INTESTINAL DELIVERY OF HETEROLOGOUS PEPTIDES WITH LACTOBACILLI

BY

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THESIS

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ABSTRACT

Oral administration of autoantigen is a promising method to induce oral tolerance in autoimmune diseases, such as multiple sclerosis. Multiple sclerosis (MS) is a complex disorder of the central nervous system. MS is caused by destruction of several brain antigens in myelin. In order to increase the efficiency of oral tolerance induction, lactobacilli were developed as a tool to deliver heterologous protein into the gastrointestinal tract. *Lactobacillus* spp. are a potential delivery vehicle for oral antigens because of their generally regarded as safe (GRAS) status, ability to persist in the acidic environment of human gastrointestinal tract and also their health benefit to the host. The goal of this study was to utilize lactobacilli as a genetic tool for heterologous protein expression and display. Three strains of lactobacilli were selected, and they are *L. acidophilus* ATCC 4356, *L. gasseri* ATCC 33323, and *L. salivarius* ATCC 11741. Two strategies have been discussed in this thesis to develop lactobacilli delivery system. The first strategy developed genetically modified lactobacilli which express myelin epitopes from proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). Two series of vectors has been constructed which direct the expression of these antigens either anchored to the cell wall or secreted into the environment. However, we only confirmed that PLP epitopes expressed from cell lysate of recombinant *L. acidophilus*, which contained plasmid with only secreted signal. We decided to develop the second strategy. The second strategy explored the non-covalent attachment of myelin epitopes to the cell wall of *Lactobacillus* spp. via cell wall binding domains. In this study, three non-covalent CWBD were selected including *L. gasseri* ATCC 33323 LysM domain-containing protein, *L. gasseri*

ATCC 33323 Lysozyme M1, Bacterial SH3 domain and C-terminal membrane anchor domain of *Lactococcus lactis* subsp. *cremoris* MG1363 AcmA protein. Through working with these domains, we found that *L. gasseri* ATCC 33323 Lysozyme M1 – bacterial SH3 domain can successfully bind on the exterior cell surface to *L. acidophilus* ATCC 4356, *L. gasseri* ATCC 33323, and *L. salivarius* ATCC 11741. Therefore, SH3 domain may be a good tool for oral administration by binding heterologous epitopes to lactobacilli. In future study, myelin epitopes and SH3 domain fusion proteins need to be displayed on the cell wall surface of lactobacilli. An animal model of multiple sclerosis is also important to evaluate an immune response of lactobacilli delivery system.

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TABLE OF CONTENTS

LIST OF FIGURES vii

LIST OF TABLES viii

CHAPTER 1 INTRODUCTION 1

1.1 Motivation 1

1.2 Objective 2

1.3 Organization of thesis 2

CHAPTER 2 LITERATURE REVIEW 4

2.1 Multiple sclerosis 4

2.2 Mucosal immunology 8

2.3 Lactobacilli 12

2.4 Mucosal delivery of therapeutic molecules using LAB 22

2.5 Future prospects 28

CHAPTER 3 RECOMBINANT LACTOBACILLI FOR SURFACE DISPLAY AND SECRETE OF MYELIN ANTIGEN 31

3.1 Introduction 31

3.2 Material and method 33

3.3 Results 37

3.4 Discussion 38

3.5 Conclusion 41

CHAPTER 4 CELL WALL BINDING OF HETEROLOGOUS PROTEIN TO THE EXTERIOR SURFACE OF *LACTOBACILLUS* SPP. 48

4.1 Introduction 48

4.2 Materials and methods 50

4.3 Results 54

4.4 Discussion 56
4.5 Conclusion 57
CHAPTER 5 SUMMARY AND FUTURE DIRECTION 63
REFERENCES 65
APPENDIX A CHAPTER 3 PLASMID MAP 81
APPENDIX B CHAPTER 4 PLASMID MAP 90

LIST OF FIGURES

Figure 2.1 The pathogenesis of multiple sclerosis (Adapted from Murphy, 2012) 29
Figure 3.1 Gene map of an expression cassette for PLP, MOG and PLP/MOG epitopes........ 45
Figure 3.2 PCR verification of the inserts........ 46
Figure 3.3 Soluble bacterial lysates from recombinant LA 4356........ 47
Figure 4.1 Gene map of an expression vectors for cell wall binding domain and genes of PLP/MOG epitopes ligated into pRSETB........ 60
Figure 4.2 SDS-PAGE of total, soluble and insoluble bacterial lysates from recombinant *E. coli* BL21 (DE3)........ 61
Figure 4.3 Fluorescence micrographs of the binding of the fusion protein EGFP_SH3 and EGFP to LG33323, LA4356, LS11741........ 62
Figure A.1 Map of pMJM-8........ 81
Figure A.2 Map of pMJM-13........ 82
Figure A.3 Map of pMJM-14........ 83
Figure A.4 Map of pMJM-19........ 84
Figure A.5 Map of pMJM-20........ 85
Figure A.6 Map of pMJM-21........ 86
Figure A.7 Map of pMJM-22........ 87
Figure A.8 Map of pMJM-23........ 88
Figure A.9 Map of pMJM-24........ 89
Figure B.1 Map of pMJM-35........ 90
Figure B.2 Map of pMJM-36........ 91
Figure B.3 Map of pMJM-37........ 92
Figure B.4 Map of pMJM-40........ 93
Figure B.5 Map of pMJM-41........ 94
Figure B.6 Map of pMJM-42........ 95
Figure B.7 Map of pMJM-44........ 96

LIST OF TABLES

Table 2.1 Recombinant Lactobacilli and Lactococci expressing autoantigens in autoimmune diseases 30
Table 3.1 Bacterial strains and plasmids 42
Table 3.2 Primers used in this study 44
Table 4.1 Bacterial strains and plasmids 58
Table 4.2 Primers used in this study 59

CHAPTER 1 INTRODUCTION

1.1 Motivation

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system, which causes multiple locations in the brain, spinal cord and other areas of the body (Awad, 1984). Work by our collaborator Dr. Mangalam has identified several important epitopes implicated in MS. These epitopes serve as the autoantigen including proteolipid protein (PLP) amino acids: 37-71, 89-154, 179-238, 264-277 and myelin-oligodendrocyte glycoprotein (MOG) amino acids: 30-150 and 181-203.

The diverse species of lactic acid bacteria (LAB) have been considered as a safe and convenient vehicle to delivery autoantigens or peptides in the human gastrointestinal tract (Pouwels, Leer, & Boersma, 1996). Initially, several mumbers of our lactobacilli culture collection were screened to identify isolates that were amenable to our genetic tools. From that group, we identified three strains that were good candidates for further study: *L. acidophilus* ATCC 4356, *L. gasseri* ATCC 33323, and *L. salivarius* ATCC 11741. These three strains were sent to Mayo Clinic where our collaborator (Ashu Mangalam) tested their inherent ability to suppress MS in a rodent model. Surprisingly, *L. gasseri* ATCC 33323 had a moderate suppressing effect; *L. salivarius* ATCC 11741 had a strong suppressing effect whereas *L. acidophilus* ATCC 4356 had no effect. Our collaborator was very excited about the inherent ability of *L. salivarius* ATCC 11741 to suppress the development of MS and we have decided to focus our future efforts on these three strains.

1.2 Objective

The objective of this study is to deliver heterologous target peptides (PLP and MOG) by lactobacilli to promote tolerance in multiple sclerosis. The lactobacilli genetic tools that enable secreted and cell-wall anchored expression or cell wall binding on the surface of cell wall were developed.

1.3 Organization of thesis

Chapter 2 is a literature review. First, background of MS disease was introduced and the mucosal immunological responses of both pathogenic infection and food protein (oral tolerance) were described. Moreover, characteristics of lactobacilli as probiotics and their applications in food were discussed. Finally, lactobacilli as intestinal delivery system that applied for oral vaccination and oral tolerance was summarized.

Chapter 3 is about constructing recombinant lactobacilli to express these epitopes (PLP, MOG or fusions of these two epitopes) under a promoter fused to a secretion signal with or without an anchoring signal. The method for plasmid construction and protein validation were described in this chapter. Some potential issues and problems for plasmid transformation and protein expression of lactobacilli in this experiment were also explained and discussed.

Chapter 4 described the second strategy of heterologous protein display on the cell wall surface of lactobacilli by cell wall binding domain (CWBD). Three different CWBD were selected to fuse with either green fluorescence protein (GFP) or our target myelin epitopes. There were two aims of this experiment. The first aim is to test the binding affinity of these CWBD by observing a number of GFP_CWBD fusion proteins. The second

aim is to bind myelin epitopes_CWBD fusion protein to the surface of lactobacilli. The methods of construct plasmid and protein validation on the surface of lactobacilli were discussed.

Finally, conclusions are listed in Chapter 5. Lactobacilli could be used as potential live carrier of heterologous protein. Different heterologous protein cell wall display strategies, expression system or CWBD system, may also be applicable for other LAB. Future studies were listed in this chapter as well.

CHAPTER 2 LITERATURE REVIEW

2.1 Multiple sclerosis

Definition of MS

Multiple sclerosis (MS) is a complex disorder of the central nervous system (CNS) (Franklin, Franklin, ffrench Constant, Edgar, & Smith, 2012). It is caused by the destruction of several neural antigens in myelin, including myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG). Its name is from the sclerotic lesion or plaques, which occur in the white matter of the central nervous system. People with MS disease develop variable symptoms, including fatigue, pain, weakness, dizziness, walking difficulties, vision problem, bladder problems and bowel problems (Kenneth Murphy, 2012). An estimated 0.1% of the population, approximately 400,000 people in United States and about 2.5 million people around the world have MS diseases, which are commonly diagnosed between the ages of 20 to 40. In United State, about 200 new cases are diagnosed each week (Multiple sclerosis in America 2013.2013).

Mechanism and risk factors

Figure 2.1 shows the pathogenesis of multiple sclerosis. Lymphocytes and other blood cells usually do not cross the blood-brain barrier. However, unknown triggers cause these cells to cross this barrier from blood vessels. Leukocytes, macrophages and blood proteins start to enter the brain. Then, activated CD4 T cells autoreactive for neural antigens migrate out of the blood vessel, and they reencounter their specific autoantigen presented by MHC class II molecules on microglical cells. Microglia are macrophage-like cells of the innate immune system resident in the central nervous system, which can act as antigen-

presenting cells. Inflammation causes increased vascular permeability and more TH17 and TH1 cells move into the brain. Cytokines and chemokines (IL-17 and IFN-g) are produced by the infiltrating effector T cells, and then recruit myeloid cells that enhance the inflammation. It recruits of T cells, B cells and innate immune cells to the site of the lesion. Autoreactive activities cause demyelination of myelin in the brain. (Kenneth Murphy, 2012).

The demyelination of myelin can be triggered by various factors. Although the cause of multiple sclerosis is still understudied, it is assumed that the cooperation of immunology, infections, genetics and environment factors are involved in MS. Current studies showed that smoking (Salzer et al., 2013), vitamin D deficiency (Martinelli et al., 2014), HLA haplotype (Callander, 2007), and Epstein-Barr virus (Salzer, Stenlund, & Sundstrom, 2014) could also heighten MS. Moreover, women are more likely develop this disease than men. The ratio of women with MS to men with MS is 2:1. Also, it will significantly raise the chance of developing the disease if their parent or sibling has MS. Additionally, MS is more prevalent in certain areas including the northern United States, southern Canada, Europe, New Zealand and southeastern Australia.

Myelin protein

The myelin sheath is a considerably extended and modified plasma membrane that surrounds nerve axons and support electrical impulses to transmit quickly and efficiently along the nerve cell (George J. Siegel, Bernard W. Agranoff, 1999). Protein components of the myelin sheath have been regarded as the targets of MS. (Ben Nun et al., 1996) reviewed possible target antigens based on their location, percent in CNS myelin, demyelinating

antibodies and T-cell reactivity in MS and identified the major myelin proteins involved in MS as PLP, MBP and MOG.

There are several reasons why PLP become a great interest in studying as a potential target antigen in MS. First, PLP is the primary protein component of myelin in CNS (>50% of total protein) and plays a significant role in myelin structure and function (Roland Martin, 2010). PLP has been confirmed to be encephalitogenic (the antigen produce experimental allergic brain disease) in various animal models. (Takashi Yamamura, 1986) demonstrated PLP-induced experimental autoimmune encephalomyelitis (EAE, animal model of MS) with significant demyelination in rat. This study had a tremendous contribution for the study of autoimmune demyelination. PLP is also encephalitogenic produce demyelination in CNS in rabbits (Selmaj, 1991), guinea pigs (Yoshimura, 1985) and mice (J. L. Trotter, 1987). Furthermore, more demyelination was observed with PLP-induced EAE compared to other myelin protein induced EAE, which infers that PLP-induced EAE is more related model to MS (Tabira, 1988). In human study, immunodominant epitopes of PLP that can be processed from whole PLP by human antigen presenting cells (APC). These epitopes typically lie within the 30-60 and 180-230 regions of PLP (Greer, 1997; J. Trotter et al., 1998).

MBP is the second most abundant protein, after PLP, in CNS. It is the most characterized human autoantigen so far, and comprising 30% of the total protein and about 10% of dry weight of myelin (Moscarello, 1997). The T-cell response to MBP has been analyzed by several studies. One of them tested a kinetic response of peripheral blood mononuclear cells from MS patients and healthy individuals. A significant response to eight

MBP regions (1-24, 30-45, 78-99, 90-114, 105-129, 120-144, 135-159 and 150-170) have been identified in a majority MS patients (Mazza et al., 2002). Moreover, the immune response of MS is also associated with the major histocompatibility complex class II phenotype DR2. Compared to the healthy group, higher frequency of MBP specific T-cell lines react with a DR2-associated region of MBP was observed in MS patients (Ota, 1990; K. J. Smith, Pyrdol, Gauthier, Wiley, & Wucherpfennig, 1998).

MOG is another important autoantigen associated to the pathogenesis of both MS and EAE. It is a minor component of the myelin sheath, comprising only about 0.05% of total myelin protein. Comparing peripheral blood lymphocytes in MS patients with control group, T-cell reactivity against MOG has been demonstrated (De Rosbo, 1993). In various animal studies, severe neurological disease was caused by immunization with MOG. The disease was similar with the clinical, pathological and immunological features of MS (Bernard et al., 1997; Linington, 1993). There are also a number of identified encephalitogenic epitopes of MOG, such as MOG 1-22, MOG 35-55 and MOG 92-106 in different animal models. Particularly, MOG 35-55 peptide is highly encephalitogenic and can induce strong T and B cell response (Amor, 1994; Ichikawa, 1996; Linington, 1993).

Treatments and drugs

In the past 30 years, a lot of effective treatments and drugs have been developed and are commercially available to slow progress of the disease or treat symptoms. Currently, there are a total number of eleven U.S. Food and Drug Administration (FDA)-approved therapies for MS, including Aubagio (teriflunomide), Avonex/ Rebif (IFNβ-1a), Betaseron/Betaferon/Extavia (IFNβ-1b), Copaxon (glatiramer acetate), Novantrone

(mitoxantrone), Tysabri (natalizumab), Gilenya (fingolimod), Plegridy (peginterferon-β1a) and Tecfidera (dimethyl fumarate) (National multiple sclerosis society.2014; Castro Borrero et al., 2012a). All of these medications could help MS patients regulate their disease and enhance their comfort and quality of life. Each of these therapies has their dose or route, mechanism of action, clinic benefit and side effects. Depending on the individual MS patient and stages of illness, different drugs are selected. For example, patients with clinically isolated syndrome have a lower risk of conversion to clinically definite MS if they receive early treatment of IFN β-1b compared to delayed treatment (Kappos et al., 2009). Therefore, IFN-β should be considered as the first treatment in newly diagnosed MS patients. For patients who fail to respond to the first-line agents, alternative treatments may be used, such as natalizumab or fingolimod (summarized by Castro Borrero et al., 2012a).

Much research is investigating this disease and developing new treatments and drugs. In particular, therapies with acceptable is long-term safety and efficacy profiles of oral, intramuscular and subcutaneous agents need to be developed. Moreover, the ideal dosing, length of treatment and side effects are also key factors to decrease inflammation and relapse.

2.2 Mucosal immunology

The importance of the mucosal system

The large internal body surface (about 400 m^2) is bounded by mucosal epithelia, such as the gastrointestinal tract, the upper and lower respiratory tract and the urogenital tract. The mucosal surface is a crucial physic-chemical barrier to protect tissues from pathogenic

microorganisms. It could also present pathogens to the immune system (Cerf Bensussan & Gaboriau Routhiau, 2010) through their physiological activities, such as gas exchange, food absorption, sensory activities of eyes, nose, mouth and throat, and reproduction. On the other hand, because of their fragility and permeability, they can be infected easily by numerous bacteria and viruses. Mucosal infections include acute respiratory infections, diarrheal diseases, human immunodeficiency virus (HIV), tuberculosis, measles, whooping cough, hepatitis B, roundworm and hookworm. All of these diseases are significant health problems in the world and cause a large number of deaths (Kenneth Murphy, 2012). For instance, seasonal flu, one of the respiratory infections is caused by seasonal influenza. According to estimated data of annual influenza-associated deaths by the Centers for Disease Control and Prevention (CDC), it showed that the flu-associated death ranged from 3,000 to about 49,000 people from year 1976-1977 seasons to the year 2006-2007 flu season (CDC, 2013).

Intestinal mucosal response to infection

The gut is the most frequent site of infection by pathogenic microorganisms, including many viruses and enteric bacteria, protozoans and multicellular helminths parasites, which can cause diseases in different ways. CDC has estimated that 31known pathogens and an unknown number of unspecified agents transmitted through food cause of 47.8 million foodborne illnesses, 127,839 hospitalizations and 3,037 deaths annually in US(CDC, 2011). Within those pathogens, *Salmonella*, *Toxoplasma gondii*, *Listeria monocytogenes*, Norovirus and *Campylobacter* spp. are the top five known pathogens contributing to foodborne illnesses resulting in death. Therefore, it is crucial to understand how they stimulate

immune responses. Essentially, pathogenic antigens are transported into the mucosa through microfold cells (M cell), which are specialized epithelial cells in a layer that separate the lymphoid tissues from the gut lumen. M cells can take up antigens from the gut lumen by endocytosis and phagocytosis. Then the M cell translocate to the subepithelial dome, which is rich in dendritic cells, T cells and B cells. After the transportation, antigens can be recognized by dendritic cell, and then T cells are activated sent out to the site of infection (Kenneth Murphy, 2012).

