiple copies) bind large numbers of contaminants to remove them from the environment. Other applications include the use of high throughput screening of protein libraries similar to phage display with the aid of fluorescence-activated cell sorting (FACS) and the engineering of proteins that have higher affinity for their substrates. A final use for surface display is for using the actual bacteria as a "whole cell antibody" for affinity chromatography applications such as the display of the heavy chain variable domains of llama antibodies for the capture of *Streptococcus mutans*. (Martín et al. 2011).

1.10 *L. casei* as a host for surface display

Currently, the surface display systems for *L. casei* have been based on the PGSA gene from *B. subtilis*, which encodes one portion of the transmembrane protein synthase complex. The N-terminus of this protein is the region that is required for anchoring (membrane association), whereas the C-terminus is extracellular and can fuse with foreign proteins. This sort of expression system has been plasmid-based. In addition, the use of transmembrane proteins may affect the viable size of the displayed protein, such as in the case of the outermembrane proteins of *E. coli*.

α-amylase (78 kDa) from *S. bovis* is one example of a successful surface display system for *L. casei* (Narita et al. 2006). This was done under the use of a lactose dehydrogenase (ldh) promoter of *L. casei* in conjunction with the untranslated leader sequence from *L. acidophilus* S-layer protein for high constitutive expression. The vector

that was used on this system has been pSECE1, in which the C-terminal part of the PGSA gene was fused to the N-terminal of the α-amylase gene. The display was done using foreign sequences aside from the lactose dehydrogenase (LDH) promoter. This bacterial system results in a starch-degrading strain of *L. casei* to ferment lactic acid from starch so it can utilize a more complex carbohydrate.

In addition, the use of the PGSA anchoring sequence has been applied to develop several vaccine delivery systems. For example, PGSA-based anchoring has been applied to anchor the K99 pilus antigen from ETEC (18 kDa) on *L. casei* (Wei et al. 2010). The expression was under the control of a high constitutive expression (HCE) promoter from *Geobacillus toebii* that controls the expression of D-amino acid transferase, resulting in constitutive gene expression. The 498bp K99 fragment was cloned into the multiple cloning site (MCS) on the C-terminal of the PGSA gene of a pLA plasmid containing a HCE promoter, all of which are foreign sequences. Similarly, another vaccine delivery system has been developed for expressing the F41 fimbrial protein of enterotoxigenic *E. coli* (ETEC) (31 kDa) on *L. casei* (Liu et al. 2009). Using the vector pHCE1LB with a HCE promoter for expression, the N-terminal of the F41 protein (834bp) was fused to C-terminal of the PGSA protein (1116 bp) to construct this surface display system of entirely foreign sequences. Another vaccine delivery system focused on the severe acute respiratory syndrome (SARS) coronavirus allowed *L. casei* to display the SA (112 residues) and SB (332 residues) spike proteins on a PSGA-based display system (Lee et al. 2006). Using an HCE promoter for induction on a pAT19 vector, the C-terminal of the PGSA was fused to the N-terminal of the SA and SB antigens respectively, consisting of

completely foreign sequences to the host. In a similar study, *L. casei* was able to display the E7 protein (16 kDa) from the human papilloma virus 16 on its surface with the use of the PGSA anchor in conjunction with an HCE promoter and pGEM-T as the vector to develop a vaccine delivery system (Poo et al. 2006). Finally, another system used the PGSA anchor to display the nucleocapsid protein (46 kDa) of porcine epidemic diarrheal virus on the surface of *L. casei* (Hou et al. 2007). Expression was mediated with the pHCE1LB that contains the HCE promoter, where the C-terminal of the PGSA (1026bp) anchor was fused to the N-terminal of nucleocapsid protein (1326bp).

Expression systems are mainly based in high copy-number plasmids for surface protein expression but may trade off stability for longer term applications as they require some type of selective pressure (usually antibiotics) to maintain the plasmids (Richter & Gescher 2012). These expression systems use protein promoters and anchoring sequences that are foreign to *L. casei*, which may affect the proper expression, secretion, and peptidoglycan anchoring, and conformation of certain proteins for surface display. Using any type of anchoring motif requires a good understanding of the protein as they often can only carry inserts within a specific size range, be susceptible to proteases, have their conformation affected by the anchor and heterologous surface display may result in growth defects of the host cell (Georgiou et al. 1997).

1.11 Research rationale

While antibiotics have been used to treat animals for veterinary purposes, they have also been significantly used in agriculture, such as with swine, cattle, and poultry, and aquaculture (Cabello 2006). Their main purpose was in prophylactic use and as

growth promoters where low levels of the antibiotics were used above the minimum inhibitory concentration (MIC) of the bacteria but maintained at a dosage lower than treatment of infection. The use of antibiotics, such as penicillin, erythromycin, and tetracycline, used to treat human infections has also been an area of concern (Barton 2000). This creates an environment that would drive evolution towards resistant strains of bacteria through mutation, uptake of DNA, and the horizontal transfer of plasmids via conjugation. The antibiotics are added to the animal's food and water supply to function in making the animal resemble a germ free state. The most immediate effect of the use of such treatments is obviously the generation of antibiotic resistant pathogens that could have severe implications in the future for veterinary medicine. In addition, some resistant foodborne pathogens, such as *Salmonella, Listeria*, *Campylobacter*, *E. coli* and *Yersina* could also affect humans as well (Mathew et al. 2007). Since the appearance of resistant strains of bacteria in human disease is due to the misuse of antibiotics, the government has been taking a precautionary approach to addressing the usage of antibiotics in animal feed. Due to this concern, there is a gradual shift towards the use of prebiotics, probiotics, bacteriophages, bacteriocins and phytotherapuetics as substitutes for feed additives (Hume 2011).

Probiotics are : “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (WHO 2001). Prebiotics are non-digestable components in food that confer a health benefit through their interaction with the host gut microbiota (Gibson et al. 2004). Bacteriocins are antimicrobial peptides produced by bacteria with activity against other bacteria that are closely related to the bacteriocin

producing strain (Heng et al. 2006). Phototherapeutics are herbal extracts that can be used to treat a variety of chronic diseases (Cravotto et al. 2010).

Since probiotics are already used with chickens, pigs and ruminants (Musa et al. 2009), proposals have come from industry for the use of genetically modified (GM) bacteria as feed additives. As such, the Canadain Food Inspection Agency (CFIA) is striving towards monitoring GM bacteria in animals in order to determine the toxicity and risk of the transgene transfer of these GM bacterial strains and their survival in the animal gut and the agricultural environment (Chesson & Flint. 1999). To this end, it was proposed to generate a strain of bacteria that would carry a reporter gene coding for a surface displayed green fluorescent protein (GFP). Since there are no current genetically modified (GM) probiotics available in the CFIA, this study aims to create a GM *L. casei* to serve as a model organism with a stable and easy to track genetic marker. Since genetic modifications need to be made in the *L. casei* genome, an investigation would have to determine a suitable site within *L. casei* that could be compatible with the insertion of the GFP reporter gene. To this end, LPXTG surface proteins were looked into because these proteins, although very little is known about them, could serve as an anchor for surface display of the GFP proteins. Surface expressed markers have an advantage that allows the bacteria to be easily recovered via methods such as bead capture or FACS, which would aid greatly in monitoring GM survival and in risk assessment.

1.12 Hypothesis and objectives

The thesis work was undertaken based on the hypothesis that one of the several selected hypothetical *L. casei* ATCC 334 LPXTG proteins is expressed at both the

mRNA level and the protein level, and thus can be used as an anchor for surface display of a foreign reporter molecule such as GFP in *L. casei* ATCC 334.

This study aims to:

1. Identify hypothetical proteins encoded by the genome *L. casei* ATCC 334 of that have an LPXTG motif and a signal peptide.
2. Determine if any of the several selected LPXTG proteins are expressed at the mRNA level and protein level.
3. Determine if any of the several selected LPXTG proteins are localized on the cell surface.
4. Determine if a knockin mutant of eGFp *L .casei* ATCC334 and the hypothetical protein emits fluorescence when exposed to an external light source.
5. Determine if a knockin mutant of eGFP *L. casei* ATCC334 and the hypothetical protein can surface display the eGFP protein using a LPXTG protein as an anchor.

1.13 Experimental design and plan

Bioinformatics would first be used to search the genome of *L. casei* ATCC334 to identify all LPXTG proteins that could be used as anchors for surface display. The parameters for identifying LPXTG proteins encoded by the genome of *L. casei* ATCC334 are the presence of N-terminal signal peptides and the C-terminal LPXTG motif that may allow for covalent attachment of the protein to the peptidoglycan cell wall via sortase A.
To detect the gene expression, at the mRNA level, reverse transcriptase polymerase chain reaction (RT-PCR) may be performed on the cells once their mRNA transcripts are extracted. PCR primers specific to the predicted open reading frame (ORF) for a hypothetical LPXTG protein would also have to be designed. Complementary DNA

(cDNA) would be synthesized using the random hexamer primers and could be further analysed for the mRNA transcript using conventional PCR.

Then, detecting gene expression at the protein level would be done by western blot analysis of the whole cell extract from *L. casei*. For detection of the protein on the cell surface, immunofluorescence microscopy would be used to detect the target protein on the surface of live bacteria with specific antibodies.

Since the hypothetical LPXTG proteins have not been characterized yet, there are no known antibodies that could react against these hypothetical proteins in the scientific community. Thus, antibodies against the candidate surface proteins would have to be generated. To achieve this, the genes coding for target antigens would have to be cloned into a protein expression vector to facilitate antigen production.

Since all LPXTG protein have a LPXTG motif, cross reaction can occur when raising antibodies against them. The pLIC C-His vector can be used (Dan et al, 2009) to express hypothetical LPXTG proteins that lack both the signal peptide and LPXTG motif, which will eliminate the cross reaction to the LPXTG motif and only produce antibodies against epitopes on the mature protein. The hexa histidine tag is fused at the C-terminal so that purification of the recombinant antigen could be done with affinity chromatography on a Nickel-agarose column (**Figure 1.7**). The recombinant antigens would then be inoculated into rabbits to generate polyclonal antibodies.

A knockin mutant would be generated with a genetic insertion consisting of enhanced GFP (eGFP) to the N-terminal of the target LPXTG gene. The insert would be sandwiched between the original signal peptide and the LPXTG motif to enable proper localization of the fusion protein. Testing for display of eGFP on the cell surface would

be done using fluorescence microscopy while determination of surface localization would be done using immunofluorescence microscopy using an to eGFP.

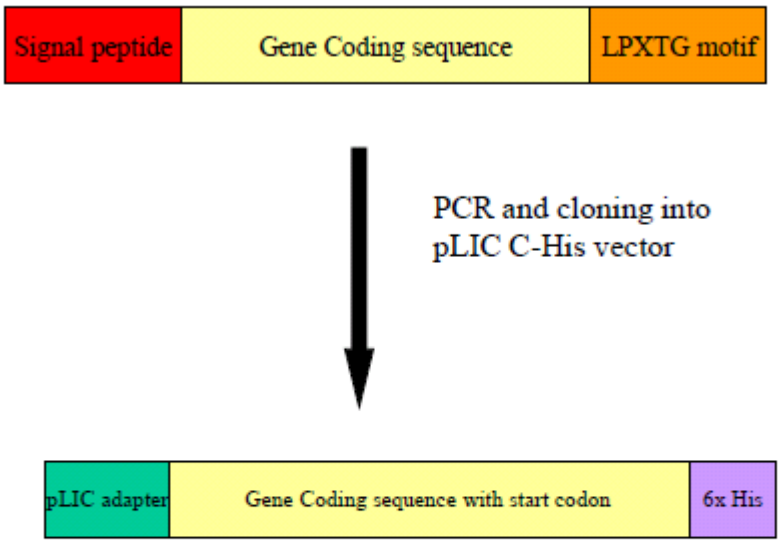


Figure 1.7 Layout of antigen for use in antibody production. The antigen used for antibody production lacks an N-terminal signal peptide and the C-terminal LPXTG motif since they are conserved in most cell wall anchored proteins. The insert also has a hexa His tag at the C-terminal for purification using nickel columns.

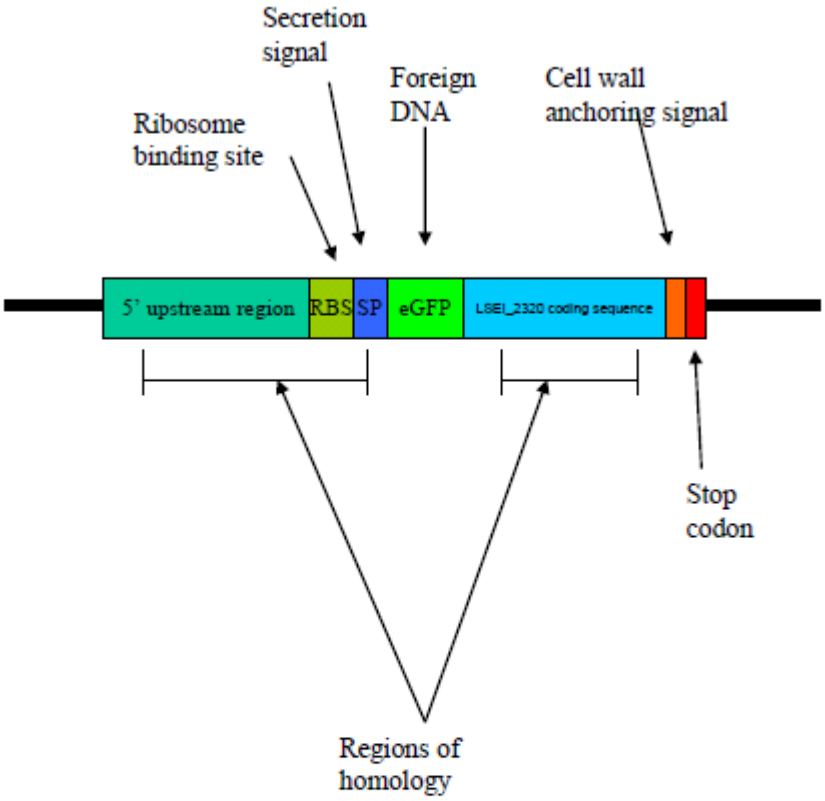


Figure 1.8a Design used for the insertion of eGFP into the LSEI_2320 gene of *L.casei*. The construct is going to contain two regions of homology to allow for RecA mediated recombination. These two regions of homology consist of the a 5' upstream region of about 1000bp that also contains the ribosome binding site (RBS) and signal peptide (SP). The 3' homologous region contains roughly a 1000bp part of the actual LSEI_ 2320 coding sequence. The insertion is going to be a fusion construct using the RBS and SP of LSEI_2320, with the eGFP being inserted right after these N-terminal features.

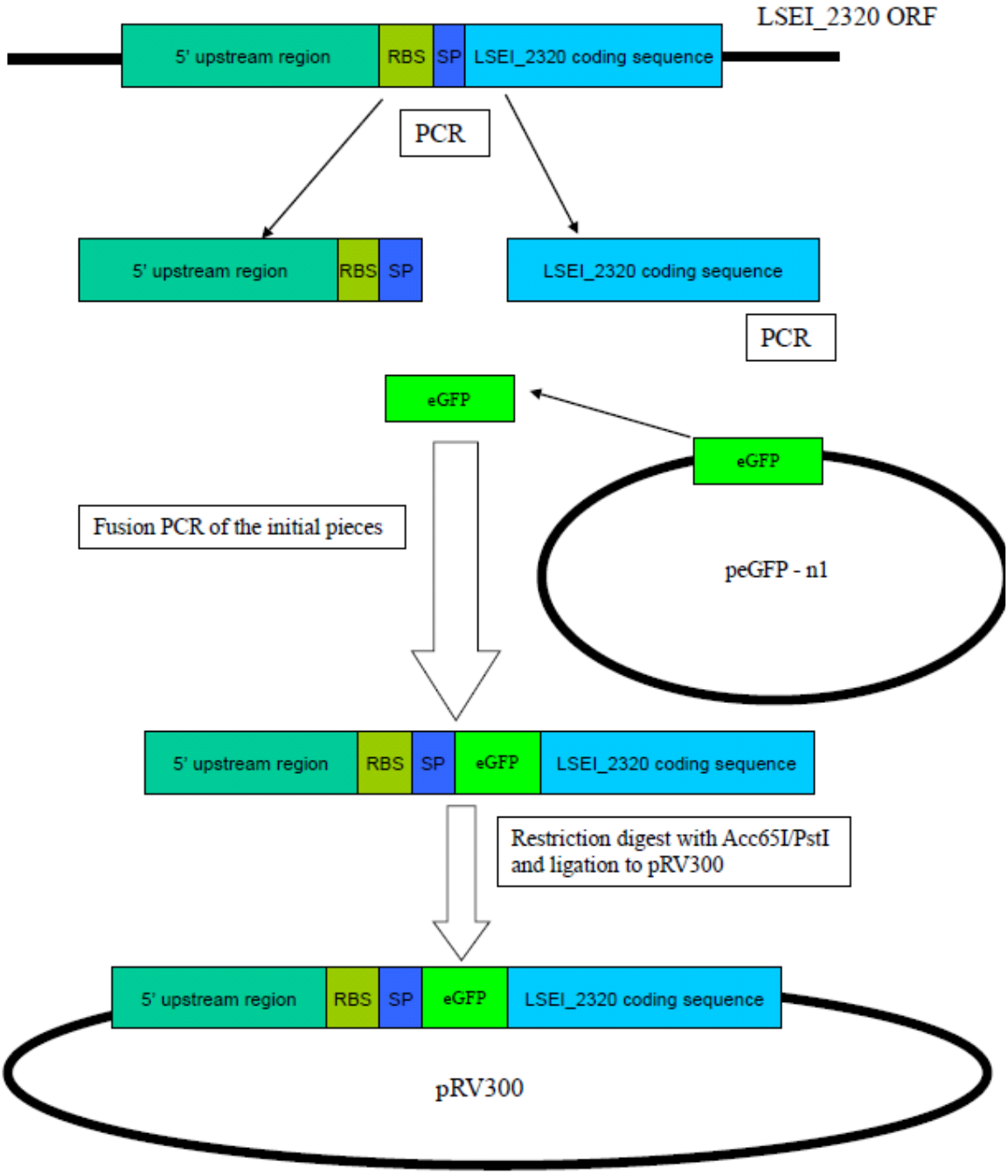


Figure 1.8b Generation of a construct for the insertion of eGFP into LSEI_2320. The insert is first created in parts from polymerase chain reaction (PCR). The three parts are the 2 regions of homology and the eGFP. The pieces are then fused together using PCR and then the insert is inserted into the pRV300 plasmid via directional cloning with Acc65I and PstI.

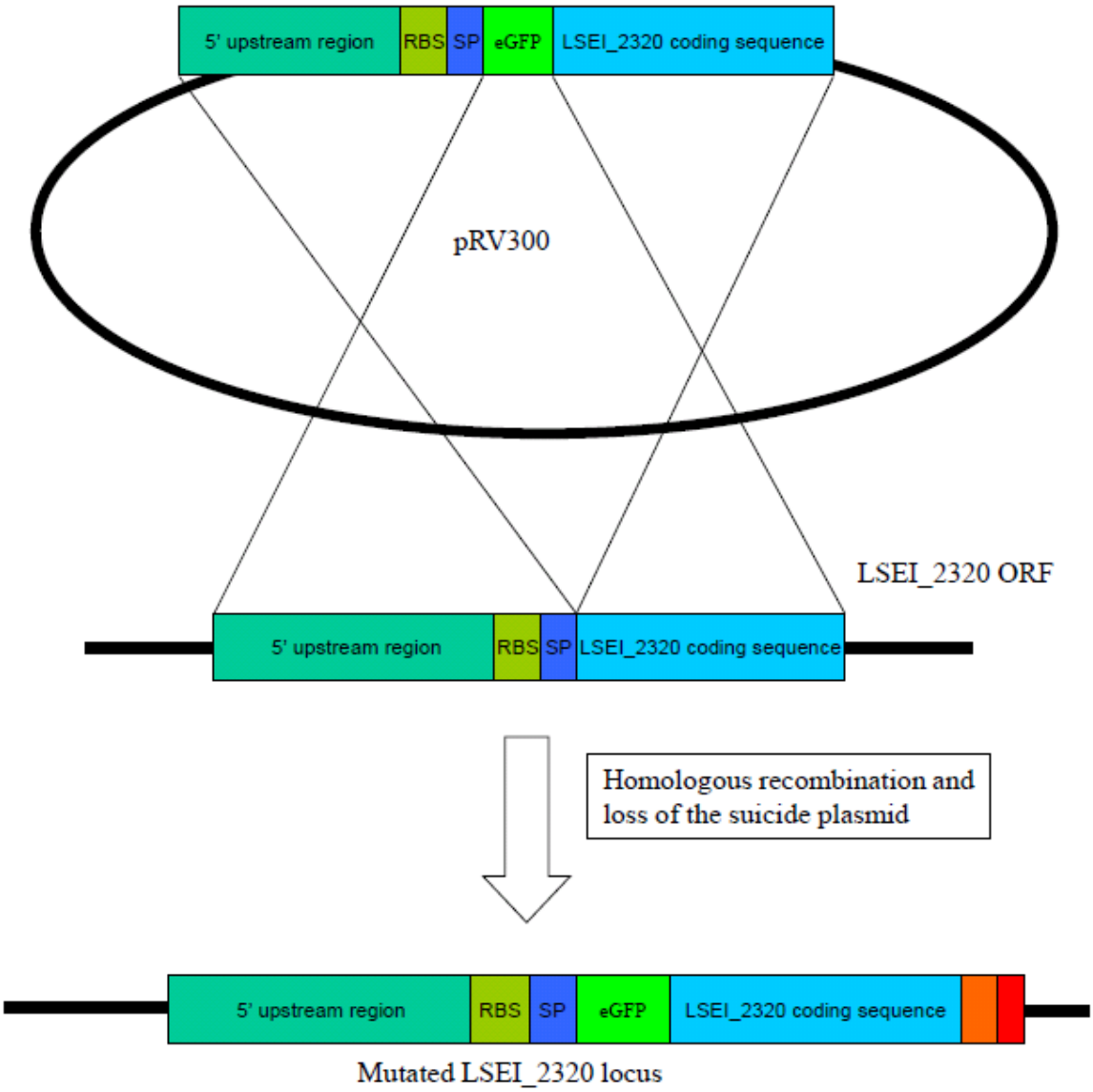


Figure 1.8c Mechanism used to generate the mutated LSEI_2320 Locus. The mutation is called a knock in mutant and occurs via double cross over homologous recombination. The entire pRV300 inserts into the *L.casei* genome during the first cross over event since the plasmid does not have a Gram-positive origin of replication. The second cross over event occurs at the second region of homology between the inserted plasmid and the host DNA. This results in the plasmid being released with a chunk of the homologous host DNA in the (multiple cloning site) MCS region.

Chapter 2: Experimental procedures

2.1 Materials and reagents

The cell types that was used for cloning was *E. coli* strain DH5α, and *E. coli* strains used for protein expression were BL21 (DE3), Rosetta. *L. casei* strains used ATCC334 (American Type Culture Collection, Manassas, VA, USA). The (De Man, Rogosa, Sharpe)MRS broth (Oxoid) was purchased from thermo fisher scientific, Whitby, The Lysogeny broth (LB) was from BD , (Missassauga, ON, Canada). Antibiotics that were used were Chloramphenicol (Sigma, Oakville, ON, Canada) Erythromycin (Fluka, Oakville, ON, Canada), and Kanamycin (Sigma). The anti-His primary antibody was purchased from Qiagen, (Louisville, Kentucky, USA). Horse radish peroxidase (HRP) anti rabbit secondary antibody conjugates from Jackson Immunoresearch, West Baltimore Pike West Grove, Pennsylvania. The Dylight 488 anti-rabbit secondary conjugates were also from Jackson Immunoresearch. Other regents, Skim milk, phenylmethanesulfonyl fluoride (PMSF), EDTA, and Imidazole, were purchased from Sigma, while Coomassie Blue, HRP Staining kit and Bradford reagent were purchased from BioRad (Missassauga, ON, Canada). Ponceu S and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Gold Biotech, (Saint Louis, Missouri, USA). Bovine serum albumin (BSA) and the RACE kit were purchased from Roche, (Laval, QC, Canada). Additional equipment used was semi dry blot from Biorad, and Fast prep (bead beater) from MP Biomedicals. Images were taken with a Olympus BX60 microscope in conjunction with a QImaging RETIGA Exi FAST 1394. The Quantity one program was used for analyzing agarose gels. The DNAstar lasergene 7 software suite

was used for primer design, gene annotation and for sequence alignment. Microscope pictures were processed using QCapture Pro 6.

2.2 Culturing *L. casei* in anaerobic conditions

L. casei ATCC334 was grown in MRS broth and sealed in an Oxoid anaerobic jar. A anaerobic indicator strip from BD was used to determine if the environment was anaerobic (white) or aerobic (blue). To replace the atmosphere in the chamber, the chamber was vacuumed to a pressure of 0.6 bar and then injected with a gas mixture 10% H_2, 10% CO_2 and 80% N_2 until the pressure was at atmospheric pressure again. This process was repeated for 5 times before the bacteria were put in an incubator at 37^{o}C for two days to reach stationary phase.

2.3 Extraction of genomic DNA from *L. casei* AATCC 334

The *L. casei* was grown anaerobically in a sealed jar with a Gas Generating Kit (Oxoid) for 2 days in 6 mL of MRS (Oxioid) media at 37^{o}C. The bacteria were spun down at 13,000 xg on a micro centrifuge in 1 mL batches. Enzymatic treatment using 10 μL of 3,000 U/mL Mutanolysin (Sigma) was carried out at a temperature of 37^{o}C for 30 min in 100 μL of 0.01 M sodium citrate buffer pH 6.7 (for 1 L: 0.2941 g sodium citrate (Sigma), 8.132 g $MgCl_2$, deionised H_2O). The cells were subsequently lysed using 0.5 mL DNAzol (Invitrogen, Burlington, ON, Canada) to extract the DNA. The mixture was spun at 13,000 rpm for 1 min on the micro centrifuge and the precipitate was discarded. The pellet was washed twice with 0.75 mL 75% ethanol. The DNA was air dried and subsequently resuspended in deionised H_2O. The DNA was precipitated with 1.5 mL of ethanol and pelleted again. The yield was verified using a 1% agarose (Sigma) gel run in

1 x TAE buffer at 80 V for 48 min. The DNA was resuspended in 50 μL sterile deionised H_2O.

2.4 Purifying Pfu polymerase

The *E. coli* was grown as per the large scale protein expression protocol (See section 2.13 for details on large scale protein expression) to an OD600nm of 0.3 -0.5. Induction with IPTG (Gold Biotech) was added at a final concentration of 0.5 mM. The induction was done at 37^{o}C for 3 h and then left overnight at 4^{o}C.

The cells were resuspended in 30mL buffer A : (for 1 L pH 7.9, Tris-HCl 6.056 g, 9.008 g dextrose, Ethylene diamine tetraacetic acid (EDTA) 0.3724 g, deionised H_2O with 4 mg/mL Lysozyme (Sigma)), and incubated at room temperature for 15 min. The cell suspension was then mixed with 30 mL of buffer B : (for 1 L : pH 7.9, Tris-HCl 1.2112g, 3.728g KCl, EDTA 0.3724g, deionised H_2O) containing 0.5% Tween 20 (Sigma), and 0.5% (v/v) Nonidet p-40 (Roche)) . The cell suspension was incubated at 75^{o}C for 1 h. The cells were pelleted down at 15,000 rpm at 4^{o}C for 20 min and the supernatant was transferred to a 100 mL flask. Subsequently, 0.04629 g of Dithioreitol (DTT), Sigma, was added to 300 mL of buffer C : (for 1 L pH 7.9: Tris-HCl 6.056 g, deionised H_2O). Then, 7.456 g KCl was added to 100 mL of the buffer C / DTT solution. A Q sepharose column (Amersham Pharmaceuticals, Baie d'Urfe, Quebec) was equilibrated with buffer C at 3-5 times the column volume. Elution was done using a 0 - 0.5M KCl gradient with the buffer C/ DTT solution. Storage solutions were mixed with the following composition: storage solution 1 : (for 1 L pH 8.0: Tris HCl 6.056g, KCl 7.456g, EDTA 0.07456 g, glycerol 500 mL, deionised H_2O) and storage solution 2 : (for 1 L pH 8.0, Tris HCl 6.056 g, KCl 7.456 g, EDTA 0.03724 g, glycerol 750 mL,deionised H_2O) were made so that

they would contain 0.5 mM DTT and 1% Triton X-100. One volume of storage solution 1 was mixed with the cell suspension and then one volume of solution 2 was added to the mixture.

2.5 Selective amplification of the hypothetical surface proteins using PCR

Primers were designed to be compatible with cloning into the pLIC C-His plasmid. Primers (**Table 2.1**) were ordered from Sigma and were used at a working concentration of 2.5 μM. The DNA fragments were amplified by PCR using a lab purified Pfu polymerase. The dNTPs were ordered from Invitrogen, and were used at a working concentration of 0.2 mM. The total reaction volume was 50 μL. The 10x reaction buffer was made as follows (for 1 L pH 8.8: Tris-HCl 20.22 g, $MgSO_4$ 2.408 g, KCl 7.456 g, $(NH_4)_2SO_4$ 13.214 g, deionised H_2O). PCR conditions were as follows: a 94ºC initial denature (2 min) was performed first, followed by 35 cycles of consisting of a 94ºC denature (30 sec), 55ºC annealing (45 sec) and a 72ºC extension (3.5 min). The PCR ended with a 72ºC final extension (10 min).

2.6 Generation of competent *E. coli* with $CaCl_2$ for heat shock protocol

The BL21 (DE) and DH5α strains of *E. coli* were initially isolated by streaking onto LB agar (BD). After overnight growth at 37ºC, a single colony was inoculated into 5 mL of LB broth (BD) and grown overnight at 37ºC with shaking at 225 rpm. The *E. coli* was subcultured at a 1/100 dilution (4 mL in 400 mL) and grown at 37ºC with 225 rpm shaking until the OD 600 reached 0.35 (10^8 cells/mL). The culture was stored in 50 mL tubes and put on ice for 10 min. The cells were pelleted at 2,700 xg for 10 min at 4ºC. The cells were dried by placing the tube on a paper towel after decanting the supernatant. The cells were then resuspended in 24 mL of ice cold solution comprising of 80 mM

Table 2.1 Primers for Ligation independent cloning (LIC)

Primer	Sequence from 5' to 3'. Underlined regions are for LIC cloning.
LSEI_0455 F	TTTAAGAAGGAGATATAAGTC*ATG*AACATTCGCCCA ACCTATCA
LSEI_0455 R	AGTGGTGGTGGTGGTGGTGAGTCGTATGCGGCAAA ATTCAAG
LSEI_1905 F	TTTAAGAAGGAGATATAAGTC*ATG*GCCAGTGACGC TGACGCAAC
LSEI_1905 R	AGTGGTGGTGGTGGTGGTGAGTCGTCTCAGGTAAA TCTTGGA
LSEI_2320 F	TTTAAGAAGGAGATATAAGTC*ATG*GCATCGACATCG CATATTCA
LSEI_2320 R	AGTGGTGGTGGTGGTGGTGAGTCGTATTCGGCAAG TAACGGCT
LSEI_2363 F	TTTAAGAAGGAGATATAAGTC*ATG*GCCGAAAGCCA GGTTGCATT
LSEI_2363 R	AGTGGTGGTGGTGGTGGTGAGTCGTCATCGGCAGC AATGTGCG
LSEI_2431 F	TTTAAGAAGGAGATATAAGTC*ATG*GGCCAGAATATC GCGGTCAC
LSEI_2431 R	AGTGGTGGTGGTGGTGGTGAGTCGTGTCAGGTAGA TGATGGGT
LSEI_2896 F	TTTAAGAAGGAGATATAAGTC*ATG*GGGACACCGAC AATTGATCA
LSEI_2896 R	AGTGGTGGTGGTGGTGGTGAGTCGTTTGCGGTAGC TGATGAGT

$MgCl_2$, 20 mM $CaCl_2$ using a shaker and a tray full of ice for 20 min. The cells were pelleted at 2,700 xg at 4°C for 10 min. The tubes were dried again with a paper towel and the pellet resuspended in 1.6 mL of ice cold 0.1 M $CaCl_2$ in 10% glycerol using a shaker and ice tray. The cells were put in 50 µL aliquots and put into -80°C for long term storage.

2.7 Cloning of hypothetical surface proteins into pLIC c-His

The Ligation independent (pLIC) plasmids (**Figure 2.1**) were purified from the DH5α strain of *E. coli* using the E/Z-10 miniprep kit (Biobasic, Markham, ON, Canada) that follows a rapid alkaline lysis protocol resulting in a yield of about 80-100 µg/mL plasmid DNA per 10 µL of prepped cells. Subsequently, the plasmids were run on a 1% agarose gel to verify amount and quality of the plasmids. For the c-His tagged pLIC plasmids, 50µL of the miniprep was cut as follows: 7 µL NEBuffer 3, 7 µL 10x BSA, 6 µL of 10 U/µL ScaI (New England biolabs, Pickering, ON, Canada). The reaction was done at 37°C overnight and heat inactived at 65°C for 20 min the next day. The cut plasmid was further purified using the Qiagen Qiaquick gel purification kit. To release their staggered ends, 30 µL of the cut plasmid was nibbled with T4 DNA polymerase (New England Biolabs) with the following conditions: 4 µL 10x NEBuffer 2, 4 µL 10x BSA, 1 µL 100 mM dCTP (Invitrogen), 1 µL T4 DNA polymerase (3 U/µL). Treatment of the insert did not require a restriction digest but still had to be nibbled with T4 DNA polymerase as follows: 4 µL 10x NEBuffer 2, 4 µL 10x BSA, 1 µL 100 mM dGTP (Invitrogen), 1 µL T4 DNA polymerase (3 U/µL). Nibbling reactions were run at room temperature for 30 min and heat inactivated at 75°C for 20 min.

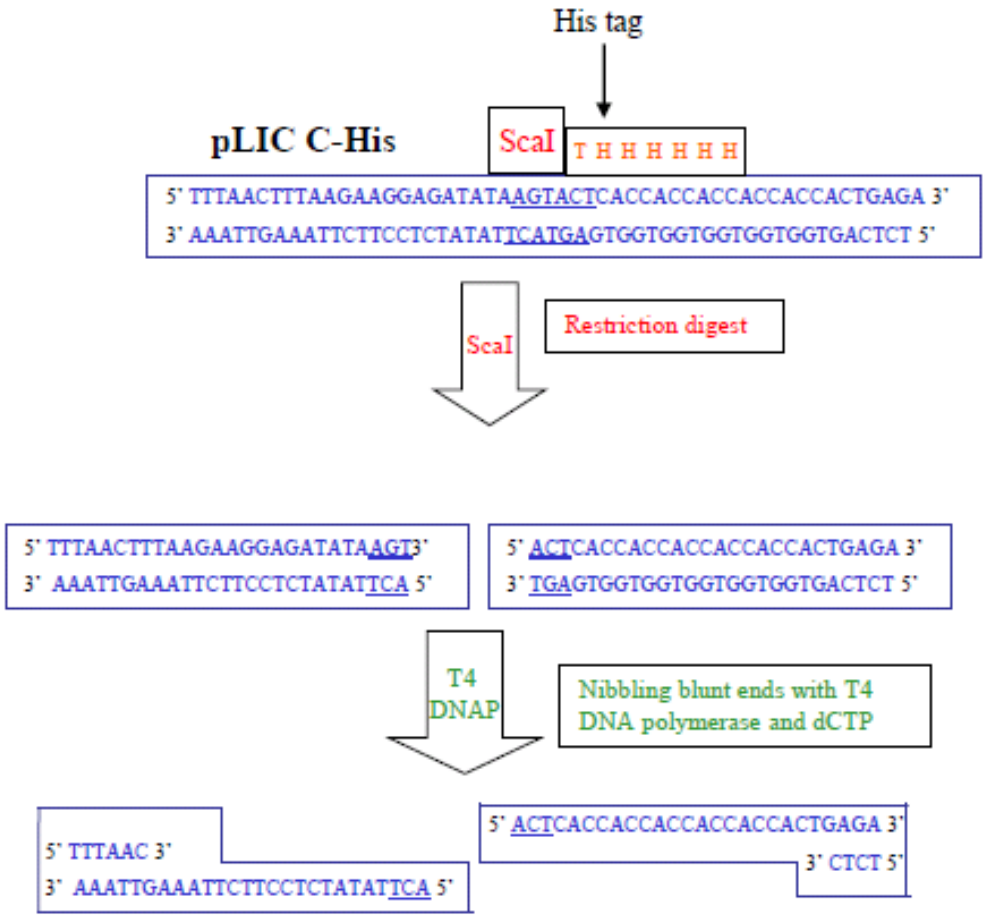


Figure 2.1a Ligation independent cloning part 1. The vector is restricted with ScaI to generate blunt ends. The blunt ends are nibbled with T4 DNA polymerase (T4 DNAP) and deoxy cytosine triphosphate (dCTP) to generate staggered ends.

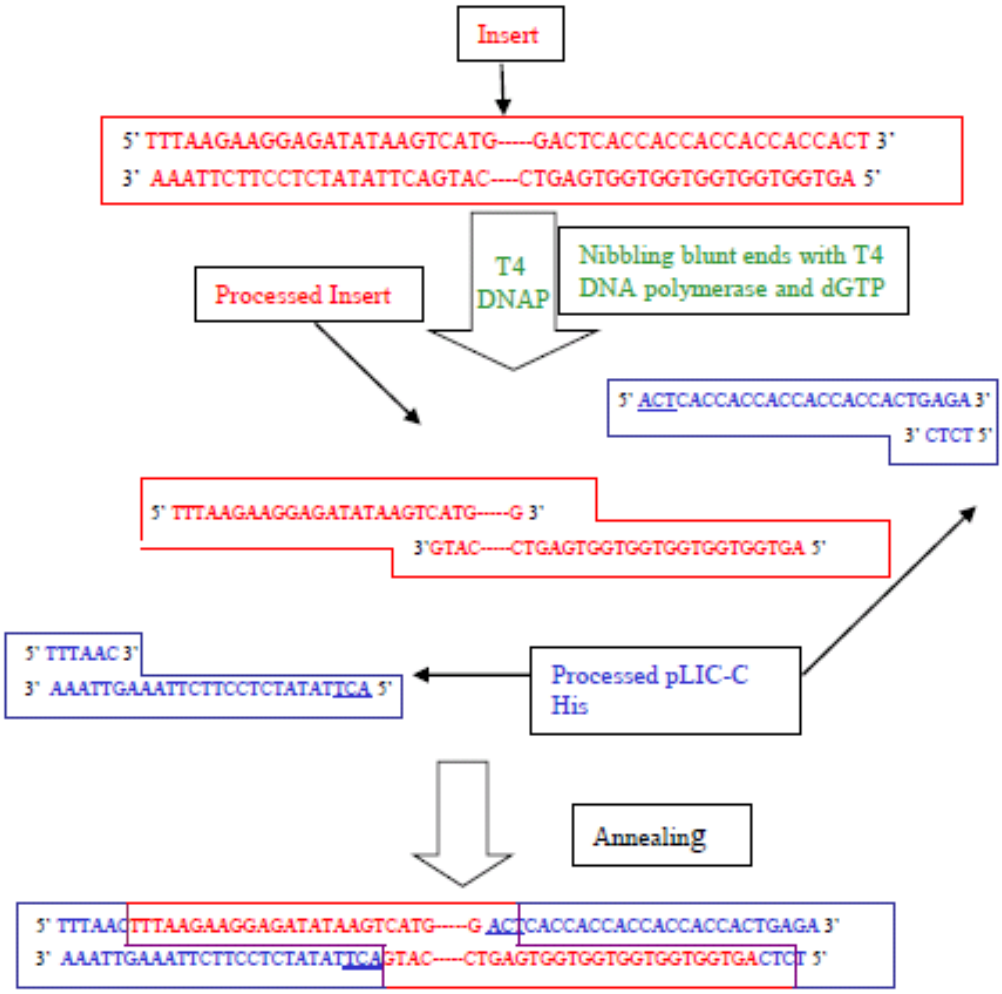


Figure 2.1b Ligation independent cloning part 2. The insert is nibbled with T4 DNA polymerase (T4 DNAP) with guanosine triphosphate (dGTP) to create staggered ends. When the processed inserts are put together they will anneal via hydrogen bonding due to complementary base pairing.

The DNA fragments were first purified in a low melt 1% agarose (Sigma) gel and subsequently extracted with the Qiaquick DNA extraction kit (Qiagen). After the fragments were nibbled to produce staggered ends, the DNA was further purified before being mixed with the trimmed pLIC C-his plasmids. The subsequent mixture (10 µL) was then added to 50 µL of competent *E. coli* DH5α cells. The heat shock procedure used was as follows:. The competent cells and plasmid mixture were incubated on ice for 30 min followed by 42ºC incubation for 45 s. A 2 minute cooling step on ice was added before the bacteria were placed into 250 µL of SOC medium (for 100 mL: 2 g Tryptone, 0.5 g Yeast extract, 0.05 g NaCl, 2 mL 1 M Glucose, deionised H_2O) and grown for 1 h at 37ºC in an incubator with shaking. The cell mixture was then spread on a LB kanamycin (50µg/ml) agar plate and grown overnight at 37ºC to acquire colonies for insert screening. Positive colonies were selected with kanamycin (Sigma) and cultured in LB broth (BD) with kanamycin. Then, they were subjected to a mini prep with the E/Z -10 spin columns. The plasmids were then tested for the presence of insert by PCR and sequencing (McGill University and Génome Québec Innovation Centre, Montreal, QC, Canada).