Even though various enteric pathogens have similar routes into lymphoid tissue, the host has quite distinct immune responses tailored for every individual pathogen. For example, *Salmonella*, an important cause of foodborne illness, can enter the gut epithelial layer by three ways, including enter through M cell and infect microphage, directly invade gut epithelial cells, and luminal capture by dendritic cells. Then, chemokines and cytokines, which are small protein that produced by macrophages, recruit neutrophils and activate them. Also, dendritic cells loaded with antigens travel to the mesenteric lymph node and trigger an adaptive immune response (Cossart, 2004). In contrast, *Shigella flexneri*, which cause bacterial dysentery, is not directly recognized by macrophages and epithelial cell after pass through M cell. Once Shigellae penetrate gut epithelium, it starts to invade and spread to epithelial cells. Then, shigella antigen is recognized by nucleotide-binding oligomerization domain (NOD) and active the NFkB pathway, which induce the expression of pro-inflammatory genes and recruit neutrophils (Carneiro et al., 2009).

Commensal bacteria and food protein in the gut

Clonal selection of lymphocytes is one of the most important principles for immunological tolerance. During lymphocyte differentiation, a large number of B- and T-cell receptors can recognize numerous self-antigens. Consequently, most of the self-reactive lymphocytes are removed by the process of central tolerance in thymus and bone marrow (Burnet, 1959). Although food protein and the microbiota contain many nonself-antigens, they can be recognized by the adaptive immune system. The immune system is extremely talented to distinguish pathogens and innocuous antigens since it is inappropriate and wasteful to target inoffensive antigens for preserving immune response. Most of these antigens together with commensal bacteria exist in the natural mucosal immune system is not only innocent but also highly advantageous to the host. This is best observed in the GIT, which is exposed over 100 g of foreign protein per day in our diet and up to 1,012 commensal microorganisms per gram in the colon. These microorganisms are from thousands of species of bacteria and live in symbiosis with their host (Kenneth Murphy, 2012).

At the moment that food protein antigen or commensal bacteria were first introduced into the GIT, like many other foreign antigens, their self-reactive T-cell was not deleted in the thymus during lymphocyte development. However, they usually do not induce inflammatory immune responses. The phenomenon that oral administration antigen induces the hyporesponsiveness in an immunogenic form is called oral tolerance (reviewed by D. W. Smith, 2005). Oral tolerance can be induced in various mechanisms in peripheral tolerance, including clonal deletion or anergy of T cells and active regulation by the

regulatory T cells (Treg). Innocuous antigens will be presented by major histocompatibility complex (MHC) class I and class II molecules on dendritic cells once they enter the gut epithelial layer. Nevertheless, peripheral tissues do not express co-stimulatory molecules, which is a significant molecule to induce inflammatory responses. Food antigen-reactive T-cell becomes angergic or eliminated to prevent clonal expansion and immune response (Schwartz, 2003). Another mechanism is active suppression by T_{reg}. CD103, a specific kind of dendritic cells, is most likely in charge of taking up food antigens after feeding to animals. Then the complex of CD 103 moves these antigens to the mesenteric lymph nodes, which lies between the layers of mesentery. This delivery action could enhance the production of gut-homing FoxP3-positive Treg cells, thereby, the systemic immune response was suppressed (Hand & Belkaid, 2010). Additionally, there are also many other important cytokines contribute to oral tolerance, such as TGF-β, IL-10 and IFN-g (Dubin & Kolls, 2008).

2.3 Lactobacilli

Lactobacillus spp. are generally defined as a group of Gram-positive, cocci or rods, non-mobile, facultatively anaerobic, fermentative, non-spore forming and catalase-negative bacteria (Makarova et al., 2006). Many lactobacilli are highly associated with food fermentation where lactic acid production is needed and they are capable surviving in an acidic environment, blow pH of 4-5 (reviewed by Anjum et al., 2014). *Lactobacillus spp.* are not only widely found in fermented foods but also in the oral cavity and gastrointestinal tracts (E. Vaughan, de Vries, Zoetendal, Ben Amor, Akkermans, & de Vos, 2002a). Lactobacilli is also one of the most popular effective probiotic organisms, which are defined

as “living micro-organisms which, when administered in adequate amounts, confer a health benefits on the host” (WHO, 2001). It is important to understand their characteristics and probiotics properties in human and animals.

Lactobacillus spp. classification

Lactobacillus spp. is one of the main genres of LAB. Its other genera are *Lactococcus*, *Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Weissella, Carnobacterium* and *Tetragenococcus* (Klein, Pack, Bonaparte, & Reuter, 1998). Similar with other LAB genre, *Lactobacillus* spp. could go through two types of fermentation, either homofermentative or heterofermentative in order to metabolize hexose sugar. Lactic acid is the primary end product during the homofermentative pathway; lactic acid, CO2, acetic acid and ethanol are produced in the heterofermentative pathway (Kandler, 1983). The Lactobacillus has been classified into three subgenera depends on carbohydrate metabolism and different temperature growth: Streptobacterium, Betabacterium, and Thermobacterium (Sharpe, 1981).

Streptobacterium are facultative heterofermentative species which can ferment glucose, and grow at 15°C, not 45°C, such as *L. plantarum, L. casei*, and *L. sakei*. Betabacterium are strict heterofermentative species, e.g. *L. brevis, L. fermentum* and *L. reuteri*, and they form CO2 by glucose fermentation, and mostly hydrolyze arginine (Schillinger, 1987). Thermobacterium are strict thermophilic and homofermentative species including *L. delbrueckii*, *L. acidophilus* several other species. Thermobacterium, which differ from other two subgenera, grow at 45°C, but 15°C, do not ferment ribose, and do not the hydrolyze arginine (BARRE, 1978; Sharpe, 1979).

Protein transport pathways

Some proteins, which are synthesized in the lactobacilli cytosol, will not be functional until they could embed or cross through the cytoplasmic membrane. There are principally seven routes for protein transport in Gram-positive bacteria. They are the secretion (Sec), twin-arginine translocation (Tat), flagella export apparatus (FEA), fimbrilin-protein exporter (FPE), holing (pore-forming), peptide-efflux ABC and the WXG100 secretion system (Wss) pathways (Desvaux, Hébraud, Talon, & Henderson, 2009). Current studies have evaluated protein secretion pathways of 13 published genomes of *Lactobacillus* spp. Based on these Lactobacillus genomes, they carry genes encoding Sec, FPE, peptide-efflux ABC and holin systems (reviewed by Kleerebezem et al., 2010).

The Sec translocase is the most important secretion mechanism that transfers protein across the cytoplasmic membrane. It consists of a protein-conducting channel (the SecYEG complex), peripherally energy (ATP-driven motor protein, SecA), an accessory protein SecDF (yajC) and YidC membrane protein. Secretory protein can be targeted to Sec translocase, specific to SecA, by their signal sequence or the assist of the molecular chaperone SecB. All Sec translocase targeted proteins contain an N-terminal signal peptide. The signal peptide contains three regions including the N region (positive charged), the H region (hydrophobic residues) and the C region (contain a signal peptidase cleavage site) (Driessen, Driessen, & Nouwen, 2008). During protein translocation, the signal peptide of precursor protein can be cleaved by signal peptidases (SPases). Different types of SPases have been found to recognize various cleavage site, such as AxAA cleavage site and lipobox cleavage site (Sutcliffe, 2002; van Roosmalen et al., 2004).

Other secretion mechanisms, FPE, peptide-efflux ABC and holin systems, contribute to different classes of protein or DNA translocation significantly. For example, holins are important integral membrane proteins that are associated with muralytic enzymes secretion pathway, which is a part of the cell lysis system (Wang, Smith, & Young, 2000). The FPE pathway is a mechanism allowing exogenous DNA transfer into naturally competent bacteria (I. Chen & Dubnau, 2004). The principal export pathway of antimicrobial peptides, including lantibiotics, bacteriocins and competence peptides, is peptide efflux ABC transporters (ATP-binding cassette transporters, (Håvarstein, Havarstein, Diep, & Nes, 1995). So far, functional ABC transporters have been identified in *L. acidophilus* and *L. plantarum* (Diep, 1996; Dobson, Sanozky Dawes, & Klaenhammer, 2007).

Cell-wall-binding domain (CWBD) of Lactobacilli

After proteins transported across the *Lactobacillus* cytoplasmic membrane, they are either secreted and released from the bacterial cell or surface-associated to the cell wall. For these surface-associated proteins, CWBD plays a role of bridge between proteins and cell wall. The CWBD could be classified into two categories based on different binding mechanisms: covalently anchored and non-covalent CWBD (reviewed by Kleerebezem et al., 2010).

Covalently anchored proteins have either a N- or C-terminal anchor sequence. As mentioned above, the Sec translocation pathway targeted protein contains N, H and C regions of signal peptides in N-terminal. During the Sec translocation process, the particular sequence of C region could be targeted by SPase, and their signal peptide can be

removed before the protein released. However, most of the Sec-translocated proteins do not contain the cleavage sites in C region (Zhou, Boekhorst, Francke, & Siezen, 2008), and will remain N-terminally-anchored to the cell membrane. In fact, a large number of N-terminally membrane-anchored proteins have been predicted in Lactobacillus genomes. The majority of these proteins are involved in of signal transductions, protein turnover, competence and cell-envelop metabolism (Kleerebezem et al., 2010). Additionally, Lactobacillus genomes also encode a minor amount of C-terminally anchored proteins, but their functions are largely unknown.

Lipoprotein is another covalent CWBD in lactobacilli secreted proteins. These proteins have a signal peptide and undergo Sec pathway. Similar with N- or C-terminally anchored protein, lipoprotein signal peptides also contain N, H and C regions, and their C region contains the lipbox motif, which is important for lipoprotein biogenesis machinery after transport. In the process of covalent bonding of lipoprotein, first, Cys-residue in lipbox is diacylglyceryl modified by the lipoprotein discylglyceryl transferase. Then, N-terminal of the Cys-residue is cleaved by SPase and anchoring protein by thioether linkage. Majority of lipoproteins that found in *Lactobacillus* spp. are components of ABC transporters and adhesion proteins (Hutchings, Palmer, Harrington, & Sutcliffe, 2009).

LPxTG-anchored proteins are a well-known family of protein as well, which can covalently attach to the peptidoglycan of cell wall. N-terminal of these proteins contains a signal sequence in its C region. The signal sequence will be removed by SPase upon secretion by Sec pathway. C-terminal of these proteins contain LPxTG (Leu-Pro-any-Thr-Gly) cell-wall-sorting motif (Boekhorst, de Been, M. W. H. J., Kleerebezem, & Siezen, 2005)

that can be recognized and cleaved by sortase enzyme. When sortase enzyme, transpeptidase, targets Thr-Gly region, transpeptidation occurs and then the protein covalently attaches the threonine carboxyl group to the peptidoglycan on the cell wall (Marraffini, DeDent, & Schneewind, 2006). A number of sortase and LPxTG-motif containing protein were predicted in different *Lactobacillus* genomes, such as *L. delbruekii bulgaricus* ATCC-BAA-365 and ATCC11842 and *L. plantarum* WFSI (Kleerebezem et al., 2010).

Non-covalent CWBD protein is another important subset of surface-associated protein. Within this subgroup, the LysM (Pfam PF01476) domain is commonly utilized. It consists of repeat units of a small LysM motif and usually able to be found in many extracellular enzymes. LysM domain has the capability to anchor proteins to the peptidoglycan layer of Gram-positive bacteria (Buist, Steen, Kok, & Kuipers, 2008). Currently, widespread utilization of the LysM domain has been used for detection of bacteria and display of enzymes and proteins on the extracellular surface of *Lactobacillus* spp. (Visweswaran et al., 2014).

The SLH domain, CWBD of S-layer protein, is typically a part of extracellular carbohydrate-binding proteins and contains 10-15 conserved amino acids (Jarosch, 2000). S-layer protein, which is paracrystalline monolayer, can non-covalently anchor to the peptidoglycan-associated polymers through an S-layer homology (SLH) domain (Fujino, 1993; Lupas, 1994). Recently, more than 40 SLH containing proteins have been found in Gram-positive bacteria (Engelhardt & Peters, 1998). The large number of S-layer proteins has also been identified in *L. acidophilus, L. helveticus* and *L. brevis* (Åvall Jääskeläinen,

AVALLJAASKELAINEN, & PALVA, 2005; Goh et al., 2009; Vilen et al., 2009). Furthermore, the SLH domain has developed into a sufficient tool to display heterologous antigens to the cell wall (Mesnage, Tosi Couture, & Fouet, 1999).

Another popular non-covalent CWBD is the eukaryotic SH3 domain. This domain can target and anchor proteins to the peptidoglycan layer by recognition of specific sequences within the cross-linking peptide bridges (Xu et al., 2011). Several proteins containing SH3 have been identified in some *Lactobacillus* spp., such as *L. brevis* ATCC 367, *L. casei* ATCC 334, *L. gasseri* ATCC 33323, *L. plantarum* WCFS1, and most of their functions are in cell wall turnover (Kleerebezem et al., 2010).

Application of Lactobacilli in food and their probiotics characteristics

The lactobacilli, generally recognized as safe (GRAS), are one of the predominant bacteria that widely utilized in food fermentation. They can be obtained from the yogurt, cheese, fermented milk, sausage and other fermented foods. *Lactobacillus* spp. are also indigenous to food habitats, like cereal grains, vegetables, fruits, and milk environments (Carr, Chill, & Maida, 2002). Their ancient anthropological role in food preservation is to ferment carbohydrate to lactic acid and provide the acidic environment for food material (Kleerebezem et al., 2003). Acidifying raw materials could enhance texture, flavor, microbial safety, increase shelf-life, control putrefactive microorganisms and resistant to microbial spoilage and food toxin (Rhee, Lee, & Lee, 2011). Lactobacilli also produce lots of other compounds to including natural antimicrobial substances, sugar polymers, sweeteners, enzymes and vitamins (Leroy & De Vuyst, 2004).

Lactobacilli are usually found in dairy and meat fermentations and even oral cavity (Ahrné et al., 1998; Colloca, 2000). Some *Lactobacillus* strains, like *L. delbrueckii subsp bulgaricus, L. acidophilus, L. rhamnosus, L. reuteri* and *L. casei*, have been used in probiotic preparations for human consumption (Fooks, Fuller, & Gibson, 1999). Lactobacilli belong to the original microflora of humans and colonize many locations of the body (Axelsson L, 2004), thus different *Lactobacillus* spp. confer health benefits in various ways. In following three paragraphs, *L. acidophilus, L. gasseri and L. salivarius* are discussed in terms of food utilization and their probiotic effects.

L. acidophilus is one of the most prevalent organisms for dietary use (N. Shah, 2007) and major commercial species of lactic acid bacteria. It is widely accessible in milk, yogurt, toddler formula and other supplements products (M. E. Sanders, 2001). It also involved in the production of fermented soymilk and different kind of cheese, such as cheddar cheese, minas fresh cheese, probiotic white cheese and Gouda cheese (reviewed by Anjum et al., 2014). Historically, *L. acidophilus* has been utilized as part of starter cultures for milk fermentation and preservation for more than 10000 years (Tamime, 2002), and 80% of the yogurts contain *L. acidophilus* in the United State (M. Sanders, 2003). This species was not only added in the milk as a part of starter culture, but also be added for additional probiotic value (N. P. Shah, 2000). Probiotic strains of lactobacilli were discovered in many commercial food and pharmaceutical products (Yeung, 2002). (N. Shah, 2007) has summarized primary commercial probiotic strains of *L. acidophilus*, including *L. acidophilus* LA-1/LA-5 (Chr. Hansen), *L. acidophilus* NCFM (Rhodia), *L. acidophilus* La1(Nestle), *L. acidophilus* DDS-1 (Nebraska Culture), *L. acidophilus* SBT-2062 (Snow Brand Milk

products). *L. acidophilus* NCFM is the most common probiotic strain and well characterized. The characteristics of the probiotic strain have been showed in both physiology experiments and the context of feeding studies (Bull, Plummer, Marchesi, & Mahenthiralingam, 2013). In these physiological studies, the probiotic possess the capabilities to be stable in food products, resist to bile, survive in low pH, attach to human colonocytes in cell culture, produce antimicrobial and induce lactase activity. Additionally, probiotic effects also were observed in feeding studies. It has been found the effects of mediation of host immune system, lowering host serum cholesterol, improving host lactose metabolism and preventing or treating infections (reviewed by Bull et al., 2013). Furthermore, the probiotic effects of *L. acidophilus* have been investigated in several clinical trials. For example, probiotics containing *L. acidophilus* NCFM could reduce bloating in patients with bowel disorders (Ringel Kulka et al., 2011), suppress cold and influenza-like symptoms in children (Leyer, Li, Mubasher, Reifer, & Ouwehand, 2009) and suppress diet-induced hypercholesterolemia (Dheeraj, Kansal, Nagpal, Yamashiro, & Marotta, 2013).

L. gasseri plays an important role for food preservation because of its production of Gassericin A, which was first described in 1991. It is produced by *L. gasseri* LA 39, comprises 58 amino acids and carries a head-to-tail peptide bond to form circular bacteriocins (Kawai et al., 2001; Kawai, 1998). Bacteriocins have been identified as antimicrobial ribosomal peptides or proteinaceous complex that is made by both Gram-positive and Gram-negative bacteria. The circular structure of Gassericin A makes it less susceptible to proteolytic cleavage and tolerant of high pH and temperature (Pandey, Malik, Kaushik, & Singroha, 2013). Gassericin A has also shown activities against several

foodborne pathogenic bacteria, such as *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* (Kawai et al., 2001; Kawai, 1998). Interestingly, only limited strains of *Lactobacillus* produce this kind of bateriocins. Gassericin A from *L. gasseri* LA 39 is the first bacteriocin that showed the inhibition of food-borne pathogenic bacteria (Itoh, 1995). Moreover, *L. gasseri* also possesses probiotics properties on human health. *L. gasseri* can colonize in many areas in humans, including the GIT, oral cavity and vagina. That indicates its prevalence as commensal bacteria in healthy adults (Delgado, 2007; HOJO et al., 2007; Pot, B., LUDWIG, W., Kersters, K., SCHLEIFER, K., 1994). Consumption of *L. gasseri* potentially contributes to maintaining of gut homeostasis. Several studies showed that consumption of *L. gasseri* in probiotics treatment could increase the concentration of butyrate (Olivares, Diaz Ropero, Martin, Rodriguez, & Xaus, 2006), increase IgA secretion in the mucosal layer to reduce fecal cytotoxicity and decrease *Salmonella choleraesuis* infection (Margreiter, 2006).