2.8 Sodium dodecylsulfate Polyacrylamide gel electrophoresis (SDS-PAGE)

The 12% separating gel was made with the following recipe: 4.0 mL 30% Acrylamide/Bis Solution (Biorad), 37.5:1, 1.5 mL Tris-HCl- pH 8.8, 3.35 mL deionised H_2O, 100 µL SDS (Sigma), 50 µL Ammonium persulfate (APS, Biorad) 5 µL Tetraethylenemethylenediamine (TEMED ,Sigma). The solution was loaded into a 0.75 mm wide gel cassette (3.5 mL each) and sealed with isopropanol for 45 min. The 4% stacking gel was prepared using the following recipe: 0.65mL Acrylamide/Bis Solution,

37.5:1, 1.25 mL 0.5M Tris-HCl – pH 6.8, 3.05 mL deionised H_2O, 50 µL 10% SDS, 25 µL 10% APS, 5 µL TEMED. This was added to the separating gel and sealed with a gel comb for 30 min. The gel itself was run at 200 V for 45 min 1x SDS running buffer (for 1L of 10x buffer : 30g Tris, 144g Glycine, 10g SDS, deionised deionised H_2O).

The SDS-PAGE gel was placed into a container containing 10mL of Coomassie Brilliant Blue R-250 (Biorad) and put on a shaker for 1 h. The staining solution was removed and 10mL destain solution (for 1 L: 400 mL methanol, 100 mL acetic acid, 500 mL deionised H_2O) was added. This was left on the shaker for 30 min and the destain solution was changed. The solution was left on the shaker for 3h. The gel was then rinsed 3 times with water and subsequently dried/preserved.

2.9 Protein transfer to a nitrocellulose membrane via semi-dry transfer cell

The protein transfer buffer (for 1L: 5.822g Tris, 2.93g Glycine, deionised H_2O) was warmed up to room temperature. The SDS-PAGE gel was placed on top of the presoaked nitrocellulose membrane (Biorad) and blotting paper. The transfer itself was run at 15 V for 30min. Verification of protein transfer was done with Ponceu S (Gold Biotech) staining using 5mL for 5min and washes with H_2O.

2.10 Western blotting with HRP staining

The Ponceu S (Gold Biotech) stained membrane was put in 10mL of 3% skim milk/1x Phosphate buffered saline (PBS) – Tween 20, Triton X-100 (TT) (recipe for 10 L of 1x PBS-TT : 400 mL 25x PBS, 5 mL Tween 20, 20 mL Triton X-100, deionised H_2O ; recipe for 1 L 25x PBS : 27.5 g dibasic sodium phosphate, 7.88 g monobasic sodium

phosphate, 212.5 g sodium chloride, deionised H_2O). The membrane was put on a shaker for 1 h at room temperature. The membrane was then washed with 10mL of 1x PBS-TT for 3 min. The primary antibody was added to 10mL 3% BSA, 1x PBS-TT and the solution was added to the membrane. Incubation was for 1 h at room temperature on the shaker. The membrane was subjected to five washes were perfomed using 10mL 1x PBS-TT for 3min. Afterwards, the secondary antibody with an HRP conjugate (Jackson Immunoresearch) is added to the 10mL of 3% BSA/ 1x PBS-TT. The membrane is then incubated for 1 h at room temperature on the shaker. The membrane was washed 5 times again and the HRP colour development substrate (Biorad) was added. The substrate composition was as follows : 9mL H_2O, 1mL 10x colour development buffer, 2mL colour Reagent A, 60µL Color Reagent B. The colour development kit was purchased from Biorad. The membrane was placed into the colour solution for 10 min with shaking at room temperature before being rinsed with water.

2.11 Small scale expression of hypothetical surface protein antigens

The pLIC vectors were transformed into the BL-21 strain of *E. coli* using the heat shock method (see above section 2.7 for the details on the heat shock method of transformation).

Cells were then grown in 5 mL of LB with kanamycin (Sigma) at 50µg/mL overnight at 37ºC at 225rpm shaking in an incubator. The cells were subcultured at 1/100 (2µL culture into 20mL of LB / 50 µg/mL kanamycin) at 37ºC at 225rpm shaking until an Optical density (OD590) of 0.6 was reached. 1mL of uninduced sample was saved while the remaining 18mL had 20 µL of 1M Isopropyl-β-D-1-thiogalactospyranoside (IPTG), (GoldBiotech), added to begin induction. The induced sample was incubated at 37ºC with

225 rpm shaking. With the exception of LSEI_1905 all the other samples were harvested after 3 h. For LSEI_1905, the sample was kept overnight at 4ºC before being harvested. The samples were pelleted at 13,000 xg for 1 min. Samples were then concentrated using PBS to an equivalent of an OD590 of 20. Then 2x SDS sample buffer (for 20 mL: 4 mL 0.5 M Tris HCl pH 6.8: 8 mL Glycerol, 4 mL β mercaptoethanol (Sigma), 0.8 g SDS, 800 µL 0.5% Bromphenol blue, 3.2 mL deionised H_2O) was added and the cells were lysed by boiling for 10 min.

The small scale protein extract was then separated on a 12% SDS PAGE gel. After transferring to a nitrocellulose membrane (Biorad) and Ponceu S (Gold Biotech) staining, the protein was blocked with 3% skim milk in PBS-TT before being incubated with Mouse anti penta-His antibodies (Qiagen) at a 1:1000 dilution. The secondary antibody was a goat anti mouse antibody conjugated to horse radish peroxidase (HRP), (Jackson), at a 1:1000 dilution. Colour development was done using the HRP substrate colour development kit (Biorad).

2.12 Large scale purification of the hypothetical surface protein antigens

The cells were grown overnight at 37ºC with 225 rpm shaking in 6mL of LB broth with 50µg/mL kanamycin (Sigma). The cells were subcultured at a 1:100 (5mL in 500mL) dilution of LB / kanamycin 50µg/mL at 37ºC with 225rpm shaking until an OD590 of at least 0.6 was reached. Induction was done with 503uL of 1M IPTG (GoldBiotech) at 37ºC with 225rpm shaking for 3 h. LSEI_1905 had to be left at 4ºC overnight before harvesting. The cells were frozen at -20ºC after being centrifuged at 8,000xg for 20min at 4ºC. 1L of cell pellets were resuspended in 20mL of PBS and 1mM

of protease inhibitor Phenylmethylsulfonylfluoride (PMSF, Sigma). Cells were kept on ice before being lysed with a French press at 1500 lb/in^2 at least three times. After cellular lysis the solution was centrifuged at 27,000xg at 4^{o}C and the soluble fraction was kept for purification using nickel column chromatography.

The Ni-NTA resin (Qiagen) was loaded into a Econo column (Biorad) of 1cm diameter to a height of 2cm.The column was equilibrated with 25mL of wash buffer pH 8.0(for 1L : 6.9g monobasic sodium phosphate monohydrate, 17.4g NaCl, 1.36g imidazole (Sigma) , deionised H_2O). The proteins were first loaded into the column resin and washed with the washing buffer. Elution was done using a high concentration imidazole buffer (for 1L: 6.9g monobasic sodium phosphate monohydrate, 17.4g NaCl, 17g imidazole, deionised H_2O). After the elution the protein fractions were combined for analysis by SDS-PAGE. The proteins were then concentrated using a filter (molecular weight cutoff 30 kDa) and then quantified using the Bradford assay.

2.13 Bradford assay

The protein assay dye reagent concentrate (Biorad) was warmed to room temperature and a set of standards were prepared with BSA stock solution (Pierce, Whitby, ON, Canada) diluted to 0.1 mg/mL. The concentration of BSA standards used are as follows: 0 (Blank),1µg/mL , 2.5 µg/mL , 5 µg/mL , 7.5 µg/mL , 10 µg/mL and 12 µg/mL . The standards were brought up to 800 µL before 200 µL of the dye was added to each standard creating a final volume of 1 mL. Protein samples were prepared as follows: Protein added : 5 µL, 10 µL and 20 µL. These were mixed with water to get a sample volume of 800 µL before 200 µL dye reagent was added. The samples were then

incubated at 10 min at room temperature before the OD595 was taken. The concentrated protein was quantified using an Absorbance vs. Concentration curve plotted by the protein standards via the Beer - Lambert Law.

2.14 Preparation of chemically competent *E. coli* with $CaCl_2$ for transformation

Female white New Zealand rabbits (Charles River, Senneville, QC, Canada) were injected subcutaneusly with 100 µg of the antigen in 0.5 µL PBS mixed with 0.5 µL Incomplete Freund's adjuvant (Sigma). The inoculation was done on days 0 with booster inoculations on days 14 and 28. Pre-immune bleeds for the rabbits were collected before the injection of antigen to act as a negative control. A test bleed was done on day 42 and the serum was collected after a positive reaction to the antigen using Western blotting. The blood was harvested from the rabbits at day 46. The serum was isolated by spinning the blood at 3,000 xg on the centrifuge for 20 min and collecting the supernatant. The pre-immune bleeds and the harvested antibodies were then blotted with the antigens they were raised against to confirm that only the harvested antibodies could bind to their respective antigen targets.

2.15 Screening for the hypothetical surface proteins using polyclonal antibodies

L. casei was grown in MRS (Oxiod) anaerobically at 37ºC for 2 days before harvesting. Extraction of *L. casei* whole cell proteins was done using treatment with mutanolysin (see section 2.3) before using a bead beater with lysing matrix B (MP Biomedicals, Solon, OH, USA). Disruption of cellular membrane was done using 2x SDS sample buffer with boiling at 10 min. The tubes were then spun down at 13,000rpm to remove the beads and cellular debris from the soluble protein.

The proteins were then loaded into a 12% SDS-PAGE gel and separated based on molecular weight. The proteins were then transferred to a Nitrocellulose membrane (Biorad) and then incubated with a 1:1,000 dilution of the polyclonal antibodies after blocking with 3% skim milk (BD) in PBS-TT. The secondary antibodies were HRP conjugated goat anti-rabbit (Jackson Immunoresearch). These were then incubated and subsequently stained using a HRP substrate kit (Biorad).

2.16 Immunofluorescence screen of live *L. casei* ATCC334 cells

L. casei ATCC334 was grown in 50 mL of MRS anaerobically at 37^{o}C for 2 days. 2 mL of cells were spun down with a micro centrifuge and subsequently resuspended in 500 µL of 5% BSA in PBS for blocking with gentle shaking for 1 h. The primary incubation was performed using the rabbit polyclonals generated from section **2.15** at a 1:2,000 dilution in 5% BSA in PBS, incubated for 1 h and washed twice with 500 µL of blocking solution before incubation with the 2^{o} antibody. Goat anti-rabbit monoclonal antibodies that were conjugated with Dylight 488 were used as secondary antibodies (Jackson immunoreserch). These were incubated at a 1:1,000 dilution in 5% BSA (Roche) in 250 µL PBS with shaking for 1 h. The sample was then washed 3 times with 500 µL PBS. Subsequently, the pellet was resuspended in 50 µL PBS. 5 µL of sample was added to a microscope slide for viewing. The cover slip and slides were sealed using cytoseal 60. The NB filter and the condenser setting PH3 were used for all images. The magnification used was 100x with an oil immersion. Images were captured at 16 µs for phase contrast and 5 s for the fluorescence using an Olympus BX60 microscope.

2.17 mRNA transcript screen of *L. casei* ATCC334

mRNA extraction from *L. casei* was facilitated by first growing the *L. casei* ATCC334 for 2 days anaerobically in MRS at 37°C. The bacteria were subcultured at a 1:10 dilution anaerobically for 1 h at 37°C in MRS. Then, 500 µL of cells were pelleted down and treated with 1 mL of RNA protect (Qiagen) in RNase free tubes followed by a 5 min incubation after mixing by vortex. The resulting mixture was then spun at 500 xg in the microfuge for 10 min. The pellet was then resuspended in 100 µL of 0.01 M sodium citrate buffer pH 6.7 (see section 2.3 for more details) before being treated with mutanolysin to weaken the cell wall. The mixture was then vortexed and left at 37°C for 30 min. 7 µL β-mercaptoethanol (Sigma) was added to buffer RLT (RNeasy kit, Qiagen). This mixture was added to the *L. casei* spheroplasts and vortexed for 10 s. The cells were then placed into lysing matrix B (MP Biomedicals) and lysed with the FastPrep bead beater machine (MP biomedicals) at setting 6 for 40 s. The extraction kit used was the RNeasy kit as per the manufacterer's instructions. Once extracted, the RNA was treated with RNase free DNAse three times to remove any genomic fragments left over. The RNA was then run on a 1% agarose gel and TAE buffer made with RNase free water (For 1 L: deionised H_2O 1 L, Diethylpyrocarbonate (DEPC) (Sigma), incubated at 37°C overnight and autoclaved to remove DEPC). For the reverse transcription, the ThermoScript kit (Invitrogen) was used. The RNA sample was first denatured at 65°C for 5 min with the following reagents: 1 µg RNA, 2 µL 10 mM dNTPs, 1 µL of 50 ng/µL random hexamers resulting in a final volume of 8 µL. The reagents were then cooled on ice for 5 min. The cDNA mix was prepared as follows : 4 µL of 5x cDNA synthesis buffer, 1 µL of 0.1 M DTT, 1 µL of RNase Out, 1 µL of RNase free water, 1 µL of

ThermoScript RT (15 U/μL). The denatured sample was then added to this and incubated at 50ºC for 35 min. The reaction was ended with heating at 85ºC for 5 min. The original template was destroyed with 1 μL of RNase H at 37ºC for 20 min. The PCR was then carried out using gene specific primers. PCR conditions are as follows: one 94ºC initial denature (2 min), followed by 35 cycles consisting of a 94ºC denature (30 sec), 55ºC annealing (45 sec), 72ºC Extension (1.5 min). A 72ºC Final extension (10 min) was carried out. See **Table 2.2** for the list of primers used for RT PCR.

2.18 Creation of a pRV300 suicide plasmid for double cross over recombination

The pRV300 plasmid was provided by Dr. Josef Deutsher from AgroParis Tech in Thiveral-Grignon. France.pRV300 was grown in 10 mL of 150 μg/mL erythromycin(Fluka) LB(BD) broth for replication in *E. coli* overnight at 37ºC with 225 rpm shaking. The plasmid extraction was done using the E/Z-10 miniprep protocol with 10 mL of culture. The plasmid was then digested using Acc65I (New England Biolabs) and PstI (New England Biolabs) restriction endonucleases before being purified using a Qiaquick gel purification kit (Qiagen) using a 1% low melt agarose gel. The PCR amplification for the insert was performed using the following primers from Sigma: 5' GCCGACTAATGGGCCCATGGTGAGCAAGGGCGAGGA 3' and 5'CCGTTGCT TGAAGCTTCTTGTACAGCTCGTCCATGC3' for the eGFP PCR product using pEGFP-N1 as a template. 5' TGCTCACCATGGGCCCATTAGTCGGCGTTGTTCC AG 3' and 5'ACAGGTACCCGTCGATATTCGTCAAGCAA3' were used to amplify the upstream recombination region with the signal peptide using *L. casei* genomic DNA as a template. Primers 5' GTGCTGCAGTAACTACTTTTCCCGACGAC 3' and

Table 2.2 Primers used for RT PCR

Primer	Sequence
16srRNA F	5' AGCTATCGCTTTTGGATGGA 3'
16srRNA R	5' CATTTCACCGCTACACATGG 3'
0455 F	5' TACCATGGCACAAAGGACAA 3'
0455 R	5' AATCCCAGTGCGATACCAAG 3'
1905 F	5' TCAACCTGAGATGAGCGATG 3'
1905 R	5' GAGCATTCGCTTGTTTGTCA 3'
2320 F	5' AGCAAGAAACTGGCGTGAAT 3'
2320 R	5' CGTCAACAGTTCCTTGAGCA 3'
2363 F	5' GTCAAGATGGGTTGGCAGTT 3'
2363 R	5' CGTTTACCGGCATACTTCGT 3'
2431 F	5' ATACAAGTCGTGGGCTGGTC 3'
2431 R	5' ACCGTCACTTGCGGTTTATC 3'
2896 F	5' GATGGCACAACGTACACTGG 3'
2896 R	5' CAATGCATCAGCAGCAATCT 3'

5’GCTGTACAAGAAGCTTCAAGCAACGGTATTGAAACA3’ were used to amplify the other recombination site using *L. casei* genomic DNA as the template. These three PCR products were then fused together using the following primer pairs via PCR: 5’ ACAGGTACCCGTCGATATTCGTCAAGCAA 3’ and 5’ GTGCTGCAGTAACTA CTTTTCCCGACGAC 3’. See T**able 2.3** for the full list of primers. The fused PCR piece was then digested with Acc65I (New England Biolabs) and PstI (New England Biolabs).

2.19 Immunofluorescence staining for the detection of 2320 eGFP mutants for surface display of eGFP using LSEI_2320 as an anchoring protein

First both the wild type and the mutant *L. casei* were grown anaerobically at 37^{o}C in 50 mL MRS for about 6 days to reach an OD600 of 1.8 that represented stationary phase. Then, 250 µL of the bacteria were pelleted at 13,000 xg and resuspended in 500 µL 1x PBS 5% BSA (Roche). A single batch of both the wild type and the mutant was then viewed using fluorescence microscopy to test for intrinsic fluorescence (5 µL of a 1:50 dilution in 1X PBS). Phase contrast pictures were taken with an exposure time of 16 ms using the Ph3 condenser while fluorescence images were taken with a NB filter and the exposure was done at 5 sec.

The other batches were then put on a shaker for 1 h at room temperature. The primary antibody was then added at a 1:500 dilution (1 µL of either the preimmune serum for LSEI_2320, the polyclonal antibodies for LSEI_2320 or the anti-eGFP rabbit antibody from Life Technologies, Burlington,ON, Canada). The incubation was at room temperature. Afterwards the cells were pelleted at 13,000 xg and washed twice with PBS. 250 µL of PBS 5% BSA (Roche) was mixed with a 1:50 dilution of goat anti-rabbit Dylight 488 conjugated antibody (Jackson Immunoresearch) and resuspended with the

Table 2.3 Primers used for cloning into the pRV300 suicide plasmid

Primer	Sequence The underlined regions are cut site for the corresponding restriction endonuclease while the bold regions correspond to the DNA sequence of LSEI_2320
eGFP F	5'GCCGACTAATGGGCCC**ATGGTGAGCAAGGGCGAGGA** 3' 2320 5' overlap/ ApaI cut site
eGFP R	5'CCGTTGCTTGAAGCTT**CTTGTACAGCTCGTCCATGC** 3' 2320 3' overlap/ HindIII cut site
5' recombination segment F	5'TGCTCACCATGGGCCC**ATTAGTCGGCGTTGTTCCAG** 3' GFP overlap/ ApaI cut site
5' recombination segment R	5'ACAGGTACC**CGTCGATATTCGTCAAGCAA** 3' Acc65I cut site
3' recombination segment F	5'GTGCTGCAG**TAACTACTTTTCCCGACGAC** 3' PstI cut site
3' recombination segment R	5'GCTGTACAAGAAGCTT**CAAGCAACGGTATTGAAACA**3' GFP overlap/ HindIII cut site

cells. The mixture was put on a shaker for 1 h at room temperature. The cells were pelleted and washed with 500 µL 1X PBS for 3 times. The cells were then resuspended in 50 µL PBS and a 1:50 dilution of this was made. 10 µL of this solution was subjected to immunofluorescence microscopy as described above.

Chapter 3: Results

3.1 Genome-wide screen for potential LPXTG proteins as surface display anchors

The complete genome sequence of *L. casei* ATCC334, publicly available, was explored to identify ORFs (Open reading frames) coding for proteins containing LPXTG motifs using the CW PRED algorithm (http://bioinformatics.biol.uoa.gr/CW-PRED/) (Fimereli et al 2012). This algorithm detects cell wall anchoring LPXTG motifs near the C-terminus of hypothetical surface proteins. Additionally, the protein candidates identified by CW PRED were scanned with the Signal P algorithm (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al. 2011), to detect signal peptides that allow protein translocation across the cytoplasmic membrane using the SecA pathway. In total, 16 hypothetical proteins, referred to as LSEI_0291, LSEI_0340, LSEI_0455, LSEI_0465, LSEI_0468, LSEI_0564, LSEI_0607, LSEI_0615, LSEI_1905, LSEI_2270, LSEI_2320, LSEI_2363, LSEI_2364, LSEI_2365, LSEI_2431, and LSEI_2896, were detected in the *L. casei* genome (**Table 3.1**).

3.2 Expression constructs for selected LPXTG proteins

It was not known if the 6 selected LPXTG candidate proteins, LSEI_0455, LSEI_1905, LSEI_2320, LSEI_2363, LSEI_2431, and LSEI_2896, could be expressed on the cell surface and serve as anchors for the surface display of foreign proteins. To probe the expression of these LPXTG proteins, expression constructs for these proteins

Table 3.1 Bioinformatics screen of the *L. casei* ATCC334 genome for potential LPXTG proteins using the CW pred and SignalP algorithms. Proteins selected as potential anchors are highlighted in yellow.

Gene Locus tag	Length of ORF (nt)	Signal Peptide position	Molecular Weight (kDa)	Description
LSEI_0291	569	32-33	61 kDa	Lacto-N-biosidase
LSEI_0340	302	None	34 kDa	Mg2+ and Co2+ cotransporter
LSEI_0455	909	40-41	97 kDa	Von willebrand factor domain, Collagen binding
LSEI_0465	1488	None	158 kDa	Hypothetical
LSEI_0468	1637	None	174 kDa	Membrane associated subtilisin – like serine protease
LSEI_0564	999	40-41	107 kDa	β-fructosidase (levanase/invertase)
LSEI_0607	125	26-27 / 31-32	13 kDa	Hypothetical
LSEI_0615	119	31-32	13 kDa	Hypothetical
LSEI_1905	531	38-39	56 kDa	Hypothetical
LSEI_2270	1902	33-34	200 kDa	lactocepin I, Serine peptidase, MEROPS family S08A
LSEI_2320	423	40-41 / 41-42	44 kDa	Hypothetical
LSEI_2363	519	34-35	55 kDa	Collagen Binding
LSEI_2364	440	27-28	48 kDa	Hypothetical
LSEI_2365	79	None	8 kDa	Hypothetical
LSEI_2431	611	40-41	64 kDa	Collagen binding
LSEI_2896	1094	20-21/ none	114 kDa	Adhesion exoprotein

were generated by PCR amplification of the genes for the production of recombinant antigens against which polyclonal antibodies can be raised, using the primer sets identified in **Table 2.1**. The sizes of the PCR product were about 2.6kbp, 1.5kbp, 1.3kbp, 1.5kbp, 2.0kbp, and 3.0kbp, respectively (**Figure 3.1**) corresponds approximately to the calculated sizes for the genes (LSEI_0455: 2613bp, LSEI_1905: 1905bp, LSEI_2320: 1078bp, LSEI_2363: 1390bp, LSEI_2431: 1651bp, and LSEI_2896: 3145bp). Since the proteins coded by these genes have regions of homology in the LPXTG motif, these anchoring signals must be excluded during cloning to prevent immunological cross reactivity to the LPXTG motif. The gene fragments were cloned into the expression vector pLIC C-His (Dan et al. 2009), yielding expression constructs for C-terminally hexa-histidine tagged proteins.

3.3 Expression and purification of recombinant LPXTG proteins

The recombinant proteins were expressed in the cytoplasm by cloning each of the 6 selected LPXTG protein genes into the pLIC C-His vector transformed into the *E. coli* BL21 (DE3), rosetta. Western blot analysis with an anti-His MAb revealed that all six LPXTG proteins were fully expressed. The sizes of the recombinant proteins estimated by Western blot analysis were approximately 116 kDa for LSEI_0455, 100 kDa for LSEI_1905, 90 kDa for LSEI_2320, 95 kDa for LSEI_2363, 100 kDa for LSEI_2431, and 150 kDa for LSEI_2896 (**Figure 3.2 A & B**). The apparent molecular weights of recombinant proteins are larger than their calculated sizes of 90 kDa, 50 kDa, 38 kDa, 50 kDa, 58 kDa, and 109 kDa, respectively. SDS PAGE analysis of the recombinant proteins purified using a nickel-agarose revealed that proteins migrated to a position close to the calculated size (**Figure 3.2 C**). Purified LPXTG proteins were used to raise polyclonal

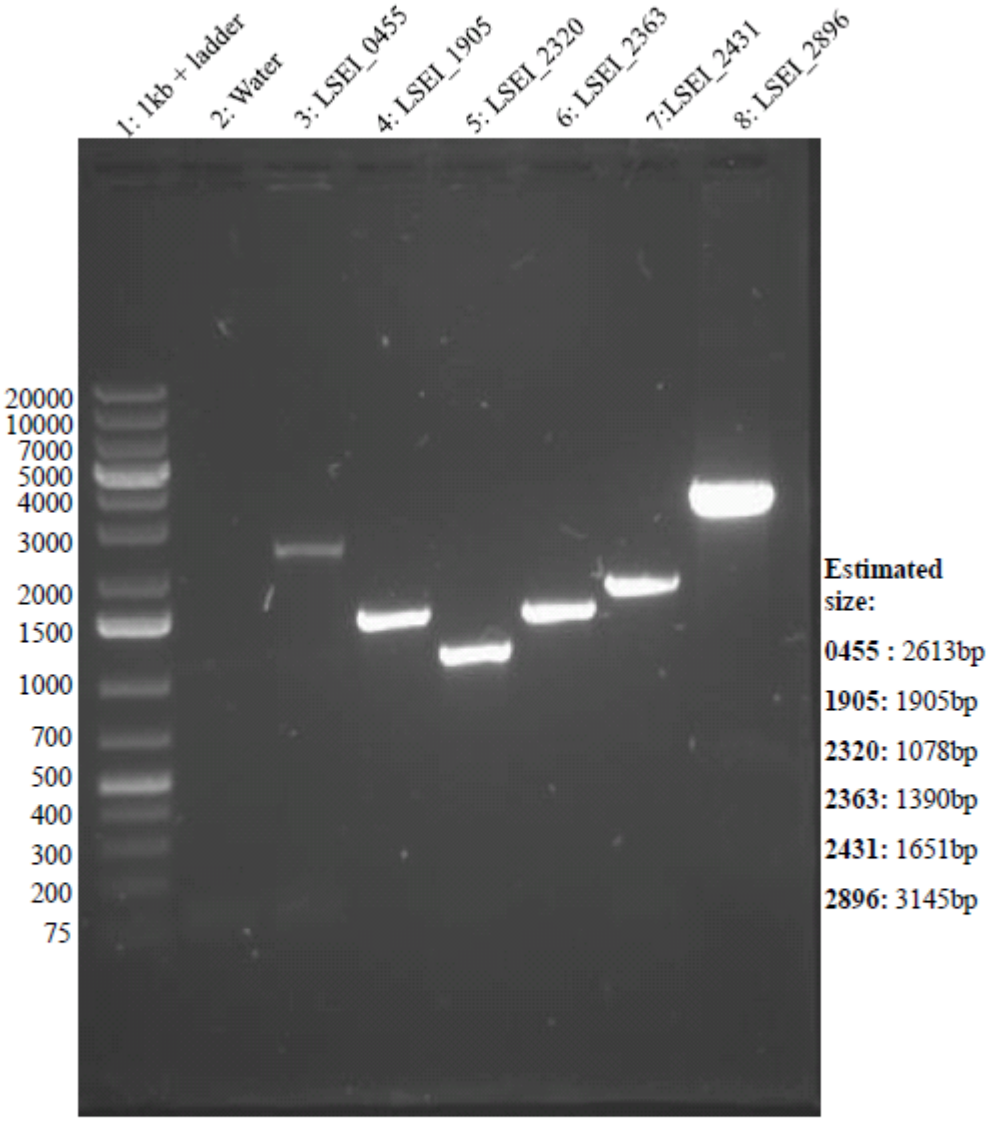


Figure 3.1 PCR amplification of six potential LPXTG protein genes from *L. casei* ATCC334 genomic DNA. The DNA samples were run on a 1% agarose gel using a SYBR® safe stain and visualized using UV light. Lane 1: 1kb plus ladder (Fisher), Lane 2: negative control for environmental contamination with water as a template, Lane 3: amplified DNA product with LSEI_0455 primers, Lane 4: amplified DNA product with LSEI_1905 primers, Lane 5: amplified DNA product with LSEI_2320 primers, Lane 6: amplified DNA product with LSEI_2363 primers, Lane 7: amplified DNA product with LSEI_2431 primers, Lane 8: amplified DNA product with LSEI_2896 primers.

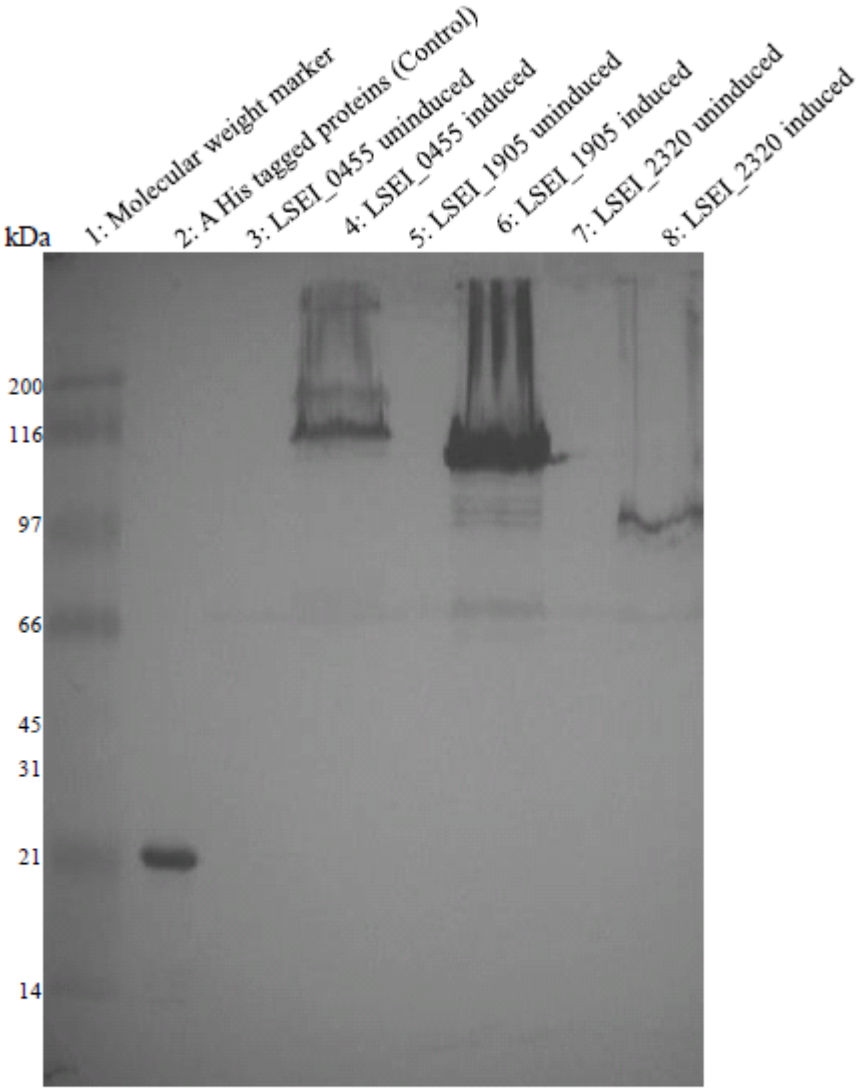


Figure 3.2a Expression of 3 LPXTG proteins in *E.coli* BL21 (DE3) Rosetta. The expressed proteins were run on a 12% SDS-PAGE gel, transferred onto a nitrocellulose membrane and then probed with a mouse monoclonal Anti-Penta-His antibody (Qiagen). Lane1: Protein molecular weight markers (Fischer), Lane 2: His tagged protein (positive control), Lane 3: uninduced LSEI_0455 cell lysate, Lane 4: induced LSEI_0455 cell lysate, Lane 5: uninduced LSEI_1905 cell lysate, Lane 6: induced LSEI_1905 cell lysate, Lane 7: uninduced LSEI_2320 cell lysate, Lane 8: LSEI_2320 cell lysate.

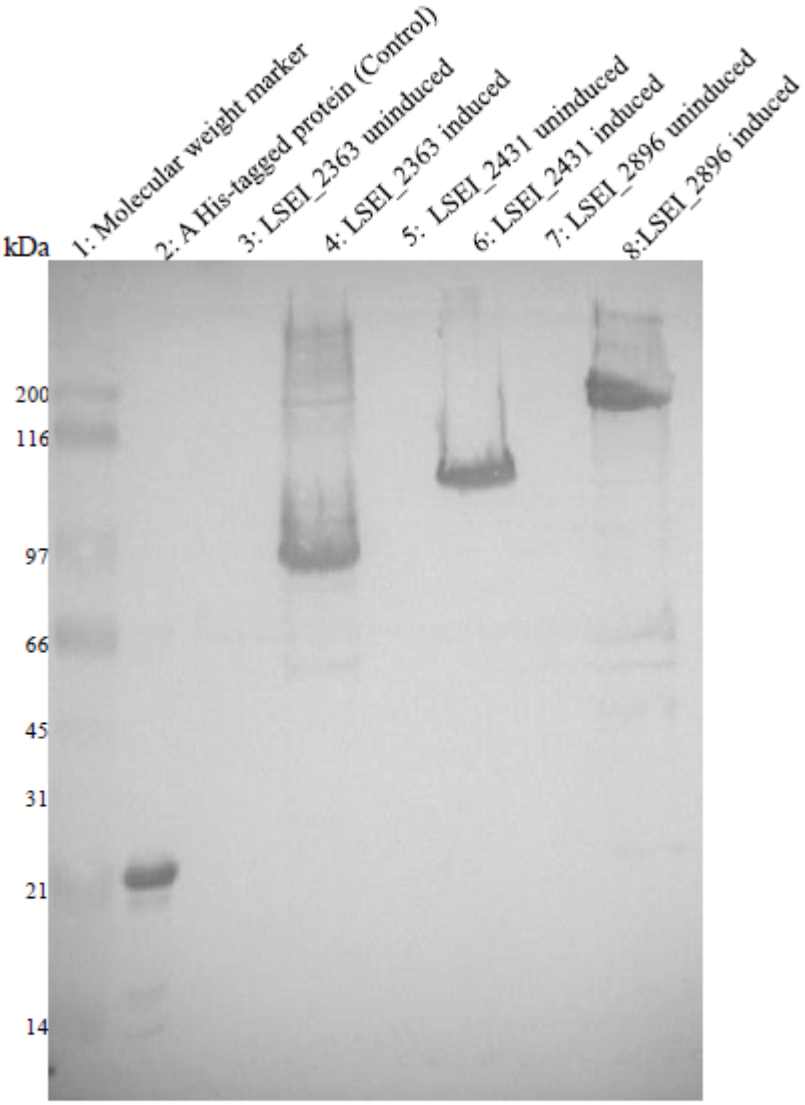


Figure 3.2b Expression of 3 LPXTG proteins in *E. coli* BL21(DE3) and Rosetta strains. The proteins were run on a 12% SDS-PAGE gel, transferred onto a nitrocellulose membrane and then probed with a mouse monoclonal Anti-Penta-His-antibody (Qiagen). Lane1: Protein molecular weight markers (Fischer), Lane 2: His tagged protein (positive control), Lane 3: uninduced LSEI_2363 cell lysate, Lane 4: induced LSEI_2363 cell lysate, Lane 5: uninduced LSEI_2431 cell lysate, Lane 6: induced LSEI_2431 cell lysate, Lane 7: uninduced LSEI_2896 cell lysate, Lane 8: induced LSEI_2896 cell lysate.

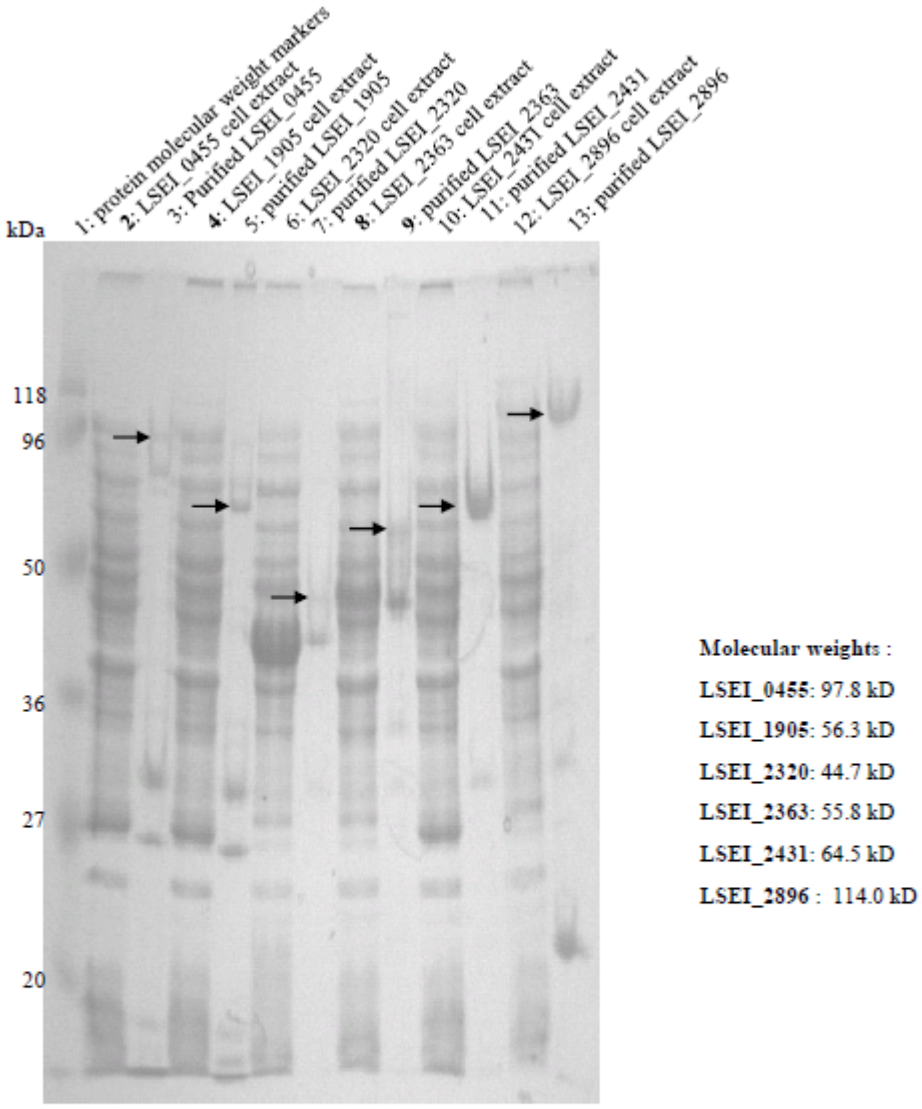


Figure 3.2c SDS-PAGE analysis of the recombinant LPXTG proteins purified by nickel agarose affinity chromatography. The Proteins were run on a 12% SDS-PAGE gel and subsequently stained with the Coomassie blue stain (Biorad). Lane1: protein molecular weight marker (Fischer), Lane 2: unpurified LSEI_0455 sample., Lane 3: purified LSEI_0455 sample., Lane 4: unpurified LSEI_1905 sample., Lane 5: purified LSEI_1905 sample., Lane 6: unpurified LSEI_2320 sample., Lane 7: purified LSEI_2320 sample., Lane 8: unpurified LSEI_2363 sample., Lane 9: purified LSEI_2363 sample., Lane 10: unpurified LSEI_2431 sample., Lane 11 purified LSEI_2431 sample., Lane 12: unpurified LSEI_2896 sample., Lane 13: purified LSEI_2896 sample.

antibodies in rabbits, which were confirmed to recognize their respective purified recombinant proteins by Western blot analysis.

3.4 Expression of LPXTG proteins in *L. casei.*

The whole cell protein extracts of *L. casei* were analysed by Western blots probed rabbit antiserum to target proteins. Out of the 6 protein candidates, only 4 of were detected in the denaturing assay, (LSEI_0455, LSEI_1905, LSEI_2320 and LSEI_2431)(**Figure 3.3A & B**), with bands appearing close to the predicted size of 89 kDa, 49 kDa, 37 kDa, and 57 kDa, respectively. To further determine whether these candidate proteins are surface exposed, live *L. casei* were probed with specific polyclonal antibodies followed by immunofluorescence staining. The immunofluorescence microscopy results revealed that only LSEI_2320 is surface-exposed **(Figure 3.4 A-F)**. In addition, the LSEI_2320 transcript was detected 1 h after subculture by using RT PCR (**Figure 3.5**).