L. salivarius is not a common species in starter cultures for food fermentation, but it is a promising probiotic species which heighten intestinal health (Neville & OToole, 2010). Five potential probiotics characteristics of *L. salivarius* have been summarized, and all of their properties indicate that *L. salivarius* could be an effective probiotic (Messaoudi et al., 2013). First, *L. salivarius* is resistant to acid and bile and able to adhere to intestinal cell. The high survival rate of *L. salivarius* CECT 5713 was obtained in an in vitro model of the human stomach and the small intestine, and the bacterium was firmly attaching to intestinal cells (Caco-2 and HT-29). The similar properties have been showed in *L. salivarius* SMXD51 as well (Maldonado et al., 2010; Messaoudi et al., 2012; Neville & OToole, 2010). Second, *L.*

salivarius should be safe to use both in vitro and vivo. For example, *L. salivarius* CECT 5713 has been demonstrated to be reliable in animal model and its behavior is similar with another probiotics *Lactobacillus* strains (Martín et al., 2006; Olivares et al., 2006). Moreover, antimicrobial activity of probiotic *L. salivarius* has been investigated in several studies. A number of *L. salivarius*, which were isolated from chicken intestine, have activity to against *Salmonella* and *Campylobacter jejuni* (Zhang et al., 2011). Another study showed the production of bacteriocin Abp 118 from *L. salivarius* UCC118 showed capacities to protect mice from infection by *Listeria monocytogenes* (Corr et al., 2007). Furthermore, probiotic bacteria could stimulate the immune system and protect the host from intestinal diseases. *L. salivarius* B1, *L. salivarius* CECT 5713 and *L. salivarius* UCC118, have been showed to improve host immunity by inducing different cytokines, such as Interleukin (IL)-10, IL-6 and IL-12 (Riboulet Bisson et al., 2012; Sierra et al., 2010; Zhang et al., 2011). *L. salivarius* SMXD51 was also found to help host fighting against pathogenic infection by the production of antimicrobial β-defensin 2 (Schlee et al., 2008).

2.4 Mucosal delivery of therapeutic molecules using LAB

LAB and other delivery systems

The primary entry for microorganism is mucosal surface (Sansonetti, 2004). Consequently, the immune system is very important at the mucosal surface (Kenneth Murphy, 2012). Mucosal immunization has been considered as an inexpensive and convenient technology to induce immunity in the mucosal surface. Several immunological studies showed that a delivery system is required to increase the uptake of antigen, avoid degradation in the gastrointestinal tract, and also stimulate adaptive immune responses

(Lavelle & OHagan, 2006; Neutra & Kozlowski, 2006). A number of live bacterial delivery systems have been developed for oral administration, including live attenuated pathogens and food-grade bacteria. Live attenuated pathogen, such as *Salmonella typhi* (Dertzbaugh, 1998) and Mycobacterium (Stover, 1993), are made by eliminating their virulence while maintaining their immunostimulation (Dertzbaugh, 1998). Over the past decades, researchers have designed vaccine strains of attenuated *Salmonella* spp. That met the balance between immunogenicity and the reactogenicity and minimized side effects in vivo (Tacket & Levine, 2007). However, the reversion of attenuated pathogens to wild-type phenotype is still considered as a potential risk in humans. Therefore, commensal and food-grade bacteria are safer alternative delivery vehicles because of their GRAS status (Dieye et al., 2003a; Zegers et al., 1999a).

Because of the unique properties of LAB that mentioned above in human gastrointestinal tract, it has been assessed as a candidate live oral delivery vector. One of the biggest advantages of LAB delivery system is their abilities to trigger the secretion of IgA response in addition to induce systemic immune response in many current vaccine (Lavelle & OHagan, 2006; Mannam, Jones, & Geller, 2004; Neutra & Kozlowski, 2006). Additionally, (Wells & Mercenier, 2008) reviewed several significant advantages of LAB as oral delivery vehicles, including survived through the human gastrointestinal tract, safety, stimulating both systemic and mucosal immune response by immigrating to Peyer's patches and expressing target molecules and adjuvants. Additionally, they can also be engineered to express heterologous antigens and adjuvants.

Vaccine delivery

In the last decade, numbers of researchers have developed recombinant LAB as genetic vaccine delivery tools for expression of heterologous protein (de Vos, 1999). Various strains of carrier, different cellular location and amount of expressed antigen could influence immune response to the host.

A single model antigen, tetanus toxin fragment C (TTFC) was investigated by expressing in three different bacterial strains, *Lactococcus lactis*, *Lactobacillus* spp. and *Streptococcus gordonii* in the murine gastrointestinal tract, oral cavity or vaginal cavity (Hanniffy et al., 2004a; Mercenier, 2000; Norton, 1996a). According to the results of several studies, the expressions of TTFC from *Lactococcus lactis* and *Lactobacillus* spp. have been shown to elicit protective immune response against tetanus toxin. However, for some perspectives, the results cannot compare with each other directly because they are inconsistent in dosage and other parameters (Wells & Mercenier, 2008). This discovery could imply that it is important to select strains of LAB when designing mucosal vaccine, because their expressed protein may perform differently to the immune system.

Moreover, the final cellular location of heterologous protein, including cytoplasmic, secreted, anchored to the cell wall, is also essential to influence the immunogenicity. For instance, the E7 antigen is a primary protein from human papillomavirus type-16. This antigen was expressed in *Lactococcus lactis*, and three cell locations of the antigen were evaluated, intracellular, secreted or anchored to the cell wall. After testing antigen immunogenicity by measuring their cellular immune responses, it has been found that the mice with cell wall anchored E7 antigen could stimulate higher level of cytokine responses

and induce highest immune responses. In contrast, the one with secreted E7 antigen have the lowest immune responses (Bermúdez Humarán & Bermudez-Humaran, 2004a). Although the results may be showed differently in other models of disease or infections, this result provided the excellent idea for future people to decide appropriate mucosal vaccine by presenting antigen in different locations.

Lastly, level of antigen expression or amount of antigen uptake is also significant for immune responses in particular studies. Evidence from existing literature has indicated that more doses of antigen could boost higher antibody response in intragastric route. For example, different level expression of TTFC from *L. lactis* has been evaluated by antibody response. They found that a larger number of antibodies were triggered with increasing amount of TTFC (Wells & Mercenier, 2008). However, another study showed that the dosage may not play an essential role in immune response. Recombinant *Lactococcus* was designed to express C-repeated region (CRR) of M protein from *Streptococcus pyogenes* to against pharyngeal infection with *S. pyogenes*. This vaccine was introduced to mice nasally and serum IgG responses were examined. The result showed that the IgG response of a fourfold-higher dosage of LL-CRR is no significant difference with the response of the lower-dosage group (Mannam et al., 2004). Therefore, depending on the immunogenicity of antigen and delivery routes, a certain amount of oral vaccination could affect the immune response differently.

Oral tolerance

Immunologic tolerance is an important mechanism of the immune system. Effective vaccination can cause immune suppression when autoimmune diseases occur. An

advanced method of antigen-specific therapy is to induce peripheral T cell tolerance via oral or nasal administration of autoantigen, which promotes mucosal tolerance (Czerkinsky et al., 1999).

Currently, autoantigens can be delivered in two different ways. The first one is to deliver purified autoantigen directly. The second method is using genetically modified organism expressing autoantigen and deliver recombinant microorganism through oral and nasal routes. The first approach has been well studied. Maassen has summarized oral tolerance induction in different models for various disease, such MS, EAE, rheumatoid arthritis, uveitis, thyroid disease, myasthenia gravis and type I diabetes by pure target antigens. Their results revealed that the autoimmune disease could be prevented and treated by oral or nasal administrated autoantigen in either human or animal model (Maassen et al., 1999).

Since oral administration of soluble antigen showed an excellent immune suppression in autoimmune diseases, delivery systems have widely grown in the past decade. Recombinant lactobacilli expressing heterologous target autoantigen is the second approach for mucosal administration. The functional recombinant *Lc. casei* has been used to display tetanus toxin TTFC on the surface, and high-level expression of TTFC has been evaluated by parenteral immunization. By adjusting this recombinant *Lc. casei*, myelin protein also can be applied for oral tolerance induction (Maassen et al., 1999). Furthermore, live lactobacilli expressing guinea pig MBP_{72-85} significantly inhibited EAE after oral administration (Maassen, 2003). This method is not limited to EAE. It could also practice as a delivery system for other autoimmune diseases, such as rheumatoid arthritis

and uveitis. Several recombinant expressing autoantigen delivery systems have been studied but not been fully developed. In table 2.1, oral administration systems with recombinant *Lactobacillus* spp. and *Lactococcus* spp. for several autoimmune diseases is summarized.

Non–GMO Gram-positive oral delivery tool

Gram-positive bacteria have a unique cell wall structure including thick peptidoglycan layer. Multiple components can attach to the peptidoglycan, such as teichoic acids, carbohydrates and protein. As mentioned above, many CWBD could recognize particular sequence or region of peptidoglycan and covalently and non-covalently bind to Gram-positive bacteria, like LPxTG, LysM, SLH and SH3. Application of CWBD could be a new cell surface display system for oral administration. This approach seems more welcome than genetically modified organism (GMO). LAB is always interesting for displaying heterologous protein on their surface because of their GRAS status (Wessels et al., 2004), but GMO LAB could lead to problems and due to acceptability by regulatory agencies (Ribelles, Rodríguez, & Suárez, 2012). Therefore, binding heterologous protein to peptidoglycan results a non-GMO oral vaccine or oral tolerance systems.

The LysM domain is an attractive CWBD. (Visweswaran et al., 2014) has reviewed that LysM –containing fusion protein is utilized for detection of bacteria and display of enzymes and antigen on the surface of Gram-positive bacteria. Most of the carriers are *Lc. lactis* and some *Lactobacillus* strains (Visweswaran et al., 2014). This could cause the direct contact between the antigen and immune system and increase immune response (Ribeiro et al., 2002). For example, antigen of Enterovirus type 71 (VP1) was displayed on the surface of

Lc. lactis and recombinant *Lc. lactis* was orally administrated. Serum antibody response was stimulated in a mouse's model (Raha, Varma, Yusoff, Ross, & Foo, 2005; Varma, 2013). Moreover, *Lb. acidophilus* was also used as a carrier to display VP1 protein of chicken anemia virus. After orally administrating non-GMO *Lb. acidophilus*, serum antibody response, virus neutralization and amount of Th1 cytokines were observed (H. Moeini, Rahim, Omar, Shafee, & Yusoff, 2011). All of the results indicated non-GMO LAB could successfully suppress immune responses.

2.5 Future prospects

Lactobacilli have been admitted as an advanced tool for mucosal delivery. Depending on the diseases and its specific objectives (vaccination or tolerance), a suitable carrier/host strain, administration pathway, delivery system, cellular location of heterologous protein, dosage of protein and model of study are very essential for oral immunological research. In the future, comparing immune responses of an individual model with orally administrated live GMO lactobacilli and live non-GMO lactobacilli will be very exciting and may guide a direction for future vaccine design.

Figure 2.1 The pathogenesis of multiple sclerosis (Adapted from Murphy, 2012)

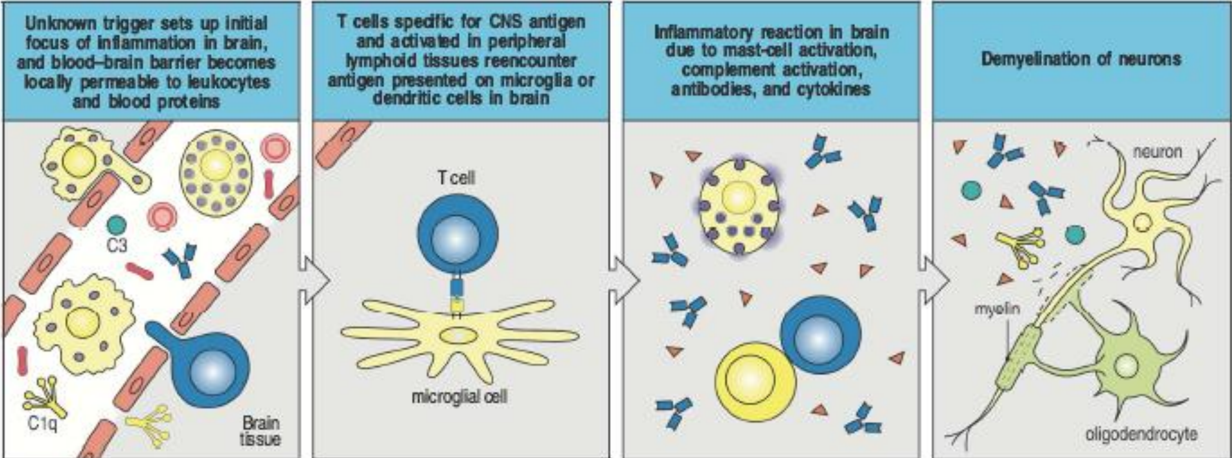


Table 2.1 Recombinant Lactobacilli and Lactococci expressing autoantigens in autoimmune diseases

Autoimmune disease	Vehicle	Tolerogen	Model	References
Multiple Sclerosis	*Lb. casei*	hMBP/ PLP		(Maassen et al., 1999)
EAE	Lactobacilli	hMBP gpMBP PLP	Lewis rat	(Maassen, 2003)
Type 1 Diabetes	*Lc. Latis*	Pancreatic β cell HSP65-P277	NOD mouse	(Ma et al., 2014; Robert & Steidler, 2014)
Cow's milk allergy	*Lc. latis*	Blg	Mice	(Adel Patient et al., 2005; Chatel et al., 2003)
Celiac disease	*Lc. Latis*	Gliadin Peptide	Mice	(Huibregtse et al, 2009)

Lb, *lactobacillus*

Lc, *Lactococcus*

hMBP, human myelin basic protein

gpMBP, guinea pig myelin basic protein

PLP, proteolipid protein peptide

HSP65-P277, HSP65 with tandem repeats of P277

Blg, bovine beta-lactoglobulin

NOD mouse, Non-obese diabetic mouse

CHAPTER 3 RECOMBINANT LACTOBACILLI FOR SURFACE DISPLAY AND SECRETE OF MYELIN ANTIGEN

3.1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system, That causes lesions in the brain, spinal cord and other areas of the body (Awad, 1984). It has been shown that MS is an autoimmune disease mediated by CD4+ Th1 and Th17 inflammatory responses. Currently, several disease-modifying drugs have been made available for MS; such as IFN-β1a, IFN-β1b, mitoxantrone and natalizumab. Unfortunately, these drugs are not fully developed and have significant side effects including, flu-like symptoms, skin site reactions, transient headache fatigue and cardiotoxicity (reviewed by Castro Borrero et al., 2012b). Therefore, additional strategies to ameliorate MS are needed. The systemic administration of an autoantigen may be an effective treatment to induce antigen-specific T cell tolerance (reviewed by Liblau, Tisch, Bercovici, & McDevitt, 1997). Mucosal administration is a promising antigen-specific therapy to promote mucosal T-cell tolerance by anergy, deletion and active suppression (Maassen, 2003). When anergy (Karpus, Kennedy, Smith, & Miller, 1996) or deletion (Y. Chen, 1995) is the desired mechanisms, the autoantigen should be known. Our collaborator Dr. Mangalam and others have identified several critical epitopes of the MS-promoting autoantigens. These epitopes inculde the proteolipid protein (PLP) amino acids: 37-71, 89-154, 179-238, 264-277 and myelin-oligodendrocyte glycoprotein (MOG) amino acids: 30-150 and 181-203.

Lactobacilli are Gram-positive lactic acid bacteria (LAB) which are commonly utilized in the food industry. Numerous lactobacillus strains are regarded as probiotics because

they provide immune-modulating and stimulating activities and contribute to health maintenance (Borchers AT, Selmi C, Meyers FJ, Keen CL, Gershwin ME, 2009). Additionally, lactobacilli have been widely used as potential live vectors for heterologous protein delivery for oral vaccine, oral tolerance and pharmaceutical applications (Cortes Perez et al., 2005; H. Moeini et al., 2011; Xu et al., 2011). (Maassen, 2003) was the first group to develop a novel method of mucosal tolerance induction by administration of recombinant lactobacilli expressing a myelin basic protein (MBP) autoantigen in an animal model of MS, experimental autoimmune encephalomyelitis (EAE). Their conclusion revealed that the live recombinant lactobacilli expressing guinea pig MBP (gpMBP) or MBP_{72-85} were able to reduce the disease significantly when administered orally. Furthermore, the system of oral delivery exercising LAB for MS is still under development. Since various lactobacilli have distinct properties, it is vital to select target lactobacilli strains carefully. We initially screened members of our lactobacilli culture collection to identify isolates that were amenable to our genetic tools. From that group, we identified three Lactobacilli that were good candidates for further study: *L. acidophilus* ATCC 4356 (LA4356), *L. gasseri* ATCC 33323 (LG33323) and *L. salivarius* ATCC 11742 (LS11742). These strains were sent to our collaborators at the Mayo Clinic to test their inherent "probiotic" ability to suppress MS in a rodent model. We observed that the LS11742 had a strong suppressive effect and LG33323 had a moderate suppressive effect; whereas, LA4356 had no suppressive effect. Therefore, LA4356, LG33323 and LS11742 were developed for recombinant autoantigen expression.

The aim of this research was to determine the best strategy for heterologous expression in lactobacilli to suppress MS in rodents. We developed genetic tools for

heterologous protein expression, using two different strategies: 1) secreted autoantigen and 2) extracellular anchoring of the autoantigen. We targeted the two previously identified MS autoantigens, PLP and MOG. Following satisfactory expression and extracellular display, the recombinant lactobacilli will be evaluated for their suppressive potential in an MS rodent model.