3.5 Generation of a pRV300 construct for egfp knockin to the LSEI_2320 gene locus

Since the LSEI_2320 protein was shown to be highly expressed on the surface of *L. casei* ATCC334, this locus was targeted for egfp knockin to create a *L. casei* mutant that would express and display the eGFP-LSEI-protein the cell surface. As shown in **Figure 1.8**.Two DNA fragments (~1kb each) derived by PCR from the genomic region containing the *L. casei* gene LSEI_2320 locus were fused together with eGFP and cloned into the suicide vector pRV300. The recombinant plasmid, designated pEGFP-2320 was confirmed to caontain a correct insert by PCR and DNA sequencing. These two 1kb regions would act as regions of homology for a double cross over event following the transformation of L. casei with pEGFP-2320. The eGFP coding sequence is inserted right after the signal peptide coding sequence, creating a fusion with the LSEI_2320 gene also

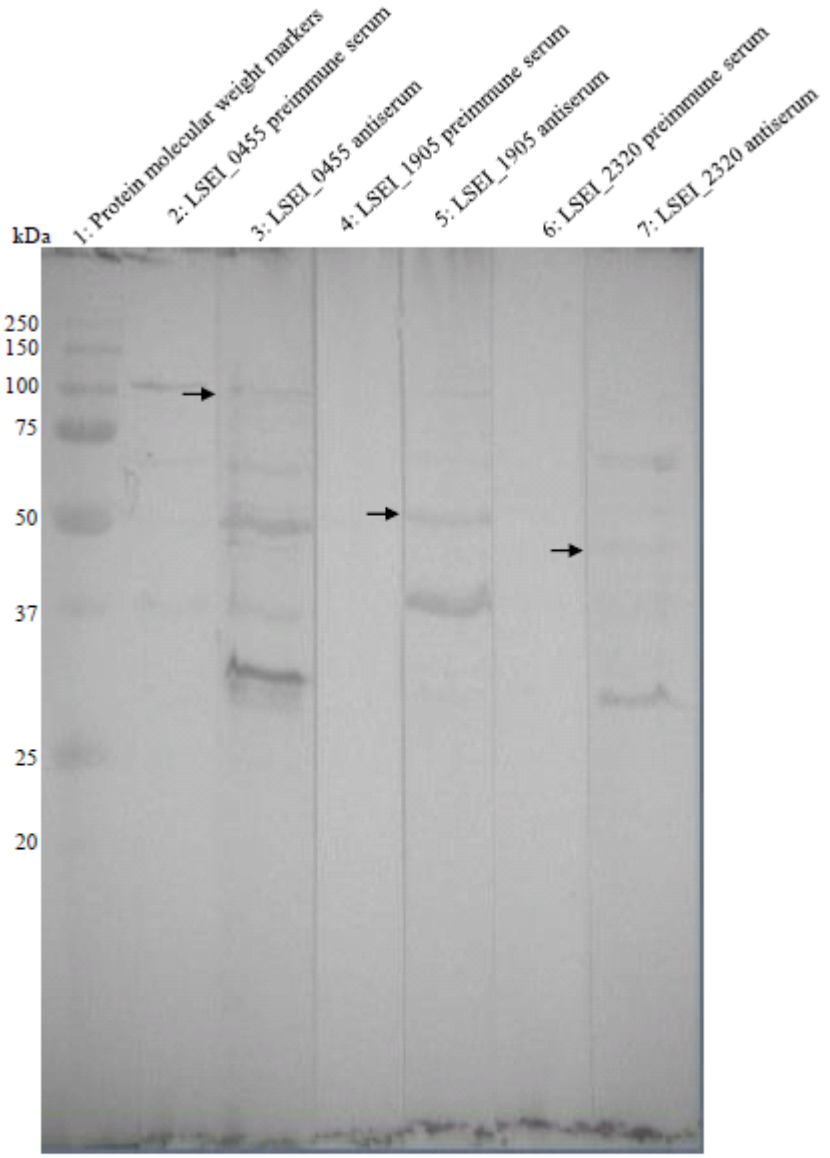


Figure 3.3a Western blot analysis of the expression of the LPXTG proteins in *L. casei* ATCC334. The protein extracts from *L. casei* ATCC334 were run on a 12% SDS-PAGE gel, transferred onto a nitrocellulose membrane and then probed with rabbit polyclonal antibodies to a target LPXTG protein. Lane1: Protein molecular weight markers (Biorad), Lane 2: probed with LSEI_0455 preimmune serum (negative control), Lane 3: probed with LSEI_0455 antiserum, Lane 4: probed with LSEI_1905 preimmune serum (negative control), Lane 5: probed LSEI_1905 antiserum, Lane 6: probed with LSEI_2320 preimmune serum (negative control), Lane 7: probed with LSEI_2320 antiserum.

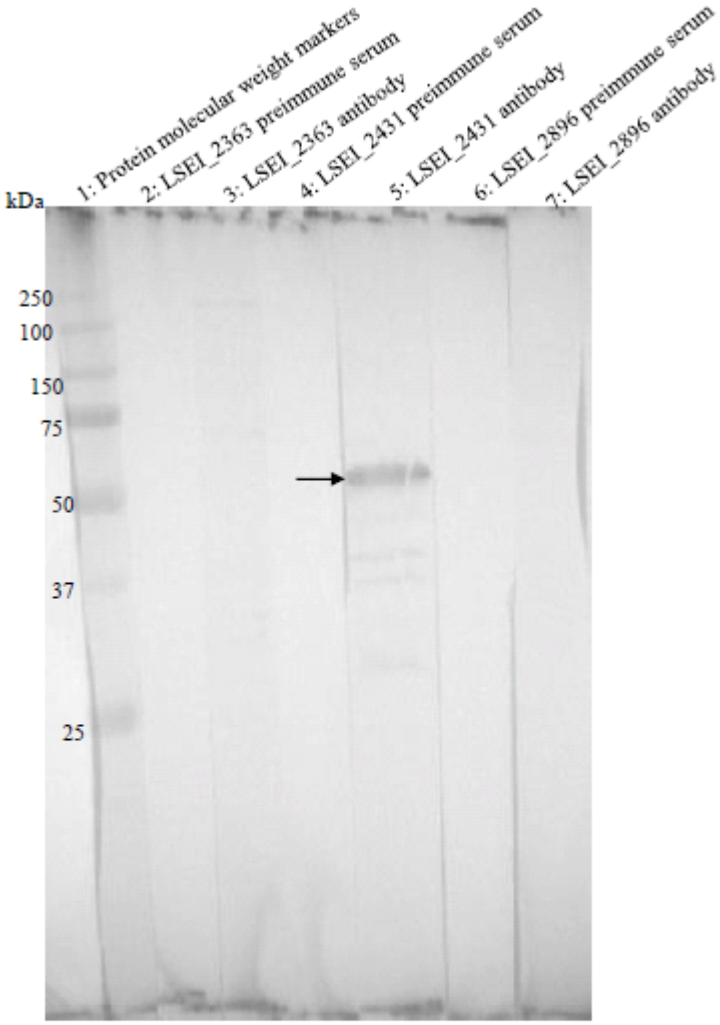


Figure 3.3b Western blot analysis of the expression of the LPXTG proteins in *L. casei* ATCC334. The Proteins were run on a 12% SDS-PAGE gel, transferred onto a nitrocellulose membrane and then probed with rabbit polyclonal antibodies against to a target LPXTG protein. Lane1: Protein molecular weight marker (Biorad), Lane 2: probed with LSEI_2363 preimmune serum (negative control), Lane 3: probed with LSEI_2363 antiserum, Lane 4: probed with LSEI_2431 preimmune serum (negative control), Lane 5: probed with LSEI_2431antiserum, Lane 6: probed with LSEI_2896 preimmune serum (negative control), Lane 7: probed with LSEI_2896 antiserum.

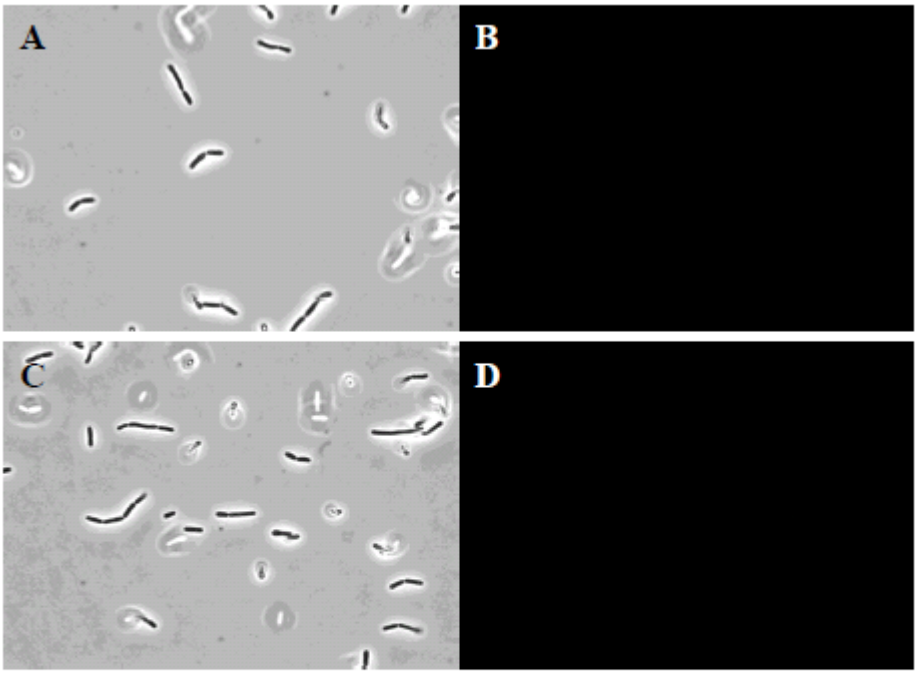


Figure 3.4a Detection of surface expression of LSEI_0455 by immunofluorescence staining. (A) Phase contrast image of *L. casei* probed with LSEI_0455 preimmune serum (negative control). (B) *L. casei* probed with LSEI_0455 preimmune serum. (C) Phase contrast image of *L. casei* probed with LSEI_0455 antiserum. (D) Fluorescent imaging of *L. casei* probed with LSEI_0455 antiserum. Both the fluorescent images (A versus B; C versus D) of the bacterial cells in the same field of view are recorded for comparison.

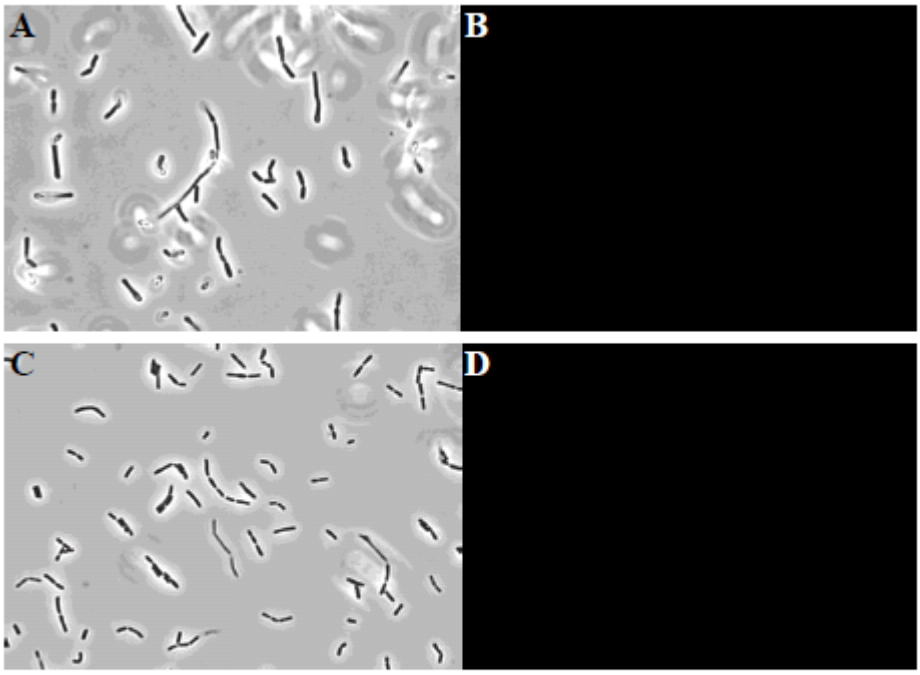


Figure 3.4b Detection of surface expression of LSEI_1905 by immunofluorescence staining. (A) Phase contrast image of *L. casei* probed with LSEI_1905 preimmune serum (negative control). (B) *L. casei* probed with LSEI_1905 preimmune serum. (C) Phase contrast image of *L. casei* probed with LSEI_1905 antiserum. (D) Fluorescent imaging of *L. casei* probed with LSEI_1905 antiserum. Both the fluorescent images (A versus B; C versus D) of the bacterial cells in the same field of view are recorded for comparison.

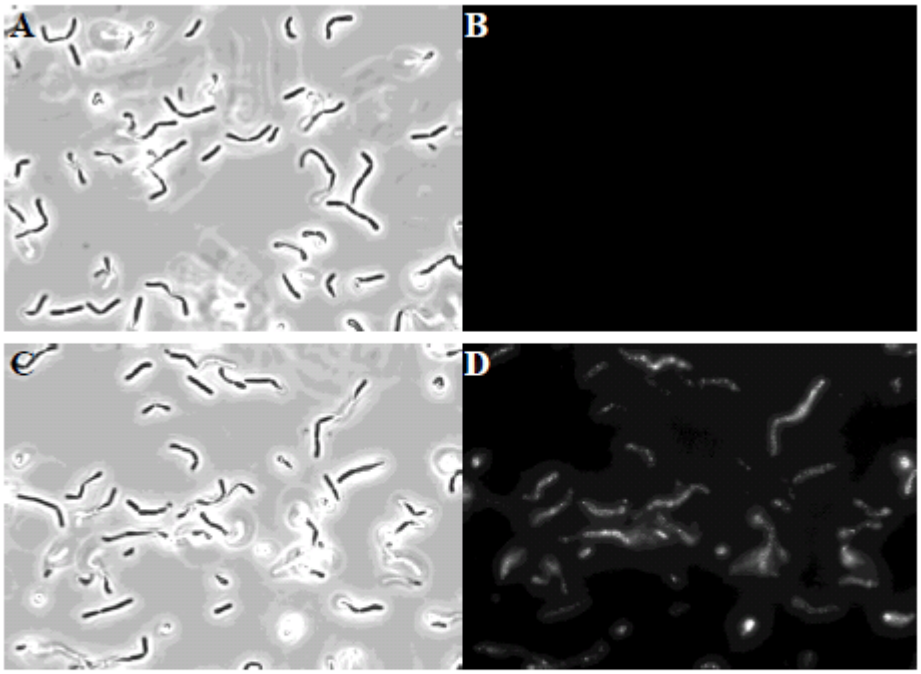


Figure 3.4c Detection of surface expression of LSEI_2320 by immunofluorescence staining. (A) Phase contrast image of *L. casei* probed with LSEI_2320 preimmune serum (negative control). (B) *L. casei* probed with LSEI_2320 preimmune serum. (C) Phase contrast image of *L. casei* probed with LSEI_2320 antiserum. (D) Fluorescent imaging of *L. casei* probed with LSEI_2320 antiserum. Both the fluorescent images (A versus B; C versus D) of the bacterial cells in the same field of view are recorded for comparison.

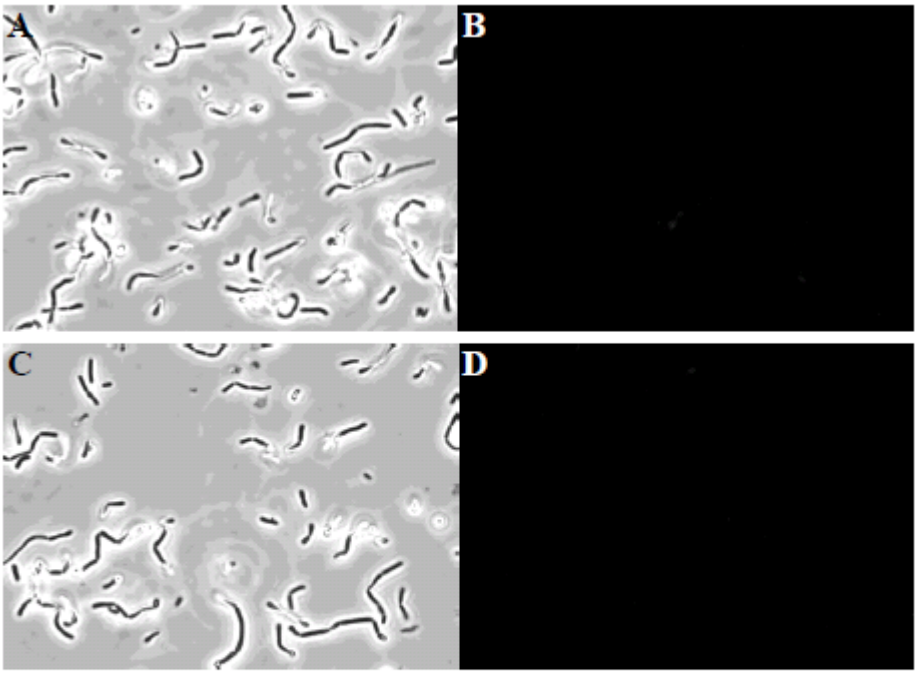


Figure 3.4d Detection of surface expression of LSEI_2363 by immunofluorescence staining. (A) Phase contrast image of *L. casei* probed with LSEI_2363 preimmune serum (negative control). (B) *L. casei* probed with LSEI_2363 preimmune serum. (C) Phase contrast image of *L. casei* probed with LSEI_2363 antiserum. (D) Fluorescent imaging of *L. casei* probed with LSEI_2363 antiserum. Both the fluorescent images (A versus B; C versus D) of the bacterial cells in the same field of view are recorded for comparison.

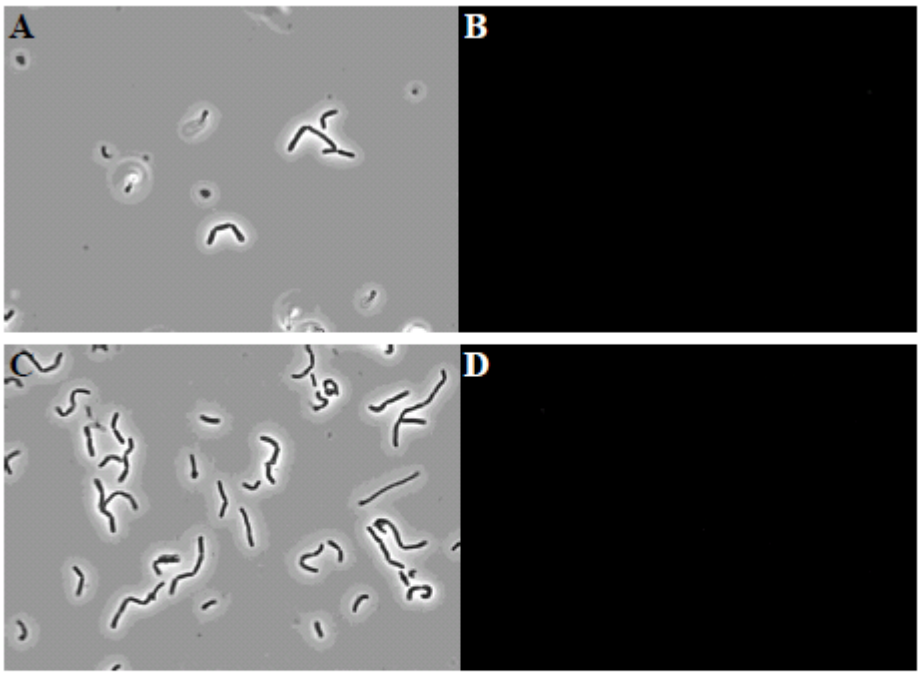


Figure 3.4e Detection of surface expression of LSEI_2431 by immunofluorescence staining. (A) Phase contrast image of *L. casei* probed with LSEI_2431 preimmune serum (negative control). (B) *L. casei* probed with LSEI_2431 preimmune serum. (C) Phase contrast image of *L. casei* probed with LSEI_2431 antiserum. (D) Fluorescent imaging of *L. casei* probed with LSEI_2431 antiserum. Both the fluorescent images (A versus B; C versus D) of the bacterial cells in the same field of view are recorded for comparison.

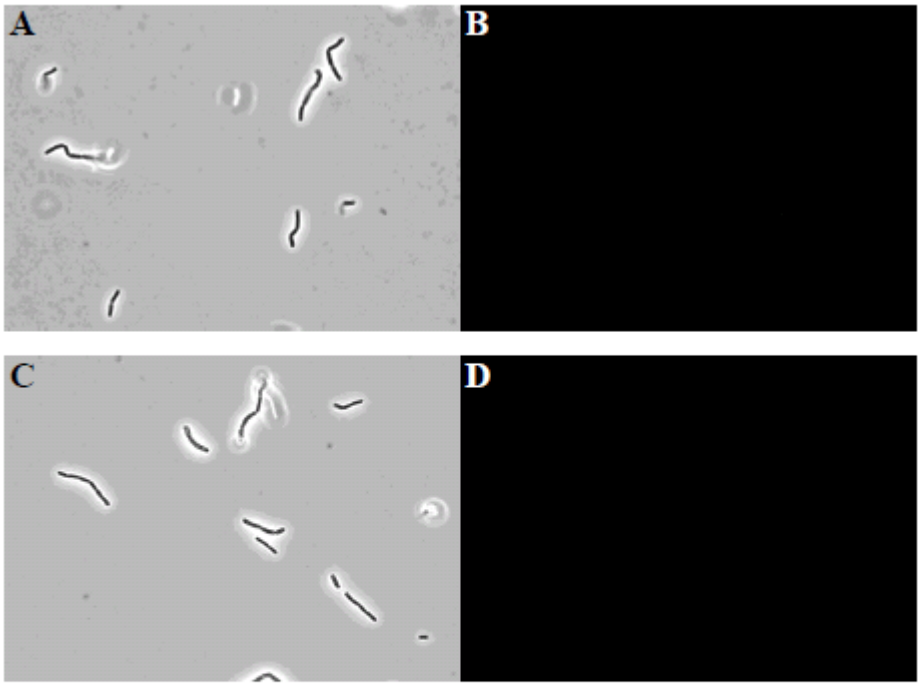


Figure 3.4f Detection of surface expression of LSEI_2896 by immunofluorescence staining. (A) Phase contrast image of *L. casei* probed with LSEI_2896 preimmune serum (negative control). (B) *L. casei* probed with LSEI_2896 preimmune serum. (C) Phase contrast image of *L. casei* probed with LSEI_2896 antiserum. (D) Fluorescent imaging of *L. casei* probed with LSEI_2896 antiserum. Both the fluorescent images (A versus B; C versus D) of the bacterial cells in the same field of view are recorded for comparison.

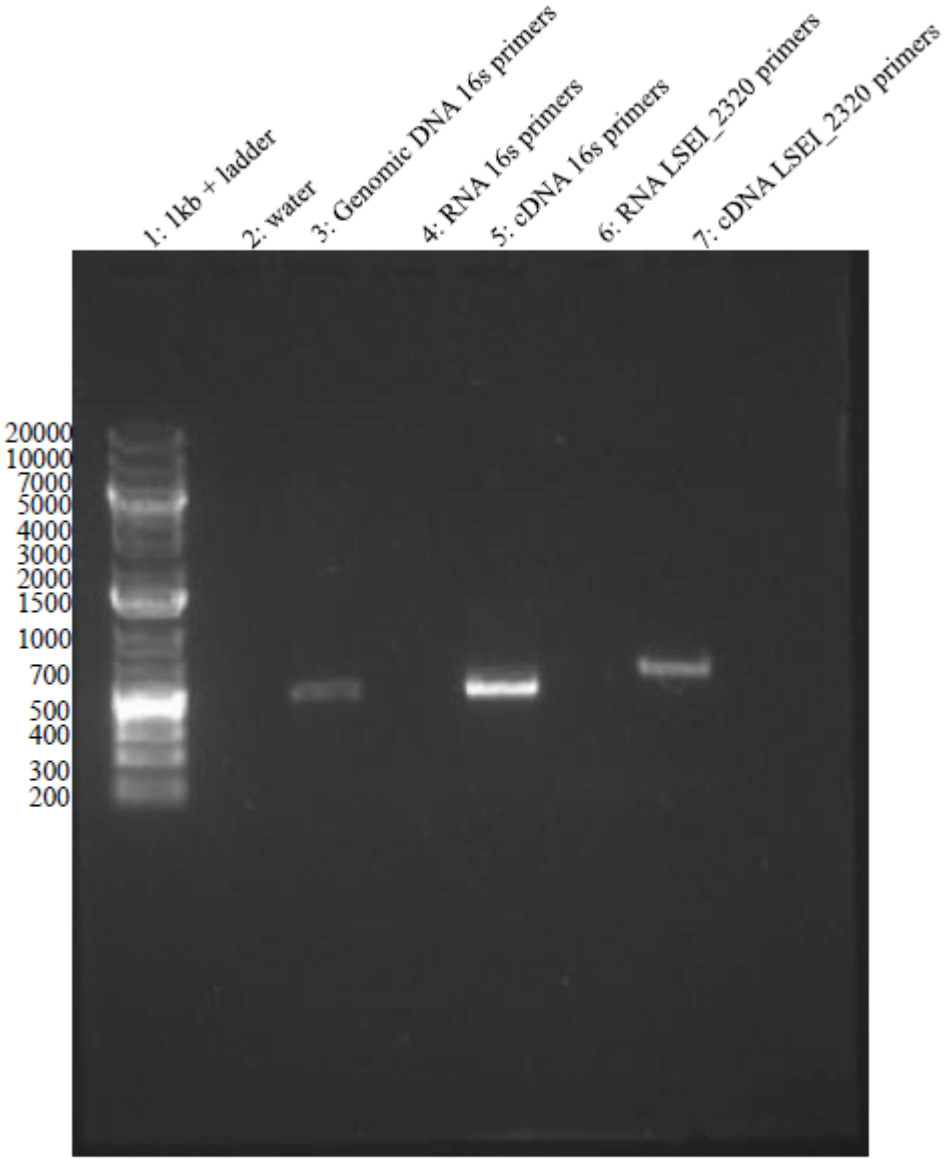


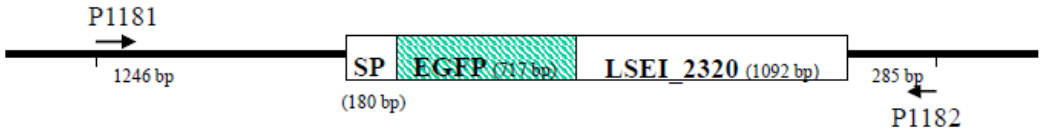
Figure 3.5 Detection of the mRNA transcript of LSEI_2320 genes in *L. casei*. The DNA was run on a 1% agarose gel using a SYBR® safe stain and visualized using UV light. Lane 1: 1kb plus ladder (Fisher), Lane 2: negative control for environmental contamination with water as a template, Lane 3: genomic DNA with 16s rRNA primers, Lane 4: RNA with 16s rRNA primers, Lane 5: cDNA with 16s rRNA primers, Lane 6: RNA with LSEI_2320 primers, Lane 7: cDNA with LSEI_2320 primers

surface display of the eGFP at the cell surface. This would allow the expressed eGFP to be displayed on the surface using LSEI_2320 as an anchor.

3.6 Generation and analysis of an eGFP knockin mutant of *L. casei*

The insertion of eGFP into the mutant strains was verified using PCR on the colonies that showed erythromycin susceptibility (**Figure 3.6**) with the primers 1181 5' GCC GAT CGT CCA TTT CTA G 3' and 1182 5' TAG TGC CAT TCG TCA AGT CC 3' that are 1200 bp upstream and down stream of the insertion site.

The PCR products were sequenced using primers 1181/1182 to confirm the presence of the eGFP insert. Since the PCR product was 3.5 kb, several sequencing reactions were performed using sets of nested primers to allow for the coverage of the whole 3.5 kb. The contigs were then aligned with the reference sequence that had the predicted eGFP insert using the seqman software (**Figure 3.7**). The results of the sequencing reads are as follows : the bold text indicates parts of the sequence that belongs to LSEI_2320 coding sequence while the underlined text represents the coding sequence of the eGFP with the start codon and stop codon removed.



Correct PCR product size for the mutant: 3.5 kb
Size of PCR product for the wild type : 2.8kb

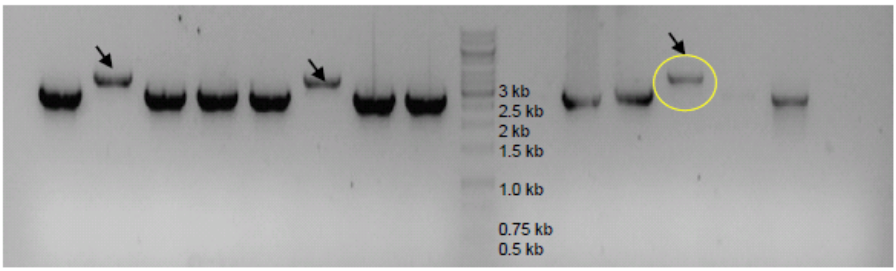
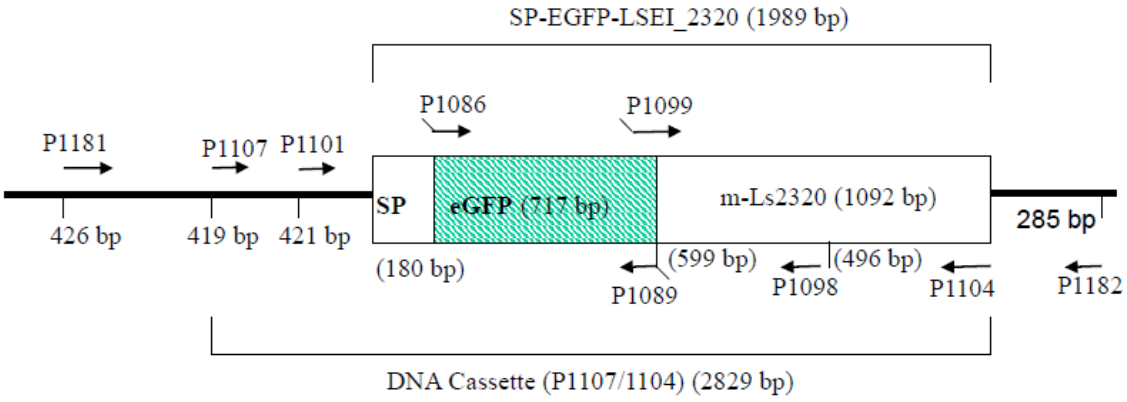


Figure 3.6 PCR analysis of insertion of the eGFP sequemce into the LSEI_2320 locus of *L.casei* ATCC334. The Colony PCR screen of *L. casei* erythromycin susceptible colonies was performed by Han Hong Dan (a research technician in Dr. Lin's Lab). The arrows point to colonies that appear to have a insertion of a 717 bp insertion into the chromosome.The PCR products were run on a 1% agarose gel and visualized with SYBR® safe stain and visualized using UV light. The molecular weight ladder used was the 1kb ladder (Fermentas).



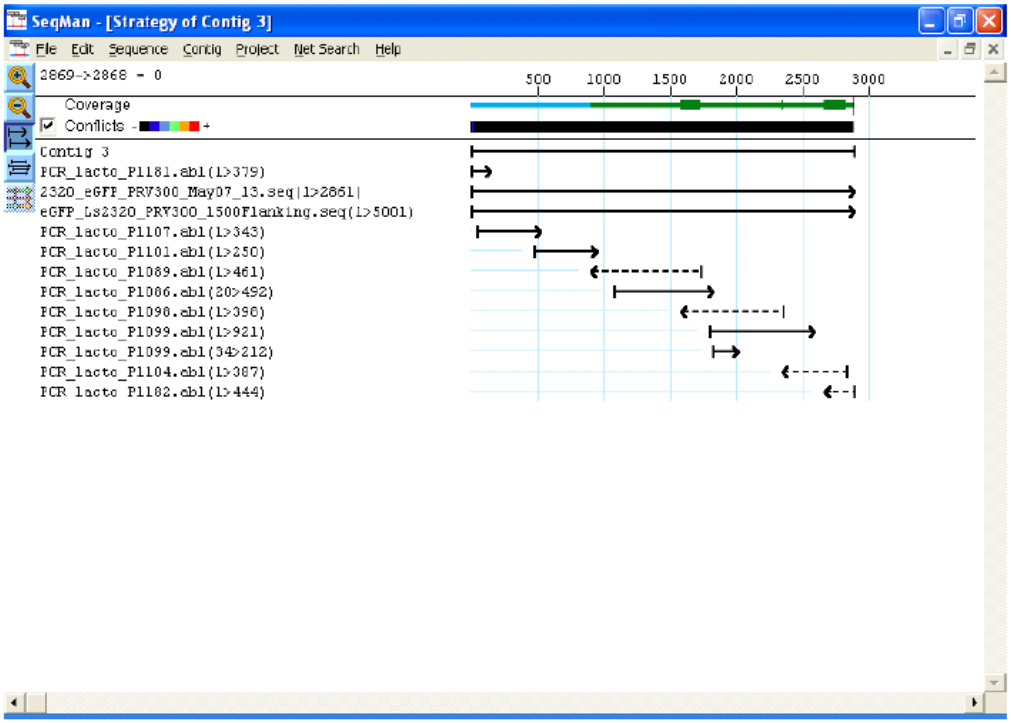


Figure 3.7 PCR and sequencing analysis of the mutant *L.casei* for proper insertion of the eGFP into the LSEI_2320 locus. Initially, primers 1181 and 1182 were used to sequence the area 1.2 kb from the insertion site. Then, primers 1104 and 1107 were designed and used as nested primers. Primers 1101 and 1098 were the next set of nested primers used for sequencing and finally, primers 1089 and 1099 were the last set used to complete the contig. The contig were then assembled with the help of seqman and then aligned with the predicted sequence that would contain an in frame eGFP insert.

Sequencing results for the LSEI_2320 eGFP knockin mutant with a 700bp insert :

5'

CGTCGATATTCGTCAAGCAAAATCTTGGCAAGCAAGTGGGTGACGCTATTGC

TATGCACGCTCGTTTTATATGCCAGTAGCTTAATAATGAGCTTATGTCTCGCA

ATAACAGTTTTTAATGGCAAGATTGATTTAGTCGCAAAGGGCATAAAAGGAC

AGCAAGTTTGGCAAATATGGTTAATGAACAACATTTCTTCATTCTTAACGCTG

ATATTCATATTGAGTTTTGTTTGTCTACTCGCAACGCTGTTTGAAAACTCAAC

GCCCGCTATCATCATTGGCCTCAGTACATACTTCATTGTTTCAATTTTTAATCA

GTTGATGTTTATGATGATCAAACAACATGAATGGATAAAATGGAATCCGGTT

AACATGATGAATTTAGGCAATCAATTGCAGAGCGCTGAACTAACTAAGTTGA

CGCGGCTACCTCTTAGTCAAATTAGTCTTGGTTATCTGATTTATGCAGCTGCC

TTTTTGGCTTTAAGTCTAGAGGTTTTCAAGTATCGTAATGAACGCTAAATAAA

AGAAGCATTTGGATTGCGGAGGTGGACTGGTCATGACTTCAGGTTTGGGTTTT

AAACAATCTAAAGAACATTGCTTTGTGGCACGTAGACGGTTTTTAAACACTC

AAAATATTAGTTAATTGATAAAGACTTTTAGACTTGGTTCTATGAAGTCTTTT

TTTGAATTGCAATACGGCAATTGAAAGAGGCTTTTTGTGCTGGAGCTACATAG

AAAAATGTCAAGTGTTATACTTGTCACCAATATGTAATATTTACTTTGTTGTTT

TCGGTTGAGTGCGTCTCGGCTTGGGTCATGAGAAGGGGGTAAAATCA**TTGCA**

GGCATTTAGTAACGAAAAGTCTCAGCATAAGATGTATAAAGCGGGTACG

CTATGGGTGGCAGCGTTGATTGGTGTGGGGATTTGTGTGGGTGGTAGTC

AAATCCAGTCAGTTGCAGCATCGACATCGCATATTCATTCAGTGGTCACC

AACGCGACTGGAACAACGCCGACTAATGGGCCCATGGTGAGCAAGGGCG

AGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGT

AAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTA

CGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCC

TGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTA

CCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGC

TACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCC

GCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGA

AGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGT

ACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACG

GCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCA

GCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG

CTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCA

ACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGAT

CACTCTCGGCATGGACGAGCTGTACAAG**AAGCTTCAAGCAACGGTATTGAA**

ACAGCAAGAAACTGGCGTGAATGCCCAGATCATTGCCGGTCATCAAACC

GGTCAATATTCGACTAAGGGTGTCACAGACGCTGGCGATGACACAACGA

GCGCTGTGCCAGACACCGATGTTTATCAGAATCGTAGTTTACCCATAACT

GGCAATGCCGTCGTCGTTAGGTGTGTTGATATAGAGGGGAATATGTTGG

CACCTGATAAGGTGATCACTGGCAAAGTCGGTGATCCTTATACGATGAC

AGCACCTGCCTTTGACTATTATGAAAATCCCCAATTAGCCACAGACAGCG

CACCAGCAAACGGGACTTTCACCAATGACGTACAGACGGTCACCTTTGT

TTATACGAAGACACCAATTCCGCGTGGCAGTGTCAACGTGAAGTTTGTT

GACGTAGACGGCAAACGGTTGGCATCTGATAAAATACTCACTGGTCAAG

CCGGTGATCCTTACGAAGCAAGACCAATTGATATGACGAATTACCAATTT

GCTCGTTTGGCGACAGGCAGTGCGCCGGAAGATGGTTCATTTACGGACG

GTACGCAAACCGTCACCTTCATCTACACTAAGAATCCAGTTGCTCAAGGA

ACTGTTGACGTGAAATATGTCGATGACAATGGCAAGCAGATTGCGCCTG

ATAAGATTCTGACCGGTCATGTCGGCGATCCGTATGCAGTTACACCAAC

GATGATCGCGAATTATCACTACTTACGATTAGCATCTGGCAGCATCGCG

GAAAATGGTGCTTTTGCAACAGGCAGTCTAACCGTGACGTTCGTTTACG

CAAAGCAAGCGACACCTGTGACCCCATCAACGCCAGTCACGCCATCAAC

ACCAACAACACCGTCAACTCAAGTTTTGCCAAGTATGTCGGATACGCCG

ACAGAATCGGCACCGCTATCCGATAATGACACCGCGCCAATCGAAGTTA

TTATCCCAACCAAGCAAATAAGCCGTTACTTGCCGAATACCGGTGATAAC

CAACGGACCAGCTTAATTGCGATCGGGGTGGCTTTGCTGCTTGCCTTGA

TCAGTTTTGGATCATTTGGACTGCGTCGTCGGGAAAAGTAGTTATTGATC

TGTAAAAAAAGAGTTTGTAAAGAGTCTCACGATGACATTTCTTGCGTGGGAC

TTTTTTTGTGCGCTTATCGCCTTTTGAGTTGTGAATTGTTGACTAAGTGAGCGT

TCGGTGTTATATACTAACTATAACAACTAGAACAGTTAGAAGGTGAA 3'.

Subsequent computational analysis showed no discrepancies between the correct sized PCR product and the reference sequence. The mutant and wild type strains of *L. casei* were then tested for fluorescence using a fluorescent microscope. Excitation at 470-490 nm of both the wild type bacteria and the mutant showed no fluorescence at the excitation range of the eGFP (**Figure 3.8a**). Probing the cell surface of the mutant and wild type strains with antibodies against eGFP did not give any signal during immunofluorescence staining for either the wild type or the eGFP knockin (**Figure 3.8b**). Staining the mutant and the wild type cells using LSEI_2320 antiserum resulted in a

signal from the wild type strain but the absence of signal from the mutant strain (**Figure 3.8c**). Whole cell extracts from the wild type and the eGFP knockin mutant were then probed with either an anti eGFP antibody (**Figure 3.9**). The eGFP protein was not detected in the wild type cell extract or the mutant eGFP-LSEI_2320 cell extract.

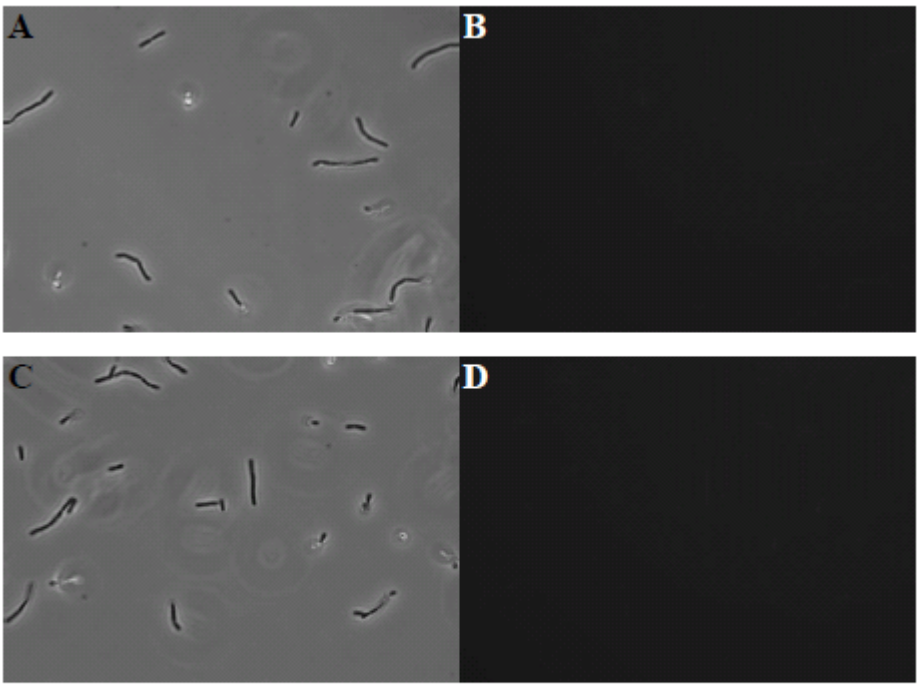


Figure 3.8a Fluorescence of the eGFP knockin mutant. (A) Phase contrast image of the eGFP knockin mutant. (B) Fluorescence imaging of the eGFP knockin mutant. (C) Phase contrast image of the wild type *L.casei*. (D) Fluorescence imaging of the wild type *L.casei*. Both phase contrast and fluorescent images (A versus B; C versus D) of bacterial cells are in the same field of view and recorded for comparison.

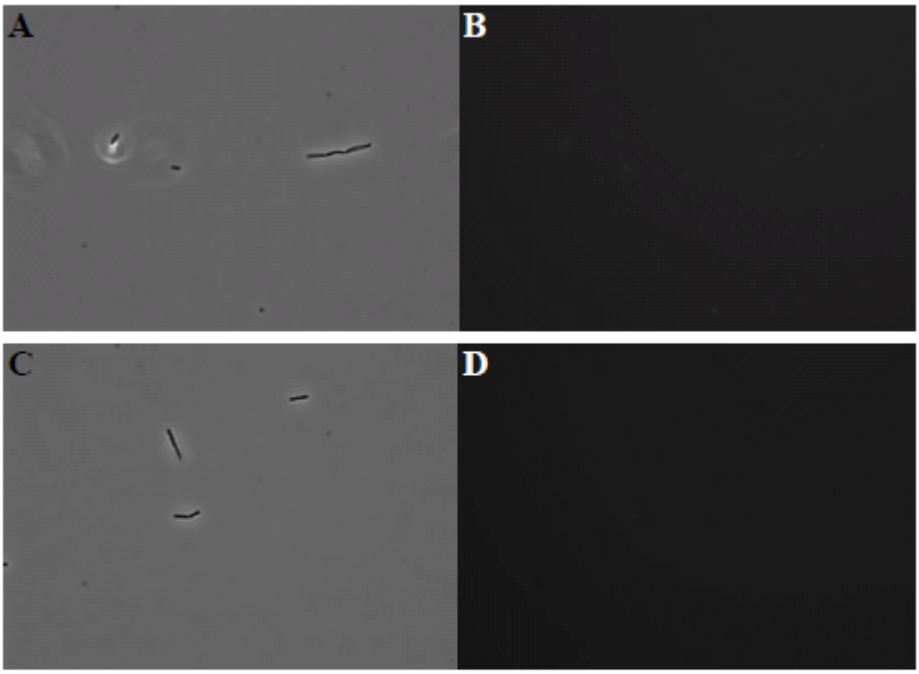


Figure 3.8b Detection of surface expression of eGFP in the knockin mutant. (A) Phase contrast image of the eGFP knockin mutant stained with anti-eGFP antibodies. (B) Fluorescence imaging of the eGFP knockin mutant stained with anti-eGFP antibodies. (C) Phase contrast image of the wild type *L. casei* stained with anti eGFP-antibodies. (D) Fluorescence imaging of the wild type *L. casei* stained with anti-eGFP antibodies. Both phase contrast and fluorescent images (A versus B; C versus D) of bacterial cells are in the same field of view and recorded for comparison.