3.2 Material and method

Microorganisms, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 3.1. Recombinant *E. coli* MC1061 and *E. coli* Top10 cells were incubated aerobically overnight at 37°C with shaking at 250 rpm in Luria-Bertani (LB) medium (Fisher Scientific, Fair Lawn, NJ) supplemented with 150 μg/ml of erythromycin (Fisher Scientific) or 50 μg/ml of ampicillin (Fisher Scientific); respectively. LA4356, LG33323 and LS11742 and other recombinant strains were grown in de Mann, Rogosa, Sharpe (MRS) broth (Difco, Sparks, MD) or on MRS agar plates and incubated overnight anaerobically (5% CO_2, 5% H_2 and 90% N_2). When necessary, MRS broth or agar plates were supplemented with either 2.5μg/ml or 5 μg/ml of erythromycin.

DNA manipulation and plasmids construction

Several genetic techniques were utilized in this study according to manufacturer's procedures. The primers listed in Table 3.2 were designed using Clone Manager 9 (Sci-Ed Software, Raleigh, NC) and purchased from Integrated DNA Technologies (IDT, Coralville, IA). PCR was carried out using Econo Taq PLUS 2X Master Mix (Lucigen, Middleton, WI) according to manufacturer standard procedure. Amplified PCR fragments were purified

using DNA Clean and Concentrator Kit (Zymo Research, Orange, CA). PCR fragments were cloned into restriction-digested plasmids using the T4 DNA Ligase (New England Biolabs, Ipswich, MA). Plasmid DNA was purified from recombinant *E. coli* (MC1061 or Top10) using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA). *Lactobacillus* spp. was transformed by electroporation according to a previously described method (Francl, Hoeflinger, & Miller, 2012). Recombinant *Lactobacillus* strains were confirmed through the plasmid DNA isolation (O'Sullivan, 1993). All plasmid DNA sequences were confirmed by Sanger sequencing by the University of Illinois Urbana-Champaign Core Sequencing Facility.

The design of expression vectors is described in Figure 3.1 (Plasmid could be found in Appendix A). Part A (secreted and anchor) was constructed to include the *pgm* promoter, an anchor signal (A1392) from the Mub gene and a secretion signal (S1709) from *L. acidophilus* NCFM and the *Campylobacter jejuni* FlpA gene. Similarly, Part B (secretion only) was constructed as Part A (S1709) except a stop codon was inserted to remove the anchor signal (A1392). Both Part A and Part B were modelled after (Kajikawa et al., 2012) and synthesized by GENEWIZ Inc. (South Plainfield, NJ). Cloning was performed using pMJM8, a derivative plasmid from pGK12 with a multi-cloning site (MCS) from pBluescript containing an erythromycin resistance gene, at the restriction enzyme sites *Sac*II and *BamH*I resulting in pFlpA_A and pFlpA_S, respectively (Berquist, 2014).

The PLP, MOG and PLP/MOG epitopes were synthesized and cloned into pUC57 vector with *Xba*I and *Spe*I restriction sites by GENEWIZ Inc. to generate plasmids pPLP, pMOG and pP/M; respectively. The genes of PLP, MOG and PLP/MOG epitopes were generated in two

different methods for cloning. First, PLP, MOG and PLP/MOG genes were isolated from pPLP, pMOG and pP/M by enzyme digestion with the *Xba*I and *Spe*I restriction enzyme sites. Then three inserts replaced the *Xba*I-*Spe*I spanning fragments (FlpA) in pFlpA_A that obtained pPLP_A, pMOG_A and pP/M_A.

Secondly, The PLP epitope was isolated from vector pPLP by PCR amplification using the primer set LZ_PLP F-*Xba*I and LZ_PLP R-*BamH*I with *Xba*I and *BamH*I sites. The MOG epitope was obtained from vector pMOG by PCR amplification using the primer set LZ_MOG F-*Xba*I and LZ_MOG R-*BamH*I with *Xba*I and *BamH*I sites. The PLP/MOG epitopes were isolated from the vector pP/M by PCR amplification using the primer set LZ_PLP F-*Xba*I and LZ_MOG R-*BamH*I with *Xba*I and *BamH*I sites. The resulting PLP, MOG and PLP/MOG amplicons were enzyme digested and ligated into pFlpA_S in place of the FlpA fragment using the *Xba*I-*BamH*I restriction enzymes. The resulting plasmids are listed in Table 3.1. All plasmids were first transformed into *E. coli* MC1061 and purified. Subsequently, all PLP, MOG, PLP/MOG plasmids were transformed into LA4356, LG33323, and LS11741.

Protein validation and SDS-PAGE PROTEIN GEL/ WESTERN BLOT

For expression analysis, the recombinant lactobacilli were grown to mid-log phase in MRS broth and cells were pelleted by centrifugation at 4000 rpm. Each bacterial pellet was resuspended in 20 μL per unit of optical density at 600 nm (OD600) with Tris-EDTA containing 3 mg/mL lysozyme, 25 U/mL DNase I, and 1x complete protease inhibitor cocktail (Roche, USA). After incubation for 1 h at 37°C, one part volume of 2 x reducing sample buffer was added, the samples were lysed by heating to 100°C for 5 min and centrifuged at 14,000 rpm for 1 min. The soluble protein fraction was separated from the

bacterial lysate by SDS-PAGE (Stoeker et al., 2011). In order to analyze the secreted protein fraction in the cellular supernatant, 5 mL of mid-log phase recombinant lactobacilli were harvest by centrifugation at 4000 rpm at 4°C. Approximately, 10 mg of sodium deoxycholate (Fisher) was added, mixed by vortex and incubated at 4°C for 30 min. The proteins were precipitated overnight at 4°C by addition of 300 µL of chilled 100% (w/v) trichloroacetic acid (TCA, Fisher). Proteins were recovered by centrifugation at 4000 rpm for 10 min at 4°C followed by washing twice with 2 mL of chilled acetone. Finally, pellets were dried at room temperature and re-solubilized by sonication in 40 µL of 1x Laemmli buffer (Sánchez, Chaignepain, Schmitter, & Urdaci, 2009).

Protein presence and size were confirmed by western blotting at the Mayo Clinic. The western blot protocol was provided by our collaborator Dr. Mangalam. Briefly, the SDS-PAGE gel was run at 7 mA constant current without cooling, and the blot apparatus was set up with the dark grid down and blotted for 45 min at 90 volts. The membrane was washed in 18 megohm water for a few minutes, and then blocked for 20 s with 4% BSA 1X TBS. About 1 mg/ml anti phoshotyrosine antibody of 30 ml mixture (2% BSA, 1% Tween-20) was added in the blot and incubate at room temperature with shaking. After that, the membrane was washed 3 times with 0.2% Tween-20 in 1X TBS. Lately, 3 mL of goat anti mouse HRP antibody in 30 mL of 0.2% Tween-20 in 1XTBS was added in the blot and gently shake for 45 min at room temperature. After washing, the membrane was exposed to film for 20 min.

3.3 Results

Construction of anchored and secreted protein vectors

The expression vectors pPLP_A, pMOG_A and pP/M_A containing both S1709 and A1392 signals were constructed with the PLP, MOG or PLP/MOG epitopes (Table 3.1 and Figure 3.1). Successful ligations were sequence confirmed using the primers AB_p13F-*Xba*I and AB_p13R-*Spe*l. Unfortunately, all of the transformants of pPLP_A, pMOG_A and pP/M_A had incorrect insertion configurations.

In the meantime the expression vectors pPLP_S, pMOG_S and pP/M_S containing only the S1709 signal were constructed with the PLP, MOG or PLP/MOG epitopes. Successful ligations were sequence confirmed using the primers AB_p13F-*Xba*I and AB_p14R-*BamH*I. The expression vectors pPLP_S, pMOG_S and pP/M_S were confirmed to harbor PCR amplicons of 717-bp, 621-bp and 1146-bp; respectively (Figure 3.2). Sequencing results confirmed that the PLP, MOG and PLP/MOG epitopes were successfully ligated in place of the FlpA fragment in the pFlpA_S vector. In addition, the expression vectors pPLP_S, pMOG_S and pP/M_S have been isolated from *Lactobacillus* strains. Thus far, we are confident that LG33323 contains pMOG_S (MJM280) and pP/M_S (MJM281), LA4356 contains pPLP_S (MJM285), pMOG_S (MJM286), pP/M_S (MJM287) and LS11741 contains pPLP_S (MJM291).

Since successful transformants were obtained using the expression vectors containing only the S1709 signal, we attempted to the A1392 signal directly into pPLP_S, pMOG_S and pP/M_S using the *Spe*I and *BamH*I restriction sites. Unfortunately, this proved futile as

mutants were consistently found in the anchor signal in all sequenced transformants. Based on these results we decided to continue exploring the secreted protein only.

Expression of the secreted proteins containing of the PLP, MOG and PLP/MOG epitopes

Since LA4356 was successfully transformed with all three epitopes, it was evaluated for its ability to express the secreted protein (Table 3.1). Protein expression was confirmed using cellular lysates and the cell-free supernatant prepared from the LA4356 containing the pPLP_S, pMOG_S and pP/M_S. Production of the target proteins were confirmed by Western blotting (Figure 3.3). The PLP epitope was detected in LA4356 containing pPLP_S cellular lysate. The estimated molecular mass of the PLP epitopes is approximately 27.4 kDa which is consistent with our results. Unfortunately, PLP or MOG was not detected in the LA4356 strains containing pMOG_S and pP/M_S. Furthermore, secreted protein fractions were unable to isolate from the cellular supernatant, which may be caused by the contamination of other membrane proteins. The smear was observed on the SDS-PAGE gel.

3.4 Discussion

Lactobacilli have the ability to attach and colonize at certain regions of intestine, which could stimulate both specific and non-specific immune response (Blomberg, 1993; E. E. Vaughan, 1999). Therefore, Lactobacilli is used as live delivery carrier for oral immunization against different infections and diseases, such as *Salmonella* infection (Rahbarizadeh et al., 2011) and anthrax (Zegers et al., 1999b). Individual *Lactobacillus* strains have different effect in oral tolerance induction. Our previous study shows that *L. gasseri* ATCC 33323 had a moderate suppressing effect on MS; *L. salivarius* ATCC 11742 had a strong suppressing effect on MS whereas *L. acidophilus* ATCC 4356 had no effect on

MS. Different suppressing effects between these three strains may be caused by variability in pathogen associated molecular patterns (PAMPs) such as peptidoglycan, cell wall polysaccharides, lipoproteins, and lipoteichoic acid (LTA) anchored in the cytoplasmic member (van Baarlen, Wells, & Kleerebezem, 2013). From molecular cloning perspective, all three strains were selected as expression host. Also, it was unknown what inherent property of the lactobacilli would work best with the autoantigens.

The number of studies on oral immunization of MS disease is very limited. Therefore, our strategy to promote tolerance of MS disease with autoantigen expression in lactobacilli using both secretion and anchored protein models. In previous studies, the immunogenicity of an antigen was shown to be strongly influenced by its final cellular location (cytoplasmic, secreted, or anchored to cell wall). Since the anchoring of an antigen to the extracellular surface showed the highest antigen immunogenicity, we chose to design an anchored protein expression vector as well as a secreted protein expression vector (Bermúdez Humarán & Bermudez-Humaran, 2004b; Norton, 1996b). In this study, three protein expression vectors were constructed to either secrete or secrete and extracellularly anchor the PLP, MOG, PLP/MOG epitopes. Since anchored vector construction was unsuccessful, alternative cell wall anchored strategy is necessary.

The PLP and MOG epitopes were chosen to be expressed independently or in combination. It is important to determine which heterologous protein (single or combined) has desired impact on the immune response. The previous study demonstrated that a heterogeneous antigen preparation like myelin is less effective than single antigen (MBP) in term of inducing tolerance (Benson, 1999). Possibly, there are immune response

differences among various heterologous proteins, but more studies are needed. A secondary outcome of this study was to determine which heterologous protein (PLP, MOG or PLP/MOG) has the highest suppressive effect on MS.

Unfortunately, expression vectors pPLP_A, pMOG_A and pP/M_A were unable to be isolated successfully. Based on the sequencing results, a majority of transformants had incorrect insertion configuration, which was caused by the same sticky ends, CTAG of enzymes XbaI (TCTAGA) and SpeI (ACTAGT). Moreover, within the minor amount of correct insertion transformants, mutants were found in both the epitope sequence and the anchor sequence on various locations in different duplications. Our conclusion is that constructs are lethal to *E. coli* and preventing our successful cloning. Interestingly, we have been able to construct pFlpA_A indicating that a different heterologous protein (FlpA) with an anchor signal can be successfully expressed in *E. coli* so there is something uniquely problematic with PLP and MOG (Berquist, 2014). Additionally, pP/M_S was unable to transform into LG33323; pMOG_S and pP/M_S were unable to transform into LS11741 as well. Strong constitutive promoter, *pgm*, may lead these unexpected mutations and failing transformation. High level of constitutive expression can be lethal to the host cell, inhibit growth, loss of the expression vector and recombinant DNA structural instability (Hanniffy et al., 2004b; Makrides, 1996).

Soluble bacterial lysates from recombinant LA 4356 (MJM285, MJM286, MJM287) were sent out for western blot. Expected sizes of MOG epitopes (24.6kDa) and PLP/MOG (44kDa) were not shown at the gel in Figure 3.3. It could be caused by multiple reasons. First, as mentioned above, MOG epitopes and PLP/MOG epitopes may not be expressed

successfully by LA 4356 due to strong constitutive promoter (Makrides 1996). Second, MOG and PLP/MOG may be in an insoluble form and have been excluded in the samples. Furthermore, the antibodies for MOG and PLP/MOG may be another reason for these proteins were not detected.

3.5 Conclusion

In this study, PLP epitope is successfully detected from LA4356 cell lysate, which indicates that *L. acidophilus* may be a candidate for PLP expression. Further research is necessary to design a vector with a functional anchored signal. While this study demonstrated protein expression of the PLP epitope in *L. acidophilus*, the animal data is critical to evaluate its potential as a therapeutic for MS. Once the animal experiments are complete, we can reevaluate our approach. For example, we will know which lactobacilli strain provides the optimal results based on our application. Efficacy of this study may be further improved by optimizing autoantigen expression level, such as replacement of the *pgm* promoter to a low expression promoter to test how protein expression levels influences the *in vivo* effects. At this time, we have abandoned anchored strategy and developed alternate strategies for cell surface localization (see chapter 4).

Table 3.1 Bacterial strains and plasmids

Bacterial strain	MJM	Plasmid	Description of plasmid	Source
E.coli Top10		NA	Cloning host	Invitrogen
	259	pPLP	Ap^R, pUC57 backbone, PLP inserted	This Study
	260	pMOG	Ap^R, pUC57 backbone, MOG inserted	This Study
	261	pP/M	Ap^R, pUC57 backbone, PLP/MOG inserted	This Study
E. coli MC1061		NA	Cloning host	Klaenhammer
	106	pMJM8	Em^R	Miller Lab
	256	pFlpA_A	Em^R, pMJM8 backbone, FlpA with secretion and anchor signals	Miller Lab
	257	pFlpA_S	Em^R, pMJM8 backbone, FlpA with secretion and stop signals	Miller Lab
	262	pPLP_A*	Em^R, pMJM13 backbone, PLP inserted	This Study
	263	pMOG_A*	Em^R, pMJM13 backbone, MOG inserted	This Study
	264	pP/M_A*	Em^R, pMJM13 backbone, PLP/MOG inserted	This Study
	265	pPLP_S	Em^R, pMJM14 backbone, PLP inserted	This Study
	266	pMOG_S	Em^R, pMJM14 backbone, MOG inserted	This Study
	267	pP/M_S	Em^R, pMJM14 backbone, PLP/MOG inserted	This Study
L. gasseri ATCC 33323		NA	Expression host	Miller Lab
	276	pPLP_A *	Em^R, pMJM13 backbone, PLP inserted	This Study
	277	pMOG_A *	Em^R, pMJM13 backbone, MOG inserted	This Study
	278	pP/M_A *	Em^R, pMJM13 backbone, PLP/MOG inserted	This Study
	279	pPLP_S *	Em^R, pMJM14 backbone, PLP inserted	This Study
	280	pMOG_S	Em^R, pMJM14 backbone, MOG inserted	This Study
	281	pP/M_S	Em^R, pMJM14 backbone, PLP/MOG inserted	This Study

Table 3.1(cont.)

L.acidophilus ATCC 4356		NA	Expression host	Miller Lab
	282	pPLP_A *	Em^R, pMJM13 backbone, PLP inserted	This Study
	283	pMOG_A *	Em^R, pMJM13 backbone, MOG inserted	This Study
	284	pP/M_A *	Em^R, pMJM13 backbone, PLP/MOG inserted	This Study
	285	pPLP_S	Em^R, pMJM14 backbone, PLP inserted	This Study
	286	pMOG_S	Em^R, pMJM14 backbone, MOG inserted	This Study
	287	pP/M_S	Em^R, pMJM14 backbone, PLP/MOG inserted	This Study
L. salivarius ATCC 11741		NA	Expression host	Miller Lab
	288	pPLP_A *	Em^R, pMJM13 backbone, PLP inserted	This Study
	289	pMOG_A *	Em^R, pMJM13 backbone, MOG inserted	This Study
	290	pP/M_A *	Em^R, pMJM13 backbone, PLP/MOG inserted	This Study
	291	pPLP_S	Em^R, pMJM14 backbone, PLP inserted	This Study
	292	pMOG_S *	Em^R, pMJM14 backbone, MOG inserted	This Study
	293	pP/M_S *	Em^R, pMJM14 backbone, PLP/MOG inserted	This Study

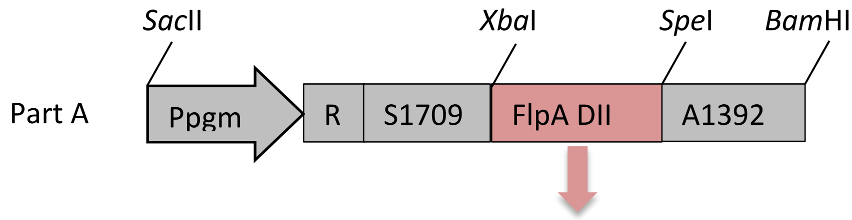
* In progress, remains to be constructed; Em^R, Erythromycin resistant

Table 3.2 Primers used in this study

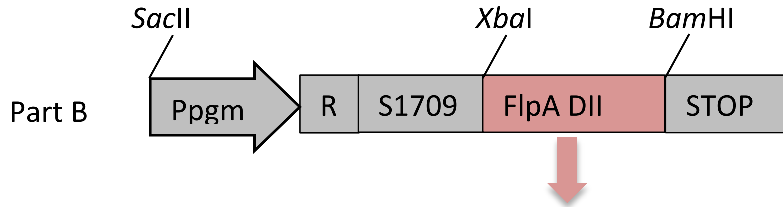
Primers	Sequence
AB_p13F-XbaI	CTGGTTTGGCTACAGTATTC
AB_p13R-SpeI	CCTAAGCCAGAGAACACT
AB_p14R-BamHI	CTTTAGTGAGGGTTAATTTCGA
LZ_PLP F-XbaI	CTAGTCTAGACATGAGGCTTTGACA
LZ_PLP R-BamHl	ACGCGGATCCGAACTTTGTTCCACGACCCAT
LZ_MOG F-XbaI	CTAGTCTAGAGGTCAGTTCCGTGTA
LZ_MOG R-BamHl	ACGCGGATCCTCTCAAGAAGTGAGGGTCGAA
LZ_A1392 F-BamHl	ACGCGGATCCACAGTTACAGTTACTTACAC
LZ_A1392 R-Apal	CTAGGGGCCCTTATTTGTCCTCCTTTCTACG
LZ_pMJM20/21 F-BamHI	GTGGAAAGTTGCGTGCAGAG
LZ_pMJM19 F-BamHI	ACTTCGCAGTATTGAAGTTG
LZ_pMJM19 R-ApaI	CAGCTATGACCATGATTACG

Restriction enzyme sites are underlined

Figure 3.1 Gene map of an expression cassette for PLP, MOG and PLP/MOG epitopes. FlpADII gene was replaced by target epitopes in Part A and obtains pPLP_A, pMOG_A and pP/M_A. FlpADII gene was replaced by target epitopes in Part B and obtains pPLP_S, pMOG_S and pP/M_S. Ppgm, promoter region of pgm gene from L. acidophilus NCFM; R, region encoding ribosome binding site of Mub from L. acidophilus NCFM (LBA1709 or LBA1392); S1709, region encoding the signal sequence of Mub (LBA1709 or LBA 1392); A1709/A1392, region encoding the anchor region of Mub (LBA1709 or LBA 1392).



Plasmid	Target protein	Size	Protein weight
pPLP_A	PLP	537 bp	41.5 kDa
pMOG_A	MOG	441 bp	38.7 kDa
pP/M_A	PLP/MOG	966 bp	58.0 kDa



Plasmid	Target protein	Size	Protein weight
pPLP_S	PLP	537 bp	27.4 kDa
pMOG_S	MOG	441 bp	24.6 kDa
pP/M_S	PLP/MOG	966 bp	44.0 kDa

Figure 3.2 PCR verification of the inserts. Primers were designed that flanked the insert. Gels show PCR products for screening pPLP_S, pMOG_S and pP/M_S. All negative are FlpA amplicons 457 bp, which means auto antigens were failed to clone into pFlpA_S. Gel#1, positive is PLP amplicon 717 bp (pPLP_S). Gel#2, positive is MOG amplicon 621bp (pMOG_S). Gel#3, positive is PLP/MOG amplicon 1146bp (pP/M_S). In all three cases, the transformants that had the correct size amplicon were sequence verified.

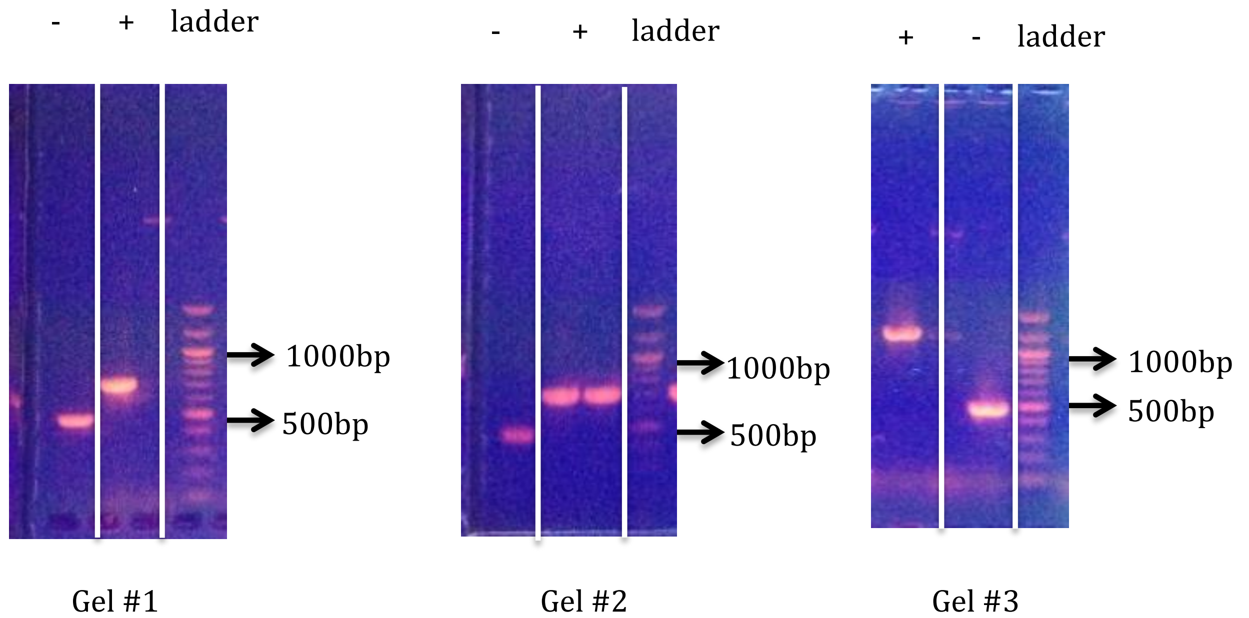
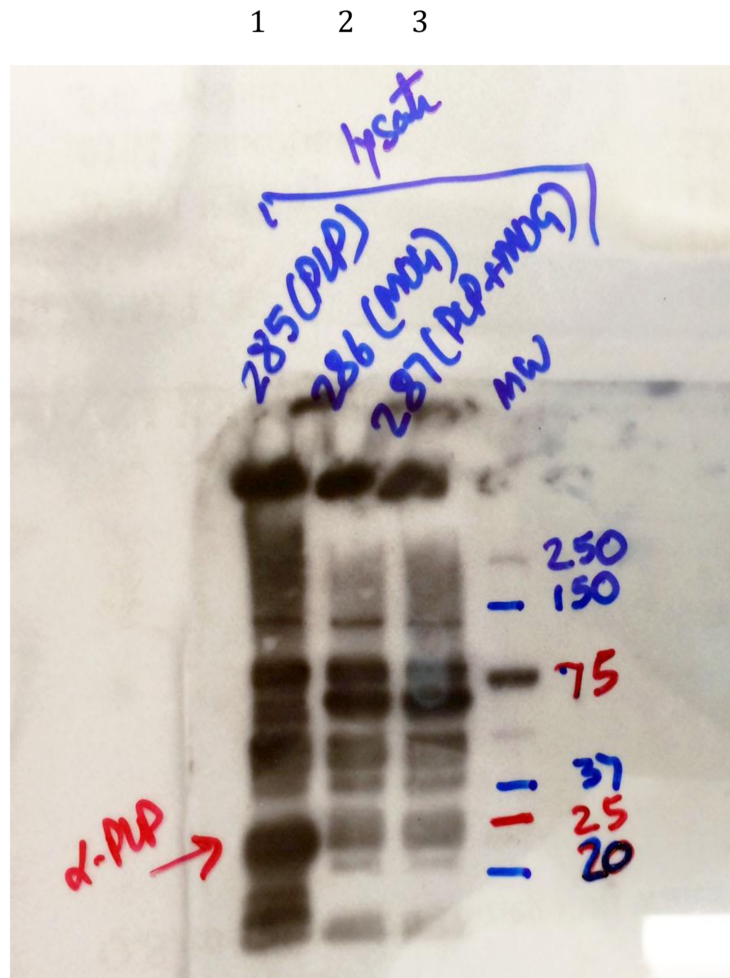


Figure 3.3 Soluble bacterial lysates from recombinant LA 4356. MJM285 expressing PLP epitopes (lane 1); MJM286 expressing MOG epitopes (lane 2); MJM287 expressing PLP/MOG epitopes (lane 3) were separated by electrophoresis on polyacrylamide gel. (Adapted from Dr. Ashutosh Mangalam, Mayo Clinic)

1 2 3



CHAPTER 4 CELL WALL BINDING OF HETEROLOGOUS PROTEIN TO THE EXTERIOR SURFACE OF *LACTOBACILLUS* SPP.

4.1 Introduction

Lactic Acid Bacteria (LAB) including *Lactobacillus* spp. are defined as a group of gram-positive, facultative anaerobic, fermentative bacteria (Makarova et al., 2006). Many *Lactobacillus* spp. are known as starter cultures for fermented food, such as yogurt, cheese, fermented milk, sausage, alcoholic beverage and other fermented foods. The natural habits of *Lactobacillus* spp. are not only in food fermentation environment but also in oral cavity and gastrointestinal tracts of human and animals (E. Vaughan, de Vries, Zoetendal, Ben Amor, Akkermans, & de Vos, 2002b). In addition, they are generally recognized as safe (GRAS) and some of them are considered as 'probiotics' strains, which provide health benefits to the host.

Over the past decade, food grade LAB, especially lactococci and lactobacilli, have been used as potential live vectors for heterologous protein delivery for oral vaccine, oral tolerance and pharmaceutical applications (Cortes Perez et al., 2005; Moeini, Hassan Rahim, Raha Omar, Abdul Shafee,Norazizah Yusoff, Khatijah, 2011; Xu et al., 2011). Previously, research had focused on the expression and anchoring of heterologous proteins to the extracellular surface using recombinant LAB. For example, the recombinant *Lactococcus lactis* expressing the envelop protein of HIV induces immune response and could be used as an HIV vaccine (Xin et al., 2003). However, this results a genetically modified (GM) lactococci. Therefore, researchers have begun developing alternative mucosal immunization strategies.

Several methods have been proposed that bind purified exogenous proteins onto the extracellular surface of non-GM LAB. The selection of which methods to use is dependent on the chosen heterologous protein. Surface-associated proteins can be bound to the extracellular surface either by covalent cell-wall binding domains (CWBD) or non-covalent CWBD. Covalent CWBDs can be a single hydrophobic N- or C-terminal domain, lipid-anchored or LPxTG cell-wall anchor; whereas non-covalent CWBD can include LysM domains, choline-binding domains, putative peptidoglycan-binding domains, S-layer protein domains, WxL domains and SH3 (Kleerebezem et al., 2010). Several studies have utilized non-covalent CWBD and have shown promising results. Particularly, the LysM (Pfam PF01476) domain is a commonly utilized non-covalent CWBD, which consists of repeat units of a small LysM motif. This domain is usually found in many extracellular enzymes and has the capability to anchor proteins in the peptidoglycan (PG) layer of Gram-positive bacteria (Buist et al., 2008). Currently, widespread utilization of the LysM domain has been used for detection of bacteria and display of enzymes and/or proteins on the extracellular surface of Gram-positive bacteria (Visweswaran et al., 2014). Another method uses the autolysin, N-Acetylmuraminidase (AcmA), produced by *Lactococcus lactis*. AcmA is responsible for cell separation and cell lysis during the stationary phase of growth (Buist, 1995). The AcmA gene of *L. lactis* MG1363 consists of three domains: an N-terminal signal sequence, an active domain and a C-terminal membrane anchor. Interestingly, the C-terminal membrane anchor possesses three repeated regions of the LysM motif (Buist, 1995). The CWBD of AcmA has strong binding capacity of proteins onto the surface of naturally occurring lactococcal strains and *Lactobacillus* spp. (Raha et al., 2005; Varma,

2013). Another popular non-covalent CWBD is the eukaryotic SH3 domain. This domain is able to target and anchor proteins to the PG layer by recognition of specific sequences within the cross-linking peptide bridges (Baba, 1996).

In this study, three non-covalent CWBD were selected including *L. gasseri* ATCC 33323 LysM domain-containing protein (LysM; NCBI Ref. YP_814716.1); *L. gasseri* ATCC 33323 Lysozyme M1, Bacterial SH3 domain (SH3; NCBI Ref. YP_814010.0); and C-terminal membrane anchor domain of *L. lactis* subsp. *cremoris* MG1363 AcmA protein (AcmA; GenBank CAL96887.1). The *L. gasseri* LysM domain and *L. gasseri* Lysozyme SH3 domains were predicted CWBD (Kleerebezem et al., 2010) based on the integrated subcellular location prediction pipeline provided by LocateP (Zhou et al., 2008). Here, we report on the expression of fusion proteins (Green fluorescent protein fused with cell wall binding anchor protein) from a recombinant *Escherichia coli*. Additionally, we describe a novel display method of these three CWBD with the target epitope (PLP/MOG) on the cell surface of *L. acidophilus* ATCC 4356 (LA4356), *L. gasseri* ATCC 33323 (LG33323), and *L. salivarius* ATCC 11741 (LS11741).

4.2 Materials and methods

Microorganisms, plasmids and culture condition

The bacteria strains and plasmids used in this study are listed in Table 4.1. *E.coli* Top 10 was purchased (Invitrogen, Carlsbad, CA) and used as the cloning host. *E.coli* BL21 (DE3) *plysS* strains (Invitrogen) was used as the *E.coli* expression host. The cloning vector pUC19 (Invitrogen) was used to clone all synthesized fragments; while pRSETB (Invitrogen) was used as a cloning and expression vector. Recombinant *E.coli* strains were

incubated overnight in LB medium (Becton, Dickenson, Franklin Lakes, New Jersey) supplemented with antibiotics (*E. coli* Top 10: 100 μg/ml of Ampicillin [Fisher, Hampton, NH], *E. coli* BL21 (DE3): 50 μg/ml of Ampicillin and 35 μg/ml of Chloramphenicol [Fisher]) at 37°C aerobically with shaking at 250 rpm. *L. gasseri* ATCC 33323, *L. acidophilus* ATCC 4356, and *L. salivarius* ATCC 11741 were incubated overnight in MRS broth (Becton, Dickenson) at 37°C anaerobically (5% CO_2, 5% H_2 and 90% N_2 atmosphere).

Construction of Plasmid pUC19 and transformation into competent *E. coli*

Three non-covalent CWBD, LysM, SH3 and AcmA, were synthesized as gBlocks® Gene Fragments with *Xho*I and *Hind*III restriction sites by Integrated DNA Technologies (IDT, Coralville, IA). The PLP/MOG peptide was also synthesized as a gBlocks® Gene Fragment with *BamH*I and *Xho*I restriction sites. The above CWBD and PLP/MOG peptide were cloned into the pUC19 digested with the blunt end restriction enzyme *Sma*I (New England Biolabs, Ipswich, MA) to generate plasmids pUC-PLP/MOG, pUC-LysM, pUC-SH3 and pUC-AcmA (Table 4.1). Each plasmid was transformed into *E.coli* Top10 competent cells and screened using the blue/white screening method. The ligations were confirmed by UIUC core sequencing facility by universal primers M13For-21and M13Rev-24 (Table 4.2).

Generation and cell binding of fused green fluorescent protein or PLP/MOG

The enhanced green fluorescent protein (EGFP) gene was isolated from the vector pEGFP-N1 (Invitrogen) by PCR amplification using forward primer LZ_EGFP_F with *BamH*I and the reverse primer LZ_EGFP R-*Xho*I with *Xho*I site. This specific EGFP gene was chosen because it contains a mutant of the wild-type GFP gene allowing for greater expression in mammalian cells and brighter green fluorescence. The EGFP amplicon was digested with

*BamH*I and *Xho*I restriction enzymes. The three CWBD were obtained by enzyme digestion from pUC-PLP/MOG, pUC-LysM, pUC-SH3 and pUC-AcmA with *XhoI* and *Hind*III restriction sites. The resulting fragments were cloned into the vector pRSETB digested with *BamH*I and *Hind*III enzymes to get a series of plasmids (pEGFP-LysM, pEGFP-SH3 and pEGFP-AcmA; Table 4.1). The resulting plasmids contained EGFP gene and each individual CWBD. An additional step exchanged EGFP with PLP/MOG and generated the plasmids pEGFP-LysM, pEGFP-SH3 and pEGFP-AcmA with the same restriction sites to get another series of plasmids (pPLP/MOG-LysM, pPLP/MOG-SH3, pPLP/MOG-AcmA; Table 4.1). The plasmids obtained were transformed into *E. coli* Top10 and sequenced using the primers LZ_pRSETB_F and LZ_pRSETB_R to ensure that the ligations were successful. Finally, each plasmid was transformed into *E. coli* BL21 (DE3) *pLysS* competent cells for protein expression. All PCRs were carried out using Econo Taq DNA Polymerase (Lucigen, Middleton, WI), and the oligonucleotides used are listed in Table 4.2.

Protein expression in *E. coli* BL 21(DE3) *plys*S

The recombinant *E. coli* BL21 (DE3) *plysS* cells were cultured as outlined previously. The overnight cultures were subcultured into a fresh 10 mL of LB medium containing ampicillin (50 μg/mL) and chloramphenicol (35 μg/mL) and incubated until mid-log phase (~OD_{600nm} = 0.6) at 30 °C before being induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, MP Biomedicals, Santa, CA) for 4 to 6 h. Cells were harvested and resuspended in 3 ml of phosphate-buffered saline (PBS). Then the cells were disrupted by sonication at 70 W for five cycles (one consists of 15 s sonication with intermission of 30 s), with interval cooling on ice. 20 µL of clear lysates were centrifuged at 15,000 rpm for 30

min at 4°C to separate soluble and insoluble protein. 20 µL of 2X reducing sample buffer (BIORAD, Hercules, CA) was added into soluble cell lysate and insoluble cell pellet was resuspended in 40 µL of 2X reducing sample buffer (BIORAD) prior to boiling at 95°C for 10 min followed by centrifugation at 10,000 g. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, BIORAD) was performed according to Laemmli (1970), using 10%–12.5% (w/v) polyacrylamide gels. An aliquot of 25 μL of total protein, soluble protein and insoluble protein were loaded onto the gel (Figure 4.2).