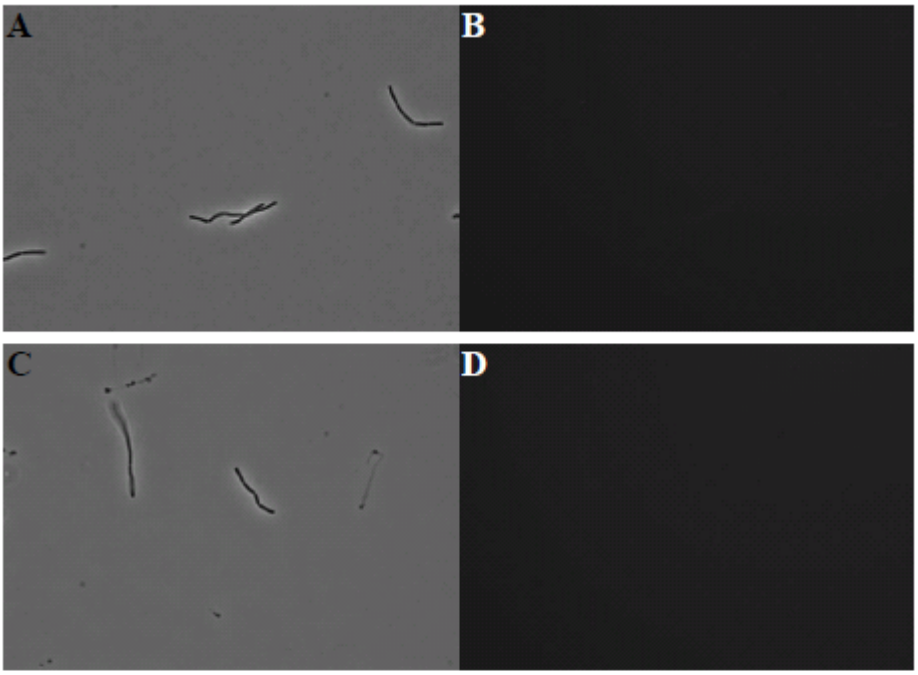


Figure 3.8c Negative control for the expression of LSEI_2320 on the surface of the knockin mutant. (A) Phase contrast image of the eGFP knockin mutant stained with LSEI_2320 pre immune serum. (B) Fluorescence imaging of the eGFP knockin mutant stained with LSEI_2320 preimmune serum. (C) Phase contrast image of the wild type *L.casei* stained with LSEI_2320 preimmune serum. (D) Fluorescence imaging of the wild type *L.casei* stained with LSEI_2320 preimmune serum. Both phase contrast and fluorescent images (A versus B; C versus D) of bacterial cells are in the same field of view and recorded for comparison.

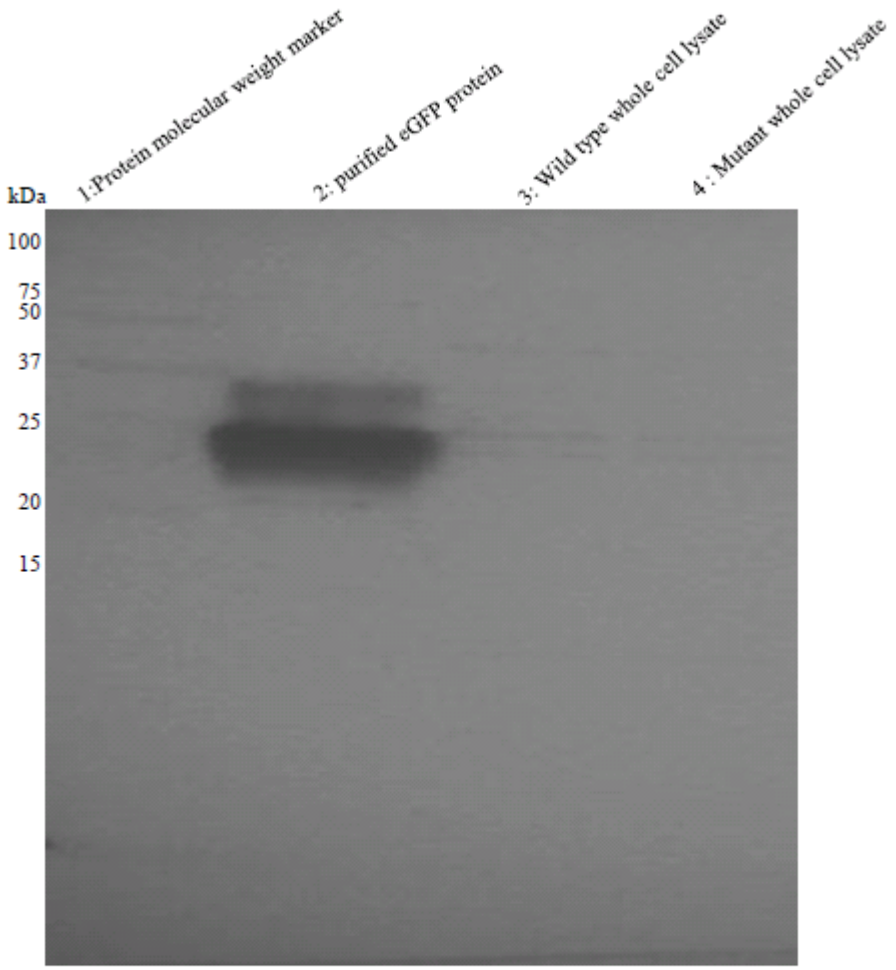


Figure 3.9 Western blot analysis of eGFP protein expression in the late stationary phase whole cell lysate. The Proteins were run on a 12% SDS-PAGE gel, transferred onto a nitrocellulose membrane and probed with an anti-eGFP antibody (Life technologies). Lane 1: Protein molecular weight marker (Biorad), Lane 2: eGFP (positive control), Lane 3: Whole cell extract of a wild type *L. casei*, Lane 4: Whole cell extract of the knockin mutant.

Chapter 4: Discussion

4.1 Discussion

Throughout this study, we were able to demonstrate one novel LPXTG protein expressed on the surface of *L. casei* out of 6 LPXTG proteins selected from all 16 proteins containing LPXTG motifs encodingd by the genome of *L. casei* ATCC334. Bioinformatics analysis of the *L. casei* genome identified 16 LPXTG proteins; six were chosen for further investigation into their expression at the protein level. Subsequently, four out of the six selected LPXTG protein candidates were found to be translated and expressed at the protein level in *L. casei*. These were LSEI_0455, LSEI_1905, LSEI_2320 and LSEI_2431. Immunofluorescence fluorescence experiments further showed that only LSEI_2320 out of the four expressed candidates was detected on the surface of *L. casei* at the stationary phase. Consistent with the expression at the protein level, the mRNA transcripts of LSEI_2320 were also detected at 1 h after subculture from the stationary phase culture.

Surface expression of LSEI_2320 in *L. casei* indicates that this protein has the potential for use as an anchor for surface display of other foreign proteins. To generate a mutant of *L. casei* using LSEI_2320 as an anchor to surface display eGFP protein, the eGFP-LSEI_2320 fusion sequence was constructed and cloned into the suicide vector pRV300. The construct was designed with the first homologous recombination region at about 800 bp long with the RBS and signal peptide (first 60 amino acids) following this sequence, while the eGFP coding sequence was added without the stop codon. The eGFP coding sequence was followed by the second homologous recombination region that

starts from the 61st amino acid to the end of the coding region (1200 bp). A GM *L. casei* strain carrying the eGFP gene inserted in frame into the LSEI_2320 locus was successfully generated and verified with sequencing of the PCR products. Unfortunately, immunofluorescence microscopy failed to detect eGFP expression (**Figure 3.7a**). Furthermore, lack of eGFP fluorescence in *L. casei* was due to the absence of both eGFP (**Figure 3.7b**) and LSEI_2320 (**Figure 3.7c**) protein components on the cell surface. While it appears that this configuration of LSEI_2320 cannot be used for surface display of a foreign protein as it interferes with expression of the eGFP-LSEI_2320 fusion localization on the cell surface, the locus itself seems to be non-essential for survival of *L. casei*. This may also be due to the fact that GFP does not fold well in *E. coli* and will end up in inclusion bodies (Crameri *et al.* 1996).

It is expected that the eGFP-LSEI_2320 fusion protein to be expressed in the cytoplasm, secreted and localized on the cell surface as the fusion protein encoded by the gnome of the mutant contains a signal peptide sequence and a LPXTG motif in the LSEI_2320 protein. Since both eGFP and LSEI_2320 components were not detected by specific antibodies on the cell surface, the possibilities may be that the LSEI_2320-eGFP fusion protein was secreted into the growth medium or degraded by the proteasomal pathway of the cell. To detect the presence of the recombinant protein in the growth media, the cells should first be filtered out with a membrane containing 0.2 μm wide pores and then the fusion protein can be purified with affinity chromatography using specific against eGFP or LSEI_2320. Western blot analysis can then be performed on the protein sample. To detect for the degradation of the fusion protein due to the, the *L. casei* proteaseome would have to be inhibited by pharmaceuticals or by knock down to

determine if the expression of the eGFP fusion can be rescued. Improvements could be made to the mutant construct by replacing the signal peptide with a TAT secretion signal since the TAT translocon can only transport correctly folded proteins across the cell membrane (Fisher *et al.* 2006). Another improvement to the mutant construct would be to insert a smaller epitope such as a His tag instead of GFP so that the fusion protein would have less of a chance at misfolding.

Expression of the other three genes, LSEI_0455, 1905 and 2431, were also determined at the protein level by Western blots; however, they were not detected on the cell surface by immunofluoresence microscopy. Currently, all these proteins have not been characterized, but the description for LSEI_0455 and 2431 did indicate some type of collagen binding domain. However, this collagen binding role does not appear to correspond to our results in this study as we showed that they were most likely cytosol-localized proteins as suggested from both the immunofluorescence data and the Western blotting analysis, and did not appear to be secreted. Further investigation into these proteins, similar to the above LSEI_2320, is necessary to characterize their individual physiological regulation patterns and functions. Furthermore, the main focus of the RT-PCR was on LSEI_2320, hence the other mRNA transcripts remains to be investigated. The reason for this discrepancy is not known. It is possible that the epitopes are not accessible to be detected with antibodies due to steric effects that can shield the epitope in the native conformation (Ubbink and Schär-Zammaretti 2005).

The GFPuv protein has been expressed with fluorescent activity by *L. casei* using a vector that controlled the expression with a lac operon (Pérez-Arellano and Pérez-

Martínez. 2003). A fusion protein produced by *E. coli* with the GFP protein fused to the LysM domain (Ly5C) of a *L.plantarum* bacteriophage was able to bind and exhibit fluorescence when mixed with several Lactobacillus species (Hu et al. 2010). So it appears that the GFP would be able to fluoresce if it were able to be expressed by *L. casei* in the cytosol. The size of the GFP protein (27 kDa) can interfere with the function and subcellular localization of the tagged protein (Tavaré et al. 2001). This could explain the absence of the LSEI_2320 from the surface of the mutant.

As only 6 proteins were screened for their expression in this study (**Table 3.1**), 10 LPXTG proteins still remain uncharacterized. Further investigation into the expression of their proteins and localization may help discover more novel LPXTG protein candidates for surface display. This can be accomplished through a similar strategy shown in this study for LSEI_2320 by cloning the target gene into a protein expression vector and then producing recombinant proteins and raising polyclonal antibodies. Subsequent detection by Western blot analysis and immunofluorescence microscopy will determine whether the LPXTG protein candidates are expressed and cell surface-anchored. In parallel, RT-PCR could be employed to confirm the transcription of the target gene. Other detection methods of for bacterial surface expression includes electron microscopy or with an enzyme-linked immunoabsorbent assay (ELISA) using *L. casei* whole cells as the target and antibody-coated plates.

This study led to the successful generation of a GM *L. casei* bearing a copy of the eGFP gene within its chromosome. This mutant strain can be a useful model for assessing the amount of transgene mobility in the host microbiota of animals fed with GM probiotic feed additives. The proposed use of this GM strain in the CFIA is to add this mutant

organism to animal feed to investigate the longevity of the GM organism in both the digestive tract and animal waste. This involves feeding the animals waith a spiked feed sample containing the GM *L. casei* and then determining the duration of time needed to clear the bacteria from the digestive tract, whether colonization occurs, and the location of colonization. Determining the longevity of the bacteria in animal waste is also important towards estimating the potential hazards associated with untreated animal waste. Furthermore, another significant aspect that must be examined is the stability of the GM *L. casei* through determining the number of growth cycles that would be needed to mutate the eGFP tag to the extent that it would not be detected by gene specific primers. This would provide an estimate on the length of time needed to isolate the GM organism to render it safe in terms of transgene mobility. In addition to the viability of the bacteria, the longevity of the modified bacteria in the environment would have to be evaluated as well to determine the time frame that the host microbiome has to uptake the transgene. Together, these experiments with the GM *L. casei* generated in this study will provide substantial knowledge of the time frame the host bacteria would have in order to uptake GM DNA from a donor strain and also the measures needed to be taken to reduce the transfer of GM DNA to the host microbiota. This could include sterilization methods and variation in the timing/frequency of the inoculation of the GM organisms into the animal feed.

Since the eGFP was not found to be fluorescent or expressed in the mutant, other strategies could be utilized to visualize the cells when observing sections of the digestive tract. In conjunction with microscopy, fluorescent in situ hybridization (FISH) can be performed on bacteria after the sections have been properly fixed and permeabilized.

Pairs of probes could be used to tag the eGFP to track the GM strain in the gut at various time points. Viability of the recovered bacteria from the passage experiment can be done in culture conditions with *L. casei* MRS growth medium. Moreover, PCR or quantitative PCR can be used to determine the amount of GM marker (ie, eGFP) in the environmental samples as it is the most reliable quantitative approach for tracking the persistence of the eGFP marker.

Alternatively, a second approach would be to look at the effectiveness of transgene uptake by the microbiota of the host. To accomplish this, the GM bacteria must initially be lysed and mixed with the general bacterial population and as well as several host specific donor strains to determine the minimum required dose to allow for transgene uptake/integration under optimal conditions. Bacteria that have taken up the GM DNA can be recovered through the use of FISH probes against eGFP and a 4',6-diamidino-2-phenylindole (DAPI) stain on fixed and permeabilized cells. The GM donor strain can be differentiated using FISH probes that anneal at the eGFP and the LSEI_2320 fusion point. The eGFP probed cells can be sorted and counted using fluorescence activated cell sorting. The advantage of this technique is that it is a culture independent method (Morgan & Huttenhower. 2012) and allows for subsequent metagenomic analysis of the bacteria to characterize the most at risk species for transgene uptake and also the mechanism for transgene uptake.

4.2 Conclusion

The work presented in this thesis demonstrates a strategy to screen and identify novel LPXTG motif-containing proteins for the surface display of foreign proteins in *L. casei.* LSEI_2320, the only one of the 6 LPXTG proteins selected for characterization,

has the potential for surface display applications as it was shown to be expressed on the cell surface. The eGFP knockin has resulted in a mutant of *L. casei* containing the eGFP gene inserted in frame into LSEI_2320 locus. This mutant was not able to express eGFP on the cell surface, may still be useful model for assessing the survival and gene transfer of GM *L. casei* to the host microbiota of animals fed with GM probiotic feed additives. Future studies are necessary to optimize the fusion protein design for surface display of foreign proteins to allow LSEI_2320 to act as an anchor.

References

1. Hessle, C., Andersson, B., Wold, A.E. 2000. Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while gram-negative bacteria preferentially stimulate IL-10 production. Infect Immun. 68(6):3581-6.

2. Rogers, H.J., Forsberg, C.W. 1971. Role of Autolysins in the Killing of Bacteria by Some Bactericidal Antibiotics, J Bacteriol. 108(3):1235-43.

3. Silhavy, T.J. Kahne, D., Walker, S. 2010. The bacterial cell envelope. Cold Spring Harb Perspect Biol. 2(5):a000414.

4. Vincent T. Lee and Olaf Schneewind. 2001, Protein secretion and the pathogenesis of bacterial infections, Genes Dev. 15: 1725-1752

5. Joachim-V. Holtje and Alexander Tomasz, Lipoteichoic Acid: A Specific Inhibitor of Autolysin Activity in Pneumococcus, Proc. Nat. Acad. Sci. USA, Vol. 72, No. 5, pp. 1690-1694, May 1975

6. Roy, H. 2009. Tuning the properties of the bacterial membrane with aminoacylated phosphatidylglycerol. IUBMB Life. 61(10):940-53.

7. Neuhaus, F.C., Baddiley, J. 2003. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev. 67(4):686-723.

8. Sára, M., Sleytr, U.B. 2000. S-Layer proteins. J Bacteriol. 182(4):859-68.

9. Llobet, E., Tomás, J.M., Bengoechea, J.A. 2008. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. Microbiology. 154(Pt 12):3877-86.

10. Anderson, G.G., Goller, C.C., Justice, S., Hultgren, S.J., Seed, P.C. 2010. Polysaccharide capsule and sialic acid-mediated regulation promote biofilm-like intracellular bacterial communities during cystitis. Infect Immun. 78(3):963-75.

11. Mora, T., Yu, H., Sowa, Y., Wingreen, N.S. 2009. Steps in the bacterial flagellar motor. PLoS Comput Biol. 5(10):e1000540.

12. Lin, J., Heuser, T., Song, K., Fu, X., Nicastro, D. 2012. One of the nine doublet microtubules of eukaryotic flagella exhibits unique and partially conserved structures. PLoS One. 7(10):e46494.

13. Kline, K.A., Fälker, S., Dahlberg, S., Normark, S., Henriques-Normark, B. 2009. Bacterial adhesins in host-microbe interactions. Cell Host Microbe. 5(6):580-92.

14. Wang, Y., Taylor, D.E. 1990. Natural transformation in Campylobacter species. J Bacteriol. 172(2):949-55.

15. Miethke, M., Marahiel, M.A. 2007. Siderophore-based iron acquisition and pathogen control. Microbiol Mol Biol Rev. 71(3):413-51.

16. O'Riordan, K., Lee, J.C. 2004. Staphylococcus aureus capsular polysaccharides. Clin Microbiol Rev. 17(1):218-34.

17. Lycklama A Nijeholt, J.A., Driessen, A.J. 2012. The bacterial Sec-translocase: structure and mechanism. Philos Trans R Soc Lond B Biol Sci. 367(1592):1016-28.

18. von Heijne, G. 1990. The signal peptide. J Membr Biol. 115(3):195-201.

19. von Heijne, G., Abrahmsén, L. 1989. Species-specific variation in signal peptide design. Implications for protein secretion in foreign hosts. FEBS Lett. 244(2):439-46.

20. Auclair, S.M., Bhanu, M.K., Kendall, D.A. 2012. Signal peptidase I: cleaving the way to mature proteins. Protein Sci. 21(1):13-25.

21. Robinson, C., Matos, C.F., Beck, D., Ren, C., Lawrence, J., Vasisht, N., Mendel, S. 2011. Transport and proofreading of proteins by the twin-arginine translocation (Tat) system in bacteria. Biochim Biophys Acta. 1808(3):876-84.

22. Bendtsen, J.D., Nielsen, H., Widdick, D., Palmer, T., Brunak, S. 2005. Prediction of twin-arginine signal peptides. BMC Bioinformatics. 2;6:167.

23. Marraffini, L.A., Dedent, A.C., Schneewind, O. 2006. Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. Microbiol Mol Biol Rev. 70(1):192-221.

24. Sutcliffe, I.C., Harrington, D.J. 2002. Pattern searches for the identification of putative lipoprotein genes in Gram-positive bacterial genomes. Microbiology. 148(Pt 7):2065-77.

25. Buist, G., Steen, A., Kok, J., Kuipers, O.P. 2008. LysM, a widely distributed protein motif for binding to (peptido)glycans. Mol Microbiol. 68(4):838-47.