Purification of the fusion proteins and binding to lactobacilli

300 mL of the cell culture were harvest after 6 h induction with IPTG. The cell were resuspended in 10 mL of lysis buffer (pH 8.0) containing 50 mM Tris-HCl, 300 mM NaCl, 10% glycerol, 2mM Dithiothreitol, 20 mM imidazole and 0.1% Triton X-100, followed by sonication at a power output of 70 W for 5 min (750 W Ultrasonic Processor, SONICS, Milpitas, CA), with interval cooling on ice. The crude cell lysate was centrifuged at 13,000×g at 4°C for 20 min. Then the supernatant containing soluble protein was loaded into a Ni^{2+} affinity column (Clonetech, Mountain View, CA). The mixture of soluble proteins and the Ni^{2+} beads were incubate at 4°C overnight with gentle agitation, and then washed five times with washing buffer (50 mM Tris-HCl, 300 mM NaCl, 10% glycerol, 2mM Dithiothreitol, 40 mM imidazole, 0.1% Triton X-100, pH 8.0). Finally, the proteins were eluted by 1.5 mL of elution buffer (50 mM Tris-HCl, 300 mM NaCl, 10% glycerol, 2mM Dithiothreitol, 300 mM imidazole, 0.1% Triton X-100, pH 6.0).

Lactobacilli preparation and binding method have been described previously (Moeini, 2011). The culture of LA4356, LG33323 and LS11741 were grown to mid-log phase in MRS

broth (OD600 of 0.5–0.7), and harvested at 13,000×g for 5 min. The cells were resuspended in 500μl of PBS, and then mixed with 100 μl of the purified proteins followed by 4 h of incubation at 30°C. The cells were precipitated at 13,000×g for 1 min and then washed with PBS five times. The binding was analyzed by immunofluorescence microscopy (Zeiss Axiovert 200M, Jena, Germany) at core facilities of the Institute for Genomic Biology at the University of Illinois at Urbana-Champaign.

4.3 Results

Construction of the recombinant plasmids

The pRSET vectors are pUC-derived expression vector designed for high-level protein expression and purification from cloned genes in *E. coli*. The plasmid contains the strong T7 promoter, initiation ATG, N-terminal 6xHis tag, N-terminal Xpress™ epitope tag, Enterokinase cleavage site, multiple cloning site, T7 terminator and Ampicillin resistance gene. The strong T7 promoter was induced by the addition of IPTG. PCR confirmed that pEGFP-LysM, pEGFP-SH3 and pEGFP-AcmA harbored fragments of 982-bp, 1534-bp and 1426-bp; respectively. Sequencing results showed that EGFP was successfully cloned in frame with three different CWBDs in the pRSETB vector. The pRSETB construct maps are shown in Figures 4.1A. Transformed cells were selected on ampicillin plates after overnight incubation. Recombinant plasmids were then transformed into *E.coli* BL21 (DE3) *plysS* cells.

The PLP/MOG peptide was obtained by enzyme digestion of pUC-PLP/MOG with *BamH*I and *Xho*I restriction enzymes and followed by Gel Extraction Kits (QIAGEN, Hilden, Germany). The pEGFP-LysM, pEGFP-SH3 and pEGFP-AcmA were treated with the same

restriction enzymes to generate compatible ligation sites. The pRSETB construct maps for pPLP/MOG-LysM, pPLP/MOG-SH3 and pPLP/MOG-AcmA are shown in Figures 4.1. Transformed cells were selected on ampicillin plates after overnight incubation. Recombinant plasmids were then transformed into *E.coli* BL21 (DE3) *plysS* cells.

Expression of EGFP fusion protein in *E.coli* BL21 (DE3) *plysS*

The expression of all EGFP and EGFP fusion protein (EGFP_LysM, EGFP_SH3, EGFP_AcmA) was detected by SDS-PAGE from the crude protein extraction of pRSETB, pEGFP, pEGFP-LysM, pEGFP-SH3 and pEGFP-AcmA transformed *E.coli* BL21 (DE3) *plysS* (Figure 4.2). In the protein extracts of *E. coli* BL21 (DE3) *plysS*, pRSETB was used as a negative control, which did not express any protein. EGFP (27KDa), EGFP_LysM (34KDa) and EGFP_SH3 (54KDa) fragments were observed in total (T), soluble (S) and insoluble (I) crude protein extractions, the size being approximately the same as the calculated size. However, majority of EGFP_AcmA (49KDa) was observed in insoluble protein extraction. It indicated that EGFP_AcmA, which was induced by the method above, was made insoluble form and cannot be used as cell surface associate binding protein.

Binding of purified fusion proteins on the cell wall surface of Lactobacilli

EGFP, EGFP_LysM and EGFP_SH3 were purified on Ni^{2+} affinity columns, but we have problem for EGFP_LysM protein purification. At this time, we may only focus on SH3 domain. EGFP_SH3 protein mixed with LA4356, LG33323 and LS11741 as described above. Immunofluorescence microscopy verified the binding of EGFP_SH3 on the cell wall surface. These three lactobacilli cells exhibited bright fluorescence on the cell surface (Figure 4.3), which indicated the presence of EGFP_SH3 on the cell wall surface. Moreover, more

fluorescence was observed on the surface of LA4356 and LG3332, which means EGFP_SH3 had higher binding efficiency to these two strains. In contrast, EGFP_SH3 has lower binding efficiency to LS11741. The control bacterial cell showed no fluorescence.

4.4 Discussion

Bacterial surface display has been widely studied and used for vaccine delivery. *Lactobacillus* spp. is gram-positive bacteria that could potentially be developed as oral delivery vehicle, because of their GRAS status (Wessels et al., 2004). Currently, numbers of studies have been conducted to display heterologous proteins on the surface of lactobacilli. Expression system in lactobacilli could present heterologous protein in three cellular locations, intracellular, secreted and anchored. Cell wall anchored protein has shown to be the most efficiency to induce specific immune responses compared to cytoplasmic or secreted protein (Reveneau, Geoffroy, Locht, Chagnaud, & Mercenier, 2002). However, translocation is always a limited step, which could control by the level of sortase and transpeptidase (Dieye et al., 2003b). Thus, it is hard to control number of protein on the cell wall and level of expression. Moreover, as we found out in the last experiment (chapter 3), it is also difficult for plasmid transformation or protein expression in lactobacilli. Based on all these defects of protein expression above, CWBD display system of heterologous protein seems more welcome than expression system in lactobacilli. We can avoid the cloning and expression problems. Moreover, recombinant lactobacilli could lead to acceptability problems by regulatory agencies (Ribelles et al., 2012). Therefore, binding heterologous protein to peptidoglycan is more convenient to develop.

In this study, we constructed plasmids vector expressing EGFP and CWBD fusion proteins. The positive color response was found for LA4356, LG33323 and LS11741 cells, especially LA4356 and LG33323, which incubated with purified EGFP_SH3 fusion protein, whereas no color response was found for LA4356, LG33323 and LS11741 incubated with EGFP only. The results indicated that this predicted SH3 could be a candidate CWBD surface display system. The number of binding sites of CWBD could be quantified by measuring fluorescence on the cell wall, and then appropriate CWBD for specific LAB could be selected for different applications.

4.5 Conclusion

In this study, predicted SH3 domain was successfully bound onto cell surface of LA4356, LG33323 and LS11741. It indicated SH3 domain may be a candidate for protein display. Moreover, we have problems to purified EGFP_lysM and EGFP_AcmA proteins. Protein expression or purification methods need to be modified in the future. Furthermore, target myelin epitopes of PLP, MOG and PLP/MOG will replace EGFP and bind to cell wall surface of lactobacilli. We were also attempting to bind SH3 or other two CWBD on the surface of other Gram-positive bacteria. This system would be attractive for the different purpose of delivery systems.

Table 4.1 Bacterial strains and plasmids

Strains	MJM#	Plasmid	Description of bacteria strain and plasmid	Source
E.coli Top10	MJM351	NA	Cloning host	Invitrogen
	MJM355	pUC19	Ap^R, cloning vector	Invitrogen
	MJM334	pUC-PLP/MOG	Ap^R, pUC19 backbone, PLP/MOG inserted	This Study
	MJM336	pUC-LysM	Ap^R, pUC19 backbone, *L.gasseri* LysM inserted	This Study
	MJM337	pUC-SH3	Ap^R, pUC19 backbone, Lysozyme SH3 inserted	This Study
	MJM338	pUC-AcmA	Ap^R, pUC19 backbone, AcmA CWBD inserted	This Study
	MJM353	pRSETB	Ap^R, expression vector	Invitrogen
E. coli MC1061	MJM349	pEGFP-N1	Km^R, encodes the GFPmut1 variant	BD Biosciences
E.coli BL21 (DE3) *plysS*	MJM326	NA	Expression host	Invitrogen
	MJM339	pPLP/MOG-LysM	Ap^R, Cam^R; pRSETB backbone, PLP/MOG and *L.gasseri* LysM inserted	This Study
	MJM340	pPLP/MOG-SH3	Ap^R, Cam^R; pRSETB backbone, PLP/MOG and Lysozyme SH3 inserted	This Study
	MJM341	pPLP/MOG-AcmA	Ap^R, Cam^R; pRSETB backbone, PLP/MOG and AcmA inserted	This Study
	MJM345	pEGFP-LysM	Ap^R, Cam^R; pRSETB backbone, EGFP and *L.gasseri* LysM inserted	This Study
	MJM346	pEGFP-SH3	Ap^R, Cam^R; pRSETB backbone, EGFP and Lysozyme SH3 inserted	This Study
	MJM347	pEGFP-AcmA	Ap^R, Cam^R; pRSETB backbone, EGFP and AcmA CWBD inserted	This Study
	MJM362	Pegfp	Ap^R, Cam^R; pRSETB backbone, EGFP inserted	This Study

Ap^R, Ampicillin resistant; Cam^R, Chloramphenicol resistant

Table 4.2 Primers used in this study

Primers	Sequence	Restriction site[1]
M13For-21	GTAAAACGACGGCCAGT	
M13Rev-24	AACAGCTATGACCATG	
LZ_EGFP_F	CGCGGATCCGATGGTGAGCAAGGG	BamHI
LZ_EGFP R-XhoI	CCGCTCGAGCTTGTACAGCTCGT	XhoI
LZ_pRSETB_F	TCGGGATCTGTACGACGATG	
LZ_pRSETB_R	CAGCTTCCTTTCGGGCTTTG	

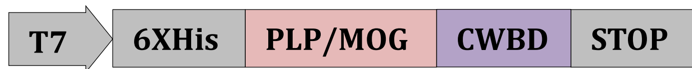
Restriction enzyme sites are underlined

Figure 4.1 Gene map of an expression vectors for cell wall binding domain and genes of PLP/MOG epitopes ligated into pRSETB. Derivative of pRSET-B, which contains T7 promoter, ribosome-binding site, His tag, EGFP, cell wall binding domain.

(A)

Plasmid	Target protein	Size of nucleotides	Protein weight
pEGFP-LysM	EGFP_LysM	916 bp	33.9kDa
pEGFP-SH3	EGFP_SH3	1468 bp	53.9kDa
pEGFP-AcmA	EGFP_AcmA	1360 bp	49.0kDa

(B)



Plasmid	Target protein	Size of nucleotides	Protein weight
pPLP/MOG-LysM	PLP/MOG_LysM	1156 bp	43.0kDa
pPLP/MOG-SH3	PLP/MOG_SH3	1707 bp	63.0kDa
pPLP/MOG-AcmA	PLP/MOG_AcmA	1599 bp	58.1kDa

Figure 4.2 SDS-PAGE of total, soluble and insoluble bacterial lysates from recombinant *E. coli* BL21 (DE3). It contains pEGFP, pEGFP_LysM, pEGFP_AcmA. T: total protein; S: soluble protein; I: insoluble protein. EGPF (27 kDa); EGFP_LysM (33.9 kDa); EGFP_SH3 (53.9 kDa); EGFP_AcmA (49.0 kDa).

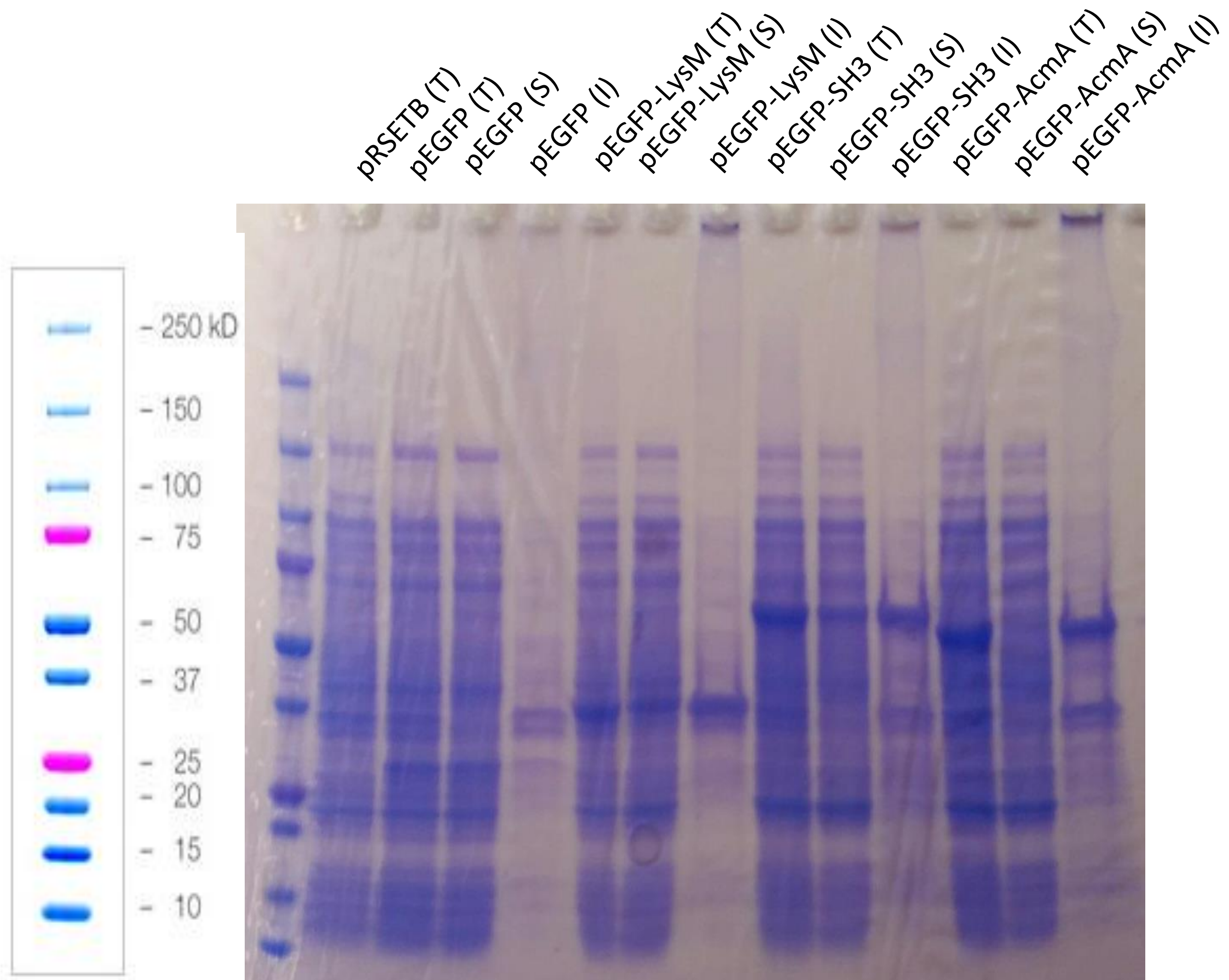
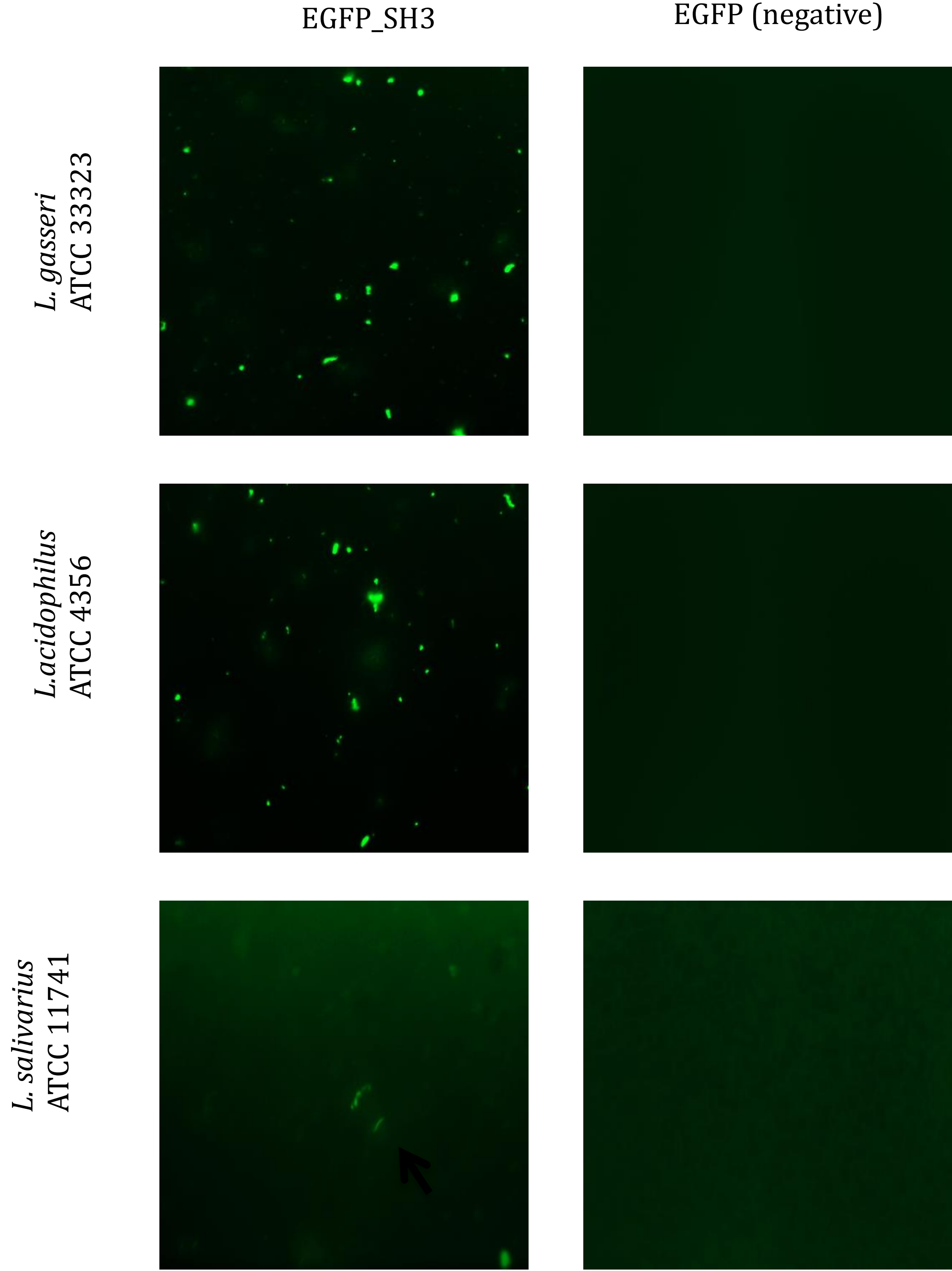


Figure 4.3 Fluorescence micrographs of the binding of the fusion protein EGFP_SH3 and EGFP to LG33323, LA4356, LS11741.



CHAPTER 5 SUMMARY AND FUTURE DIRECTION

Therapies of MS disease are still limited, and most of the current clinic treatments have side effects. *Lactobacillus* spp. as an oral delivery system is a promising method for oral tolerance induction for MS. *Lactobacillus* spp. could be developed as a potential oral delivery vehicle because of their GRAS status. There are two strategies that have been discussed in this thesis. Recombinant lactobacilli could be used as a suitable candidate of heterologous epitopes. We described the construction of antigen-presenting plasmid, which either secreted myelin epitopes to the environment or anchored them to the exterior cell surface of recombinant *Lactobacillus* spp. However, there are many problems to make lactobacilli to express protein, such as cloning, construction, transformation and protein expression. An alternate strategy explored the non-covalent attachment of myelin protein to the cell wall of *Lactobacillus* spp. via cell wall binding domains. Through working with these domains, we found that *L. gasseri* ATCC 33323 Lysozyme M1 – bacterial SH3 domain can successfully bind to the exterior cell surface to *L. acidophilus* ATCC 4356, *L. gasseri* ATCC 33323, and *L. salivarius* ATCC 11741. Therefore, SH3 domain may be a good tool for oral administration by binding heterologous epitopes to lactobacilli.

Through working with this project, we summarized several points for future study. First, the strong constitutive promoter could cause difficulties of cloning and protein expression in the first strategy (chapter 3). It may be better to switch *pgm* promoter to a lower expression level promoter. In the second strategy (chapter 4), the stability of fusion proteins on the cell surface to lactobacilli is very significant at different temperatures and pH. Lately, myelin epitopes need to replace GFP and be displayed on the cell surface of

lactobacilli. Moreover, the animal data is critical to assess its potential as a therapeutic for MS. Once the animal experiments are complete, we can reevaluate our approach. For example, we will know which lactobacilli strain provides the optimal results based on our application. Ultimately, the clinical trial of lactobacilli as oral delivery system is necessary to evaluate the usability for human.

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APPENDIX A CHAPTER 3 PLASMID MAP

(Sequence of the plasmids can be found in S:\Miller Lab\Luyu Zhang\Chapter 3\Final plasmid)

Figure A.1 Map of pMJM-8. Multiple Cloning Site (MCS) showed by restriction enzymes sites. Em, Erythromycin.

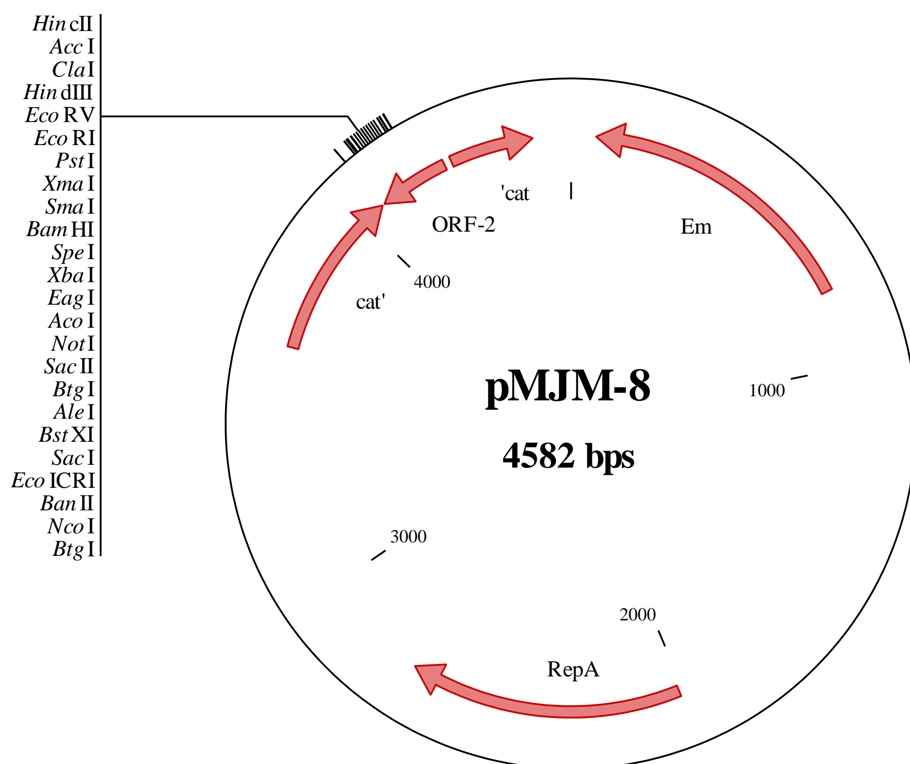


Figure A.2 Map of pMJM-13. pFlpA_A; Secreted and anchored C. jejuni FlpA DII. ORF-1 represents C. jejuni FlpA DII and anchored genes.

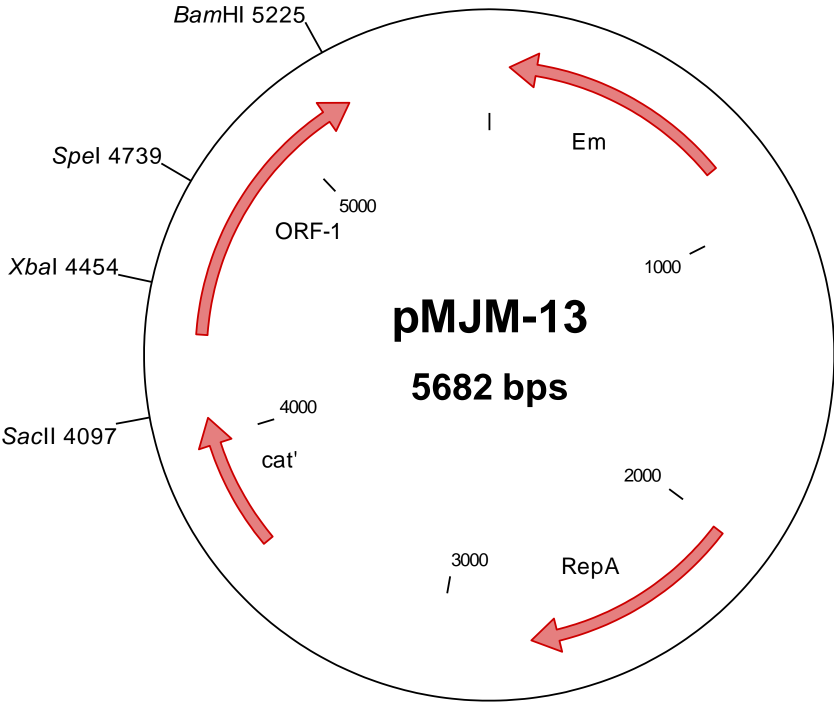


Figure A.3 Map of pMJM-14. pFlpA_S; Secreted and anchored *C. jejuni* FlpA DII. ORF-1 represents *C. jejuni* FlpA DII and anchored genes.

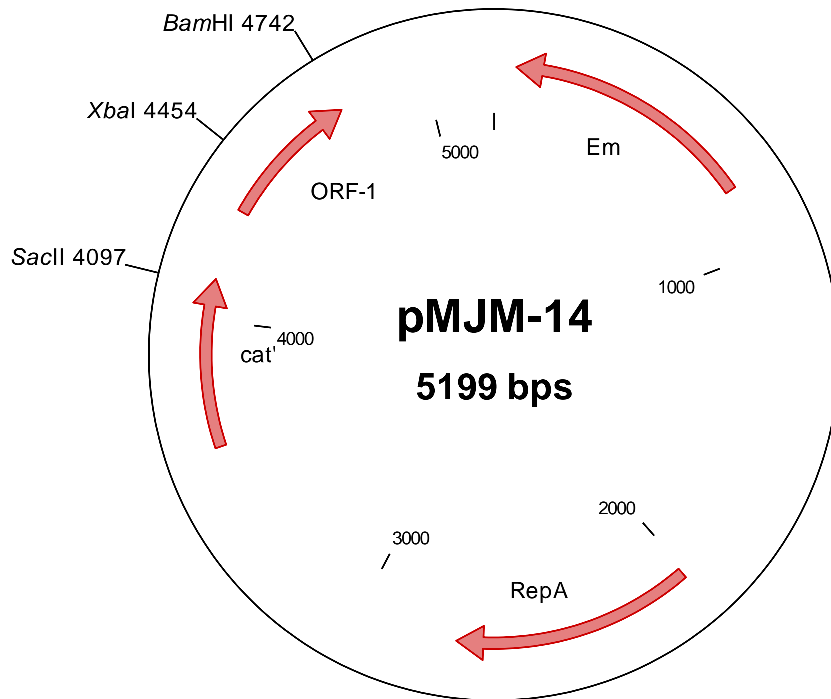


Figure A.4 Map of pMJM-19. pPLP_A; Secreted and anchored PLP epitopes. ORF-1 represents PLP epitopes and anchored genes.

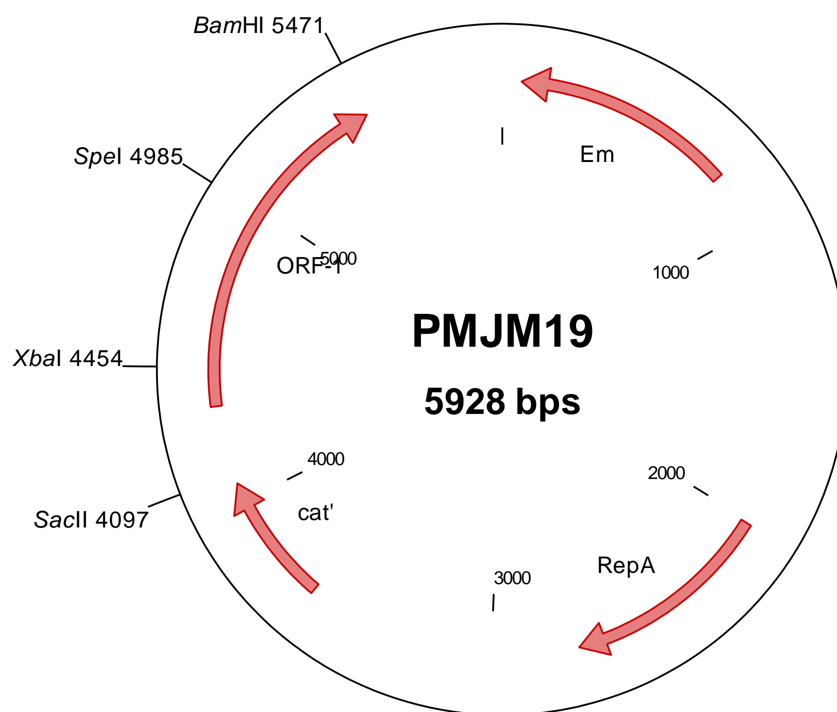


Figure A.5 Map of pMJM-20. pMOG_A; Secreted and anchored MOG epitopes. ORF-1 represents MOG epitopes and anchored genes.

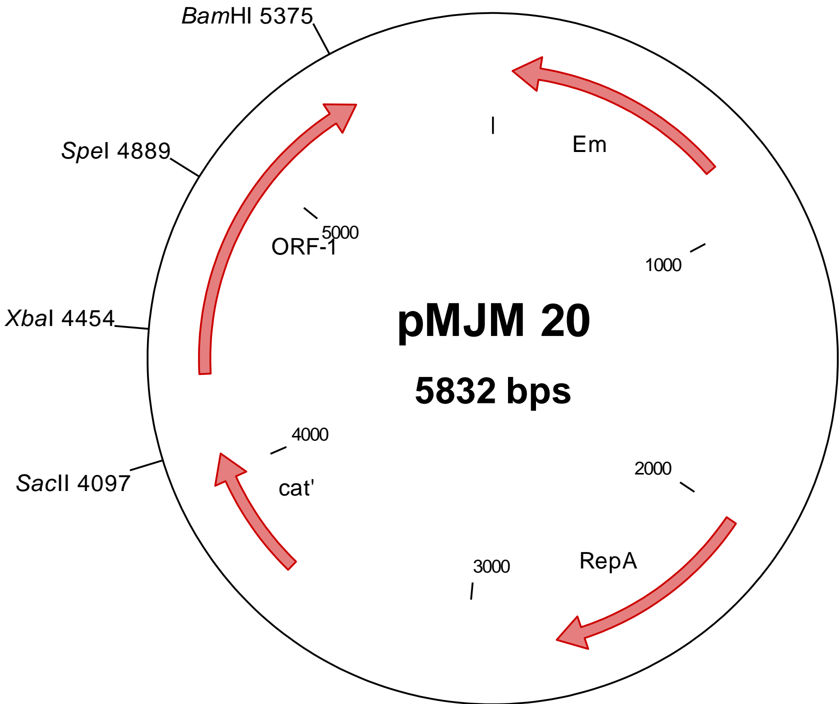


Figure A.6 Map of pMJM-21. pPLP/MOG_A; Secreted and anchored PLP/MOG epitopes. ORF-1 represents PLP/MOG epitopes and anchored genes.

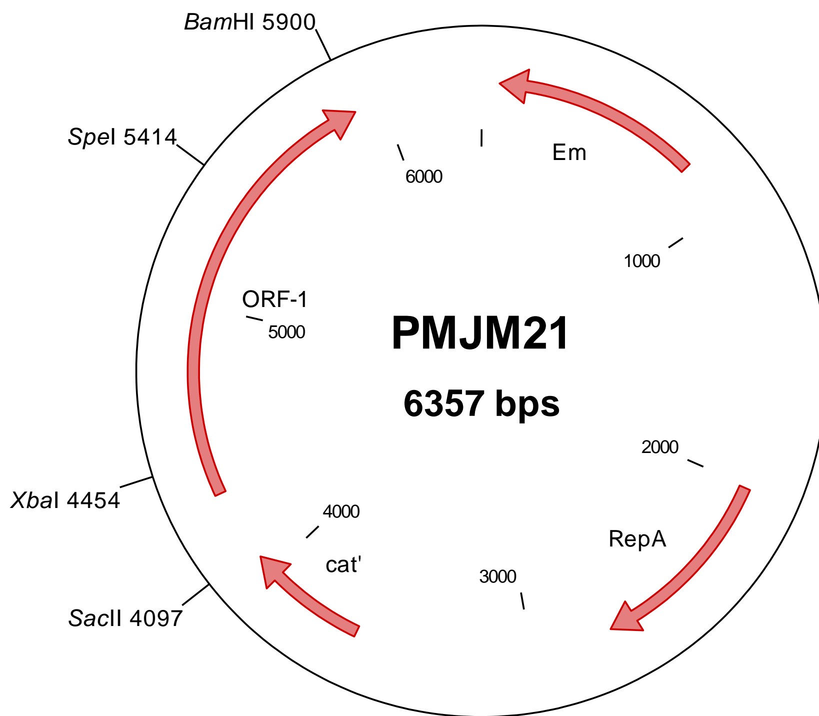


Figure A.7 Map of pMJM-22. pPLP_S; Secreted PLP epitopes. ORF-1 represents PLP epitopes.

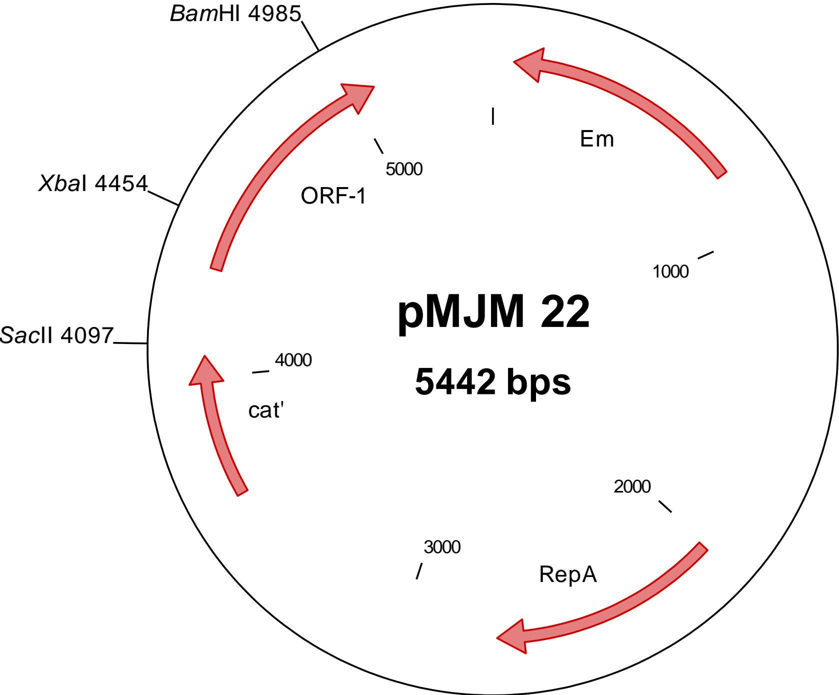


Figure A.8 Map of pMJM-23. pMOG_S; Secreted MOG epitopes. ORF-1 represents MOG epitopes.