26. Renier, S., Micheau, P., Talon, R., Hébraud, M., Desvaux, M. 2012. Subcellular localization of extracytoplasmic proteins in monoderm bacteria: rational secretomics-based strategy for genomic and proteomic analyses. PLoS One. 7(8):e42982.

27. Maresso, A.W., Schneewind, O. 2008. Sortase as a target of anti-infective therapy. Pharmacol Rev. 60(1):128-41.

28. Oh, S.Y., Budzik, J.M., Schneewind, O. 2008. Sortases make pili from three ingredients. Proc Natl Acad Sci U S A. 105(37):13703-4.

29. Kovacs-Simon, A., Titball, R.W., Michell, S.L. 2011. Lipoproteins of bacterial pathogens. Infect Immun. 79(2):548-61.

30. Welte, T., Kudva, R., Kuhn, P., Sturm, L., Braig, D., Müller, M., Warscheid, B., Drepper, F., Koch, H.G. 2012. Promiscuous targeting of polytopic membrane proteins to SecYEG or YidC by the Escherichia coli signal recognition particle. Mol Biol Cell. 23(3):464-79.

31. Xie, K., Dalbey, R.E. 2008. Inserting proteins into the bacterial cytoplasmic membrane using the Sec and YidC translocases. Nat Rev Microbiol. 6(3):234-44.

32. Ebbes, M., Bleymüller, W.M., Cernescu, M., Nölker, R., Brutschy, B., Niemann, H.H. 2011. Fold and function of the InlB B-repeat. J Biol Chem. (17):15496-506.

33. Desvaux, M., Dumas, E., Chafsey, I., Hébraud, M. 2006. Protein cell surface display in Gram-positive bacteria: from single protein to macromolecular protein structure. FEMS Microbiol Lett. 256(1):1-15.

34. Blocker, A., Komoriya, K., Aizawa, S. 2003. Type III secretion systems and bacterial flagella: insights into their function from structural similarities. Proc Natl Acad Sci U S A. 100(6):3027-30.

35. Tanskanen, J., Korhonen, T.K., Westerlund-Wikström, B. 2000. Construction of a multihybrid display system: flagellar filaments carrying two foreign adhesive peptides. Appl Environ Microbiol. 66(9):4152-6.

36. van Bloois, E., Winter, R.T., Kolmar, H., Fraaije, M.W. 2011. Decorating microbes: surface display of proteins on Escherichia coli. Trends Biotechnol. 29(2):79-86.

37. Pozzi, G., Contorni, M., Oggioni, M.R., Manganelli, R., Tommasino, M., Cavalieri, F., Fischetti, V.A. 1992. Delivery and expression of a heterologous antigen on the surface of streptococci. Infect Immun. 60(5):1902-7.

38. Kajikawa, A., Nordone, S.K., Zhang, L., Stoeker, L.L., LaVoy, A.S., Klaenhammer, T.R., Dean, G.A. 2011. Dissimilar properties of two recombinant Lactobacillus acidophilus strains displaying Salmonella FliC with different anchoring motifs. Appl Environ Microbiol. 77(18):6587-96.

39. van Pijkeren J.P., Canchaya C., Ryan K.A., Li Y., Claesson M.J., Sheil B., Steidler L., O'Mahony L., Fitzgerald G.F., van Sinderen D., O'Toole P.W. 2006. Comparative and functional analysis of sortase-dependent proteins in the predicted secretome of Lactobacillus salivarius UCC118. Appl Environ Microbiol. Jun;72(6):4143-53.

40. Hess, G.T., Cragnolini, J.J., Popp, M.W., Allen, M.A., Dougan, S.K., Spooner, E., Ploegh, H.L., Belcher, A.M., Guimarae,s C.P. 2012. M13 bacteriophage display framework that allows sortase-mediated modification of surface-accessible phage proteins. Bioconjug Chem. 23(7):1478-87.

41. Verhoeven, G.S., Alexeeva, S., Dogterom, M., den Blaauwen, T. 2009. Differential Bacterial Surface Display of Peptides by the Transmembrane Domain of OmpA. PLoS ONE. 4(8)e6739.

42. Taylor, J.L., Harrison, K.N., Timmis, C. D., O'Connor, C.D. 1990. The TraT lipoprotein as a vehicle for the transport of foreign antigenic determinants to the cell surface of Escherichia coli K12: structure-function relationships in the TraT protein. Molecular Microbiology. 4(8):1259–1268.

43. Westerlund-Wikström, B., Tanskanen, J., Virkola, R., Hacker, J., Lindberg, M., Skurnik, M., Korhonen, T.K. 1997. Functional expression of adhesive peptides as fusions to Escherichia coli flagellin, Protein Engineering. 10(11):1319-1326.

44. Van Die, I., Wauben, M., Van Megen, I., Bergmans, H., Riegman, N., Hoekstra, W., Pouwels, P., Enger-Valk, B. 1988. Genetic Manipulation of Major P-Fimbrial Subunits and Consequences for Formation of Fimbriae. J Bacteriol. 170(12):5870-6.

45. Bingle, W.H., Nomellini, J.F., Smit, J. 1997. Cell-surface display of a Pseudomonas aeruginosa strain K pilin peptide within the paracrystalline S-layer of Caulobacter crescentus. Molecular Microbiology. 26(2):277–288.

46. Samuelson, P., Hansson, M., Ahlborg, N., Andréoni, C., Götz, F., Bächi, T., Nguyen, T.N., Binz, H., Uhlén, M., Ståhl, S. 1995. Cell Surface Display of Recombinant Proteins on Staphylococcus carnosus. J Bacteriol. 177(6):1470-6.

47. Stover, C.K., Bansal, G.P., Hanson, M.S., Burlein, J.E., Palaszynski, S.R., Young, J.F., Koenig, S., Young, D.B., Sadziene, A., Barbour, A.G. 1993. Protective Immunity EHcited by Recombinant Bacille Calmette-Guerin (BCG) Expressing Outer Surface Protein A (OspA) Lipoprotein: A Candidate Lyme Disease Vaccine. J. Exp. Med. 178(1):197-209.

48. Mesnage, S., Weber-Levy, M., Haustant, M., Mock, M., Fouet, A.. 1999. Cell Surface-Exposed Tetanus Toxin Fragment C Produced by Recombinant Bacillus anthracis Protects against Tetanus Toxin. Infect Immun. 67(9):4847-50.

49. Ciabattini, A., Parigi, R., Isticato, R., Oggioni, M.R., Pozzi, G. 2004. Gianni Pozzi, Oral priming of mice by recombinant spores of Bacillus subtilis. Vaccine. 22(31-32):4139-43.

50. Felis, G.E., Dellaglio, F. 2007. Taxonomy of Lactobacilli and Bifidobacteria. Curr Issues Intest Microbiol. 8(2):44-61.

51. Walter, J. 2008. Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and biomedical research. Appl Environ Microbiol. 74(16):4985-96.

52. Mosbah, A. & Mesbah, M.R. 2009. Study of the role of hydrogen peroxide production by lactobacilli in preterm labor. International Journal of Medicine and Medical Sciences. 1(9): 388-395.

53. Bernardeau, M., Guguen, M., Vernoux, J.P. 2006. Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. FEMS Microbiol Rev. 30(4):487-513.

54. Reid, G. 1999. The scientific basis for probiotic strains of Lactobacillus. Appl Environ Microbiol. 65(9):3763-6.

55. Neal-McKinney, J.M., Lu, X., Duong, T., Larson, C.L., Call, D.R., Shah, D.H., Konkel, M.E. 2012. Production of organic acids by probiotic lactobacilli can be used to reduce pathogen load in poultry. PLoS One. 7(9):e43928.

56. Maqueda, M., Sánchez-Hidalgo, M., Fernández, M., Montalbán-López, M., Valdivia, E., Martínez-Bueno, M. 2008. Genetic features of circular bacteriocins produced by Gram-positive bacteria. FEMS Microbiol Rev. 32(1):2-22.

57. Isolauri, E., Sütas, Y., Kankaanpää, P., Arvilommi, H., Salminen, S. 2001. Probiotics: effects on immunity. Am J Clin Nutr. 73(2 Suppl):444S-450S.

58. Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M., Roy, N.C. 2011. Regulation of tight junction permeability by intestinal bacteria and dietary components. J Nutr. 141(5):769-76.

59. Johansson, M.A., Sjögren, Y.M., Persson, J.O., Nilsson, C., Sverremark-Ekström, E. 2011. Early colonization with a group of Lactobacilli decreases the risk for allergy at five years of age despite allergic heredity. PLoS One. 6(8):e23031.

60. Rooj, A.K., Kimura, Y., Buddington, R.K. 2010. Metabolites produced by probiotic Lactobacilli rapidly increase glucose uptake by Caco-2 cells. BMC Microbiol. 10:16.

61. Naaber, P., Smidt, I., Stsepetova, J., Brilene, T., Annuk, H., Mikelsaar, M. 2004. Inhibition of Clostridium difficile strains by intestinal Lactobacillus species. J Med Microbiol. 53(Pt 6):551-4.

62. Myllyluoma, E., Veijola, L., Ahlroos, T., Tynkkynen, S., Kankuri, E., Vapaatalo, H., Rautelin, H., Korpela, R. 2005. Probiotic supplementation improves tolerance to Helicobacter pylori eradication therapy--a placebo-controlled, double-blind randomized pilot study. Aliment Pharmacol Ther. 21(10):1263-72.

63. Snydman, D.R. 2008. The Safety of Probiotics. Clin Infect Dis. 46 Suppl 2:S104-11.

64. Narita, J., Okano, K., Tateno, T., Tanino, T., Sewaki, T., Sung, M.H., Fukuda, H., Kondo, A. 2006. Display of active enzymes on the cell surface of Escherichia coli

using PgsA anchor protein and their application to bioconversion. Appl Microbiol Biotechnol. 70(5):564-72.

65. Ashiuchi, M., Nawa, C., Kamei, T., Song, J.J., Hong, S.P., Sung, M.H., Soda, K., Misono, H. 2001. Physiological and biochemical characteristics of poly gamma-glutamate synthetase complex of Bacillus subtilis. Eur J Biochem. 268(20):5321-8.

66. Huang, S.J., Chen, M.J., Yueh, P.Y., Yu, B., Zhao, X., Liu, J.R. 2011. Display of Fibrobacter succinogenes ß-Glucanase on the Cell Surface of Lactobacillus reuteri. J. Agric. Food Chem. 59(5):1744–1751.

67. Martín, M.C., Pant, N., Ladero, V., Günaydin, G., Andersen, K.K., Alvarez, B., Martínez, N., Alvarez, M.A., Hammarström, L., Marcotte, H. 2011. Integrative expression system for delivery of antibody fragments by lactobacilli. Appl Environ Microbiol. 77(6):2174-9.

68. Avall-Jääskeläinen, S., Kylä-Nikkilä, K., Kahala, M., Miikkulainen-Lahti, T., Palva, A. 2002. Surface Display of Foreign Epitopes on the Lactobacillus brevis S-Layer. Appl Environ Microbiol. 68(12):5943-51.

69. Narita, J., Okano, K., Kitao, T., Ishida, S., Sewaki, T., Sung, M.H., Fukuda, H., Kondo, A. 2006. Display of alpha-Amylase on the Surface of Lactobacillus casei Cells by Use of the PgsA Anchor Protein, and Production of Lactic Acid from Starch. Appl Environ Microbiol. 72(1):269-75.

70. Wei, C.H., Liu, J.K., Hou, X.L., Yu, L.Y., Lee, J.S., Kim, C.J. 2010. Immunogenicity and protective efficacy of orally or intranasally administered recombinant Lactobacillus casei expressing ETEC K99. Vaccine. 28(24):4113-8.

71. Liu, J.K., Hou, X.L., Wei, C.H., Yu, L.Y., He, X.J., Wang, G.H., Lee, J.S., Kim, C.J. 2009. Induction of Immune Responses in Mice after Oral Immunization with Recombinant Lactobacillus casei Strains Expressing Enterotoxigenic Escherichia coli F41 Fimbrial Protein. Appl Environ Microbiol. 75(13):4491-7.

72. Lee, J.S., Poo, H., Han, D.P., Hong, S.P., Kim, K., Cho, M.W., Kim, E., Sung, M.H., Kim, C.J. 2006. Mucosal Immunization with Surface-Displayed Severe Acute Respiratory Syndrome Coronavirus Spike Protein on Lactobacillus casei Induces Neutralizing Antibodies in Mice. J Virol. 80(8):4079-87.

73. Poo, H., Pyo, H.M., Lee, T.Y., Yoon, S.W., Lee, J.S., Kim, C.J., Sung, M.H., Lee, S.H. 2006. Oral administration of human papillomavirus type 16 E7 displayed on Lactobacillus casei induces E7-specific antitumor effects in C57/BL6 mice. Int J Cancer. 119(7):1702-9.

74. Hou, X.L., Yu, L.Y., Liu, J., Wang, G.H. 2007. Surface-displayed porcine epidemic diarrhea viral (PEDV) antigens on lactic acid bacteria. Vaccine. 26(1):24-31.

75. Richter, K., Gescher, J. 2012. The molecular toolbox for chromosomal heterologous multiprotein expression in Escherichia coli. Biochem Soc Trans. (6):1222-6.

76. Georgiou, G., C. Stathopoulos, P. S. Daugherty, A. R. Nayak, B. L. Iverson,and R. Curtiss III. 1997. Display of heterologous proteins on the surface of microorganisms: from the screening of combinatorial libraries to live recombinant vaccines. Nat. Biotechnol. 15:29–34.

77. Cabello, F.C. 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ Microbiol. 8(7):1137-44.

78. Barton, M.D. 2000. Antibiotic use in animal feed and its impact on human healt. Nutr Res Rev. 13(2):279-99.

79. Mathew, A.G., Cissell, R., Liamthong, S. 2007. Antibiotic resistance in bacteria associated with food animals: a United States perspective of livestock production. Foodborne Pathog Dis. 4(2):115-33.

80. M. E. Hume, 2011, Food Safety Symposium: Potential Impact of Reduced Antibiotic Use and the Roles of Prebiotics, Probiotics, and Other Alternatives in Antibiotic-Free Broiler Production, Poultry Science 90 :2663–2669

81. Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, October 2001

82. Food Including Powder Milk with Live Lactic Acid Bacteria, October 2001

83. Gibson G.R., Probert H.M., Loo J.V., Rastall R.A., Roberfroid M.B. 2004, Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev 17:259–75.

84. Heng N.C., Ragland N.L., Swe P.M., Baird H.J., Inglis M.A., Tagg J.R., Jack R.W. 2006, Dysgalacticin: a novel, plasmid-encoded antimicrobial protein (bacteriocin) produced by Streptococcus dysgalactiae subsp. equisimilis. Microbiology 152: 1991–2001

85. Cravotto, G., Boffa, L., Genzini, L. and Garella, D. 2010, Phytotherapeutics: an evaluation of the potential of 1000 plants. Journal of Clinical Pharmacy and Therapeutics, 35: 11–48.

86. Musa H.H., Wu S.L., Zhu C.H., Seri H.I. and Zhu G.Q., 2009, The Potential Benefits of Probiotics in Animal Production and Health, J. Anim. Vet. Adv., 8(2): 313-321

87. Chesson A., Flint H.J. 1999, Genetically modified ingredients in animal nutrition : Their safety and future. In : Brufau J. (ed.), Tacon A. (ed.). Feed manufacturing in

the Mediterranean region: Recent advances in research and technology. Zaragoza : CIHEAM, p.49-60 (Cahiers Options Méditerranéennes; n.37)

88. Petersen, T.N., Brunak, S., von Heijne G., Nielsen, H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions, Nature Methods, 8:785-786

89. Dan, H., Balachandran, A., Lin, M. 2009. A pair of ligation-independent Escherichia coli expression vectors for rapid addition of a polyhistidine affinity tag to the N- or C-termini of recombinant proteins. J Biomol Tech. 20(5):241-8.

90. Fimereli DK, Tsirigos KD, Litou ZI, Liakopoulos TD, Bagos PG and Hamodrakas SJ. 2012,CW-PRED: A HMM-based method for the classification of cell wall-anchored proteins of Gram-positive bacteria, Springer's Lecture Notes in Computer Science, Volume 7297/2012, p. 285-290

91. Tavaré JM, Fletcher LM, Welsh GI. 2001. Using green fluorescent protein to study intracellular signalling. J Endocrinol. Aug;170(2):297-306.

92. Ubbink J, Schär-Zammaretti P. 2005. Probing bacterial interactions: integrated approaches combining atomicforce microscopy, electron microscopy and biophysical techniques, Micron. 36(4):293-320. Epub 2005 Feb 17.

93. Crameri A., Whitehorn E.A., Tate E, Stemmer W.P.1996. Improved green fluorescent protein by molecular evolution using DNA shuffling.Nat Biotechnol. Mar;14(3):315-9.

94. Fisher A.C., Kim W., DeLisa M.P. 2006. Genetic selection for protein solubility enabled by the folding quality control feature of the twin-arginine translocation pathway. Protein Sci 15: 449–458.

95. Pérez-Arellano I, Pérez-Martínez G. 2003. Optimization of the green fluorescent protein (GFP) expression froma lactose-inducible promoter in Lactobacillus casei, FEMS Microbiol Lett. May 16;222(1):123-7.

96. Hu S, Kong J, Kong W, Guo T, Ji M. 2010. Characterization of a novel LysM domain from Lactobacillus fermentum bacteriophage endolysin and its use as an anchor to display heterologous proteins on the surfaces of lactic acid bacteria, Appl Environ Microbiol. Apr;76(8):2410-8. doi: 10.1128/AEM.01752-09. Epub 2010 Feb 19.

97. Morgan XC, Huttenhower C 2012. Chapter 12: Human Microbiome Analysis. PLoS Comput Biol 8(12): e1002808. doi:10.1371/journal.pcbi.1002808

Colin Ho Lim Chan

M.Sc Candidate • Microbiology & Immunology Department • University of Ottawa

EDUCATION

2010-2014 University of Ottawa Ottawa, ON
Masters of Science, Microbiology

2006–2010 University of Toronto Toronto, ON
Baccalaureate of Science, Specialist in Cell and Molecular Biology

WORK/VOLUNTEER EXPERIENCE

Winter 2013 Research assistant at the Hong Kong university of Science and Technology

Metagenomics research on cold seep bacteria in Dr. Pei-Yuan Qian's lab

- Learned organization, critical thinking and practical skills.
- Learned to correct scientific literature for academic journals
- Learned proper techniques for analyzing and quantifying the yield of DNA in low biomass sample to send out for pyro sequencing.

Fall 2010 Master's student at Dr. Min Lin's lab

- Research project on the development of GM bacteria for risk assessment in food safety.
- Learned organization, critical thinking and practical skills.
- Learned to write scientific literature for academic journals

Summer 2010 **Chemical Safety and WHMIS course**

- Learned about work safety and organizational skills

Research project with Dr. Xiao-an Zhang

- Learned organization, critical thinking and practical skills.
- Learned how to find literature and do academic writing

Winter 2010 **Science engagement student for Organic chemistry**

- Taught students basic lab skills and some course material
- Posted handouts and announcements over the black board site
- Gain communication, organization, teamwork and leadership skills

Volunteered at Dr.Harrison's lab

- Quantified Osteoclasts for osteoporosis studies

- Learned about Fluorescence imaging, cell development and interpreting slides.

Fall 2009 **Volunteer at the faculty of chemistry in UTSC**

- Helped in a chemistry demonstration for high school students
- Helped in the lab preparation
- Experience gained in organization, teamwork and social skills

Volunteer at BIOSA
UTSC, ON

- Helped in the lab coat sale
- Helped in tutoring chemistry
- Helped in a fusion radio feature
- Experience in teamwork and social skills

Volunteer at the 2009 UTSC Orientation

- Helped serve food and register new students
- Experience in teamwork and organization

Summer 2009 **Tutor(Volunteer)**
Yuen Long, Hong Kong

- Helped teach students English
- Experience in communication and organization skills

Summer 2008 **Activity Organizer (Volunteer)**
Yuen Long, Hong Kong

- Organized children's activities for the Yuen Long town hall
- Experience in leadership and organizational skills

2007 **Mentoring (Volunteer)**
Toronto, ON

- Mentored students in math and science
- Helped students build a better understanding of math/sciences
- Experience in communication and social skills
-

March 2003 **Volunteer for Arts and Crafts (Volunteer)**
Kanata, ON

- Supervised the arts and crafts for children sponsored by the Kanata Food Cupboard
- Experience in working in a team

Summer 2003 **Food Drive Organizer/Food distribution (Volunteer)**
Kanata, ON

- Organized food drives for the Kanata Food Cupboard

- Helped with the organization and distribution of food for the Kanata Food Cupboard
- Experience in organizational skills and teamwork

ACHIEVEMENTS/AWARDS

- Graduated on with distinction from the University of Toronto 2010
-
- Volunteer Training Certificate 2008
- Ontario Scholar Certificate 2006
- National Biology Scholar Certificate 2006
- Earl of March Secondary School Honour Role 2006
- Earl of March Secondary School Honour Role 2005
- Earl of March Secondary School Honour Role 2004

INTERESTS

- Playing piano
- Reading both fictional and non-fictional novels
- Running/jogging, swimming, and scuba diving

LANGUAGES

- Fluent in English and Cantonese (spoken)