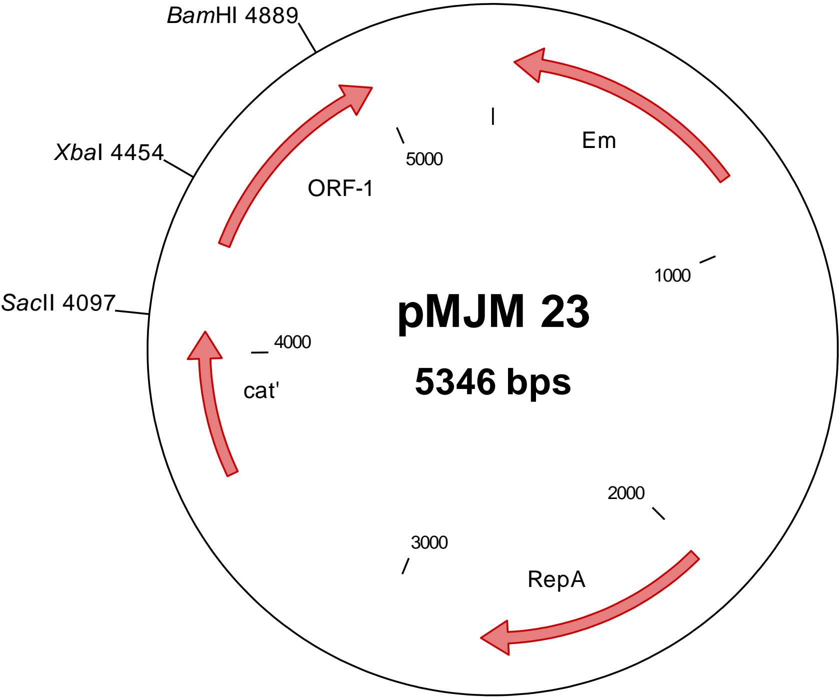
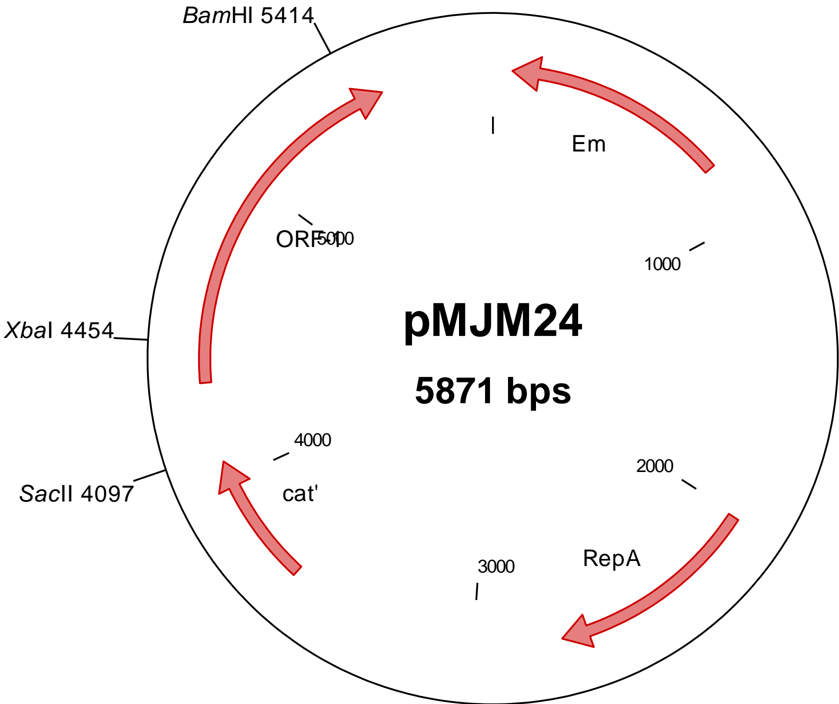


Figure A.9 Map of pMJM-24. pPLP/MOG_S; Secreted PLP/MOG epitopes. ORF-1 represents PLP/MOG epitopes.



APPENDIX B CHAPTER 4 PLASMID MAP

(Sequence of the plasmids can be found in S:\Miller Lab\Luyu Zhang\Chapter 4\Codon Optimized Genes\Ligation Products 5-21-2014)

Figure B.1 Map of pMJM-35. pPLP/MOG_SH3; Derivative of pRSET-B with PLP/MOG epitopes gene and Lysozyme domain SH3 from *L. gasseri*. ORF-1 represents insert.

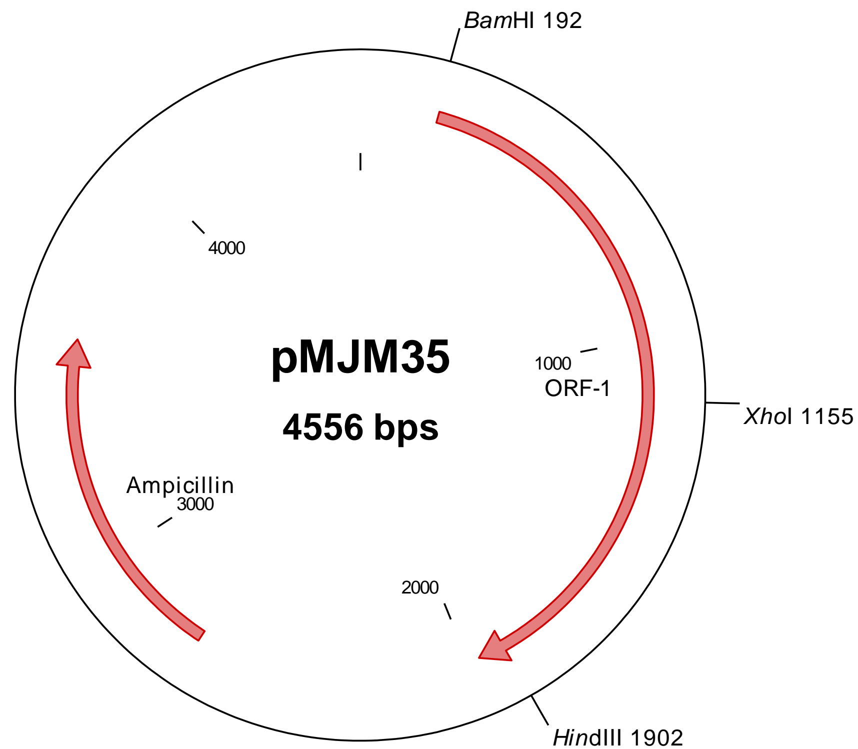


Figure B.2 Map of pMJM-36. pPLP/MOG_AcmA; Derivative of pRSET-B with PLP/MOG epitopes gene and AcmA domain from *L. lactis subsp. cremoris*. ORF-1 represents insert.

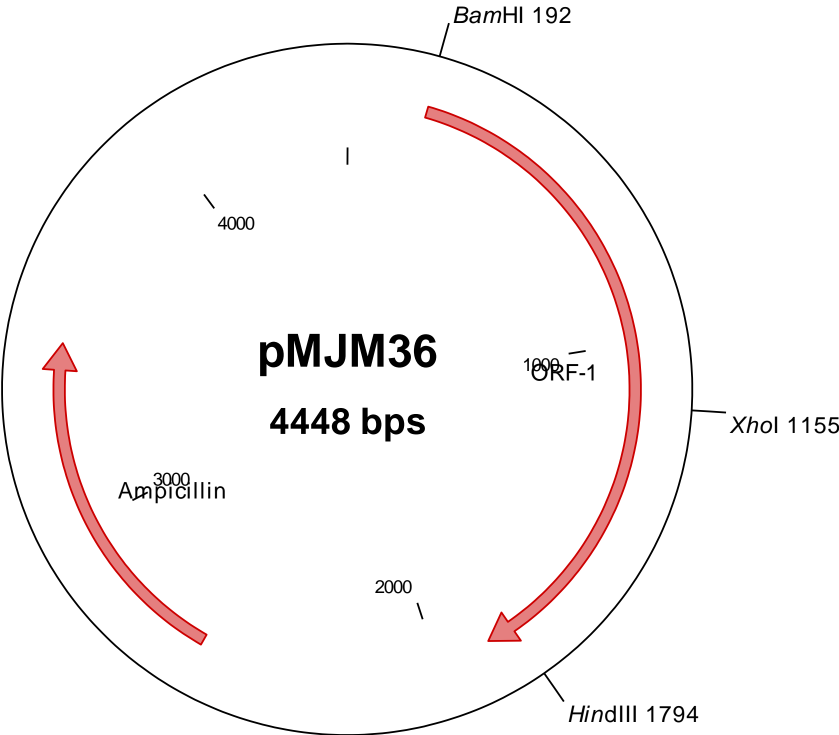


Figure B.3 Map of pMJM-37. pPLP/MOG_LysM; Derivative of pRSET-B with PLP/MOG epitopes gene and LysM, LysM domain from *L. gasseri*. ORF-1 represents insert.

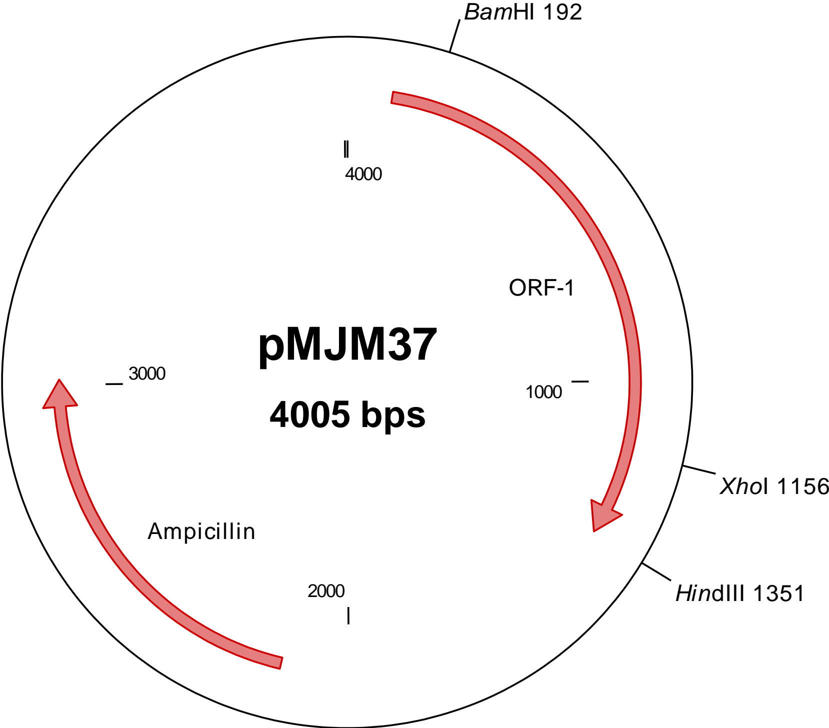


Figure B.4 Map of pMJM-40. pEGFP_LysM; Derivative of pRSET-B with EGFP gene from pEGFP-N1 and LysM domain from *L. gasseri*. ORF-1 represents EGFP gene.

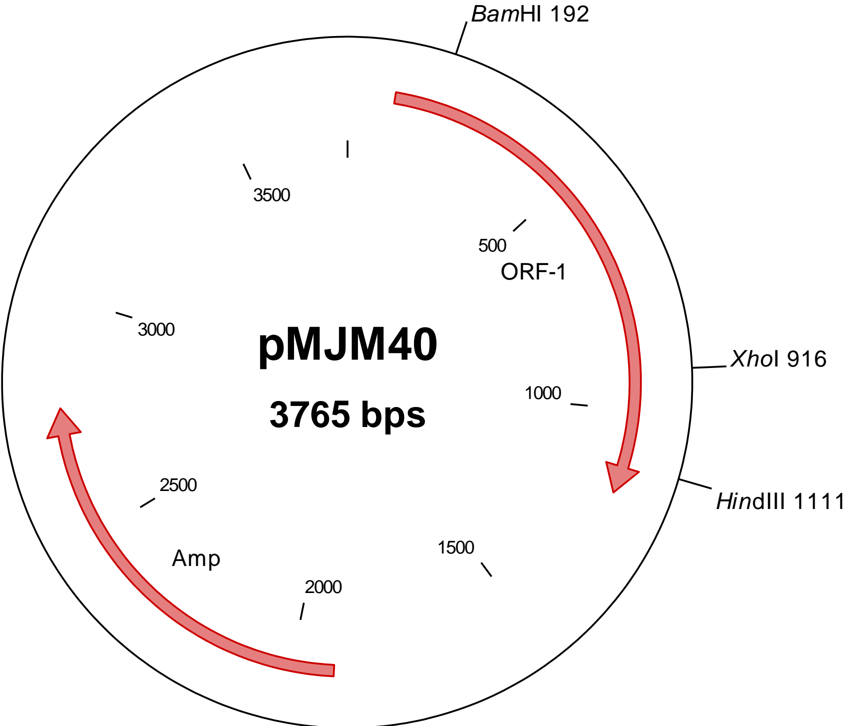


Figure B.5 Map of pMJM-41. pEGFP_SH3; Derivative of pRSET-B with EGFP gene from pEGFP-N1 and Lysozyme SH3 domain from *L. gasseri*. ORF-1 represents EGFP gene.

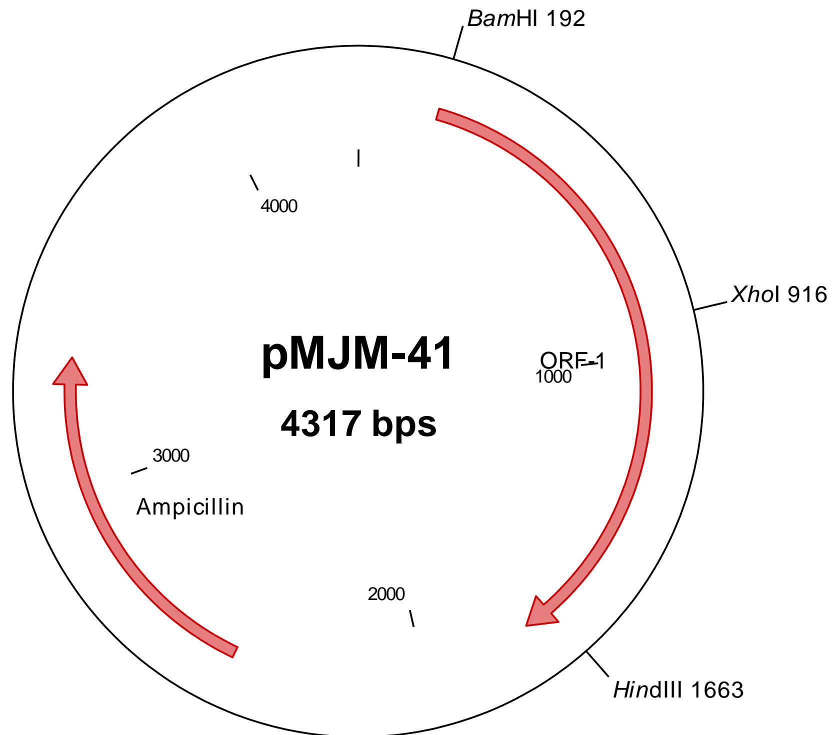


Figure B.6 Map of pMJM-42. pEGFP_AcmA; Derivative of pRSET-B with EGFP gene from pEGFP-N1 and AcmA domain from L. lactis subsp. cremoris. ORF-1 represents EGFP gene.

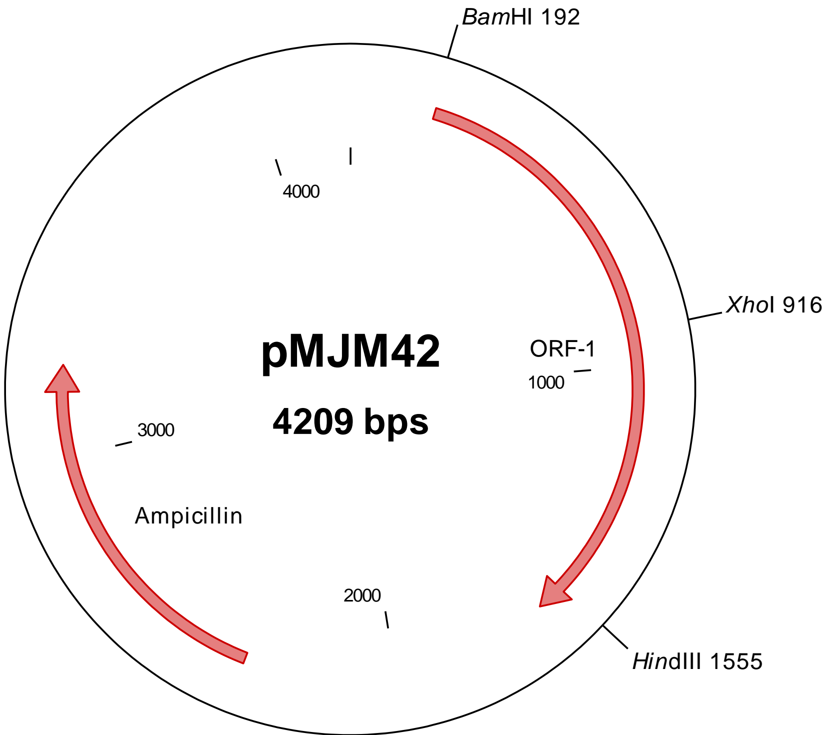


Figure B.7 Map of pMJM-44. pEGFP; Derivative of pRSET-B with EGFP gene from pEGFP-N1 ORF-1 represents EGFP gene.