## ABSTRACT

JOHNSON, BRANT ROBERT. Identification and functional analysis of S-layer associated proteins in *Lactobacillus* species. (Under the direction of Dr. Todd R. Klaenhammer).

Beneficial microorganisms such as probiotics are defined by the FAO/WHO as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host." *Lactobacillus acidophilus* NCFM is a generally recognized as safe, industrially significant lactic acid bacterium which has been sold commercially and consumed in various probiotic food formulations for over 35 years. Predicated by the availability of a fully sequenced and annotated genome, *L. acidophilus* NCFM is one of the most studied and well characterized probiotic bacteria.

Health-promoting aspects attributed to probiotic microbes, including adhesion to intestinal epithelia and modulation of the host mucosal immune system, are mediated by proteins found on the bacterial cell surface. Notably, certain probiotic and commensal bacteria contain an S-layer as the outermost stratum of the cell wall. S-layers are semi-porous, crystalline arrays of self-assembling, proteinaceous subunits called S-layer proteins (SLPs). In *Lactobacillus acidophilus* NCFM, the SLP, SlpA, has been implicated in mucosal immunomodulation and adhesion to host intestinal epithelium. As such, it is critical to explore the properties of S-layers and secreted cell surface proteins as a functional interface for probiotic activity. One such approach has focused on the characterization of novel S-layer associated proteins (SLAPs), which are extracellular proteins, non-covalently co-localized at the cell surface along with the S-layer.

Here, we describe the proteomic identification of SLAPs in *L. acidophilus* NCFM and other strains of the closely related *L. acidophilus* homology group. Exoproteomic characterization of the S-layer forming species of the group, including *L. crispatus*, *L.*

*helveticus*, and *L. amylovorus*, revealed conserved SLAPs. However, no SLAPs were found within the exoproteomes of the non-S-layer forming species of the homology group, including *L. gasseri*, *L. johnsonii*, and the taxonomic progenitor, *L. delbrueckii* subsp. *bulgaricus*. Using *L. acidophilus* NCFM as a model system, three genes encoding SLAPs were characterized: *lba1029*, encoding an uncharacterized surface protein; *acmB*, encoding a β-*N*-acetylglucosaminidase; and *prtX*, encoding a serine protease. Each gene was successively deleted from the chromosome of *L. acidophilus* NCFM to make the isogenic mutant strains NCK2258 (Δ*lba1029*), NCK2395 (Δ*acmB*), and NCK2282 (Δ*prtX*) for phenotypic analysis compared to a parent strain (NCK1909).

Phenotypic characterization of the Δ*lba1029* mutant demonstrated that the SLAP LBA1029 contributes to a pro-inflammatory TNF-α response from murine DCs. Chromosomal deletion of *acmB* resulted in aberrant cell division, autolysis, and autoaggregation. Complementation of *acmB* in the Δ*acmB* strain restored the wild-type phenotype, confirming the role of this SLAP in cell division. The absence of AcmB within the exoproteome had a pleiotropic effect on the S-layer, which led to a decrease in the ability of the Δ*acmB* strain to bind to mucin and extracellular matrices, *in vitro*. The Δ*prtX* mutant demonstrated an altered cellular morphology, which lead to a pleiotropic increase in adhesion to mucin and fibronectin, *in vitro*. Furthermore, Δ*prtX* demonstrated increased immunomodulation of TNF-α, IL-6, and IL-10 compared to wild-type, when exposed to mouse dendritic cells. Finally, *in vivo* colonization of germ-free mice with Δ*prtX* resulted in increased epithelial barrier integrity.

These data describe a new subset of proteins non-covalently co-localized at the cell surface along with S-layers. Here, we have shown that SLAPs play a critical role in cell

physiology, including cell division, autolytic activity, and extracellular protein turnover, as well as mediate intimate probiotic-host interactions, such as adhesion to mucin and extracellular matrices, immunomodulation, and maintenance of gastrointestinal epithelial barrier integrity. Despite these preliminary results, there is still a dearth of information regarding the structure and function of S-layers and SLAPs. Continued efforts characterizing the S-layer will undoubtedly reveal their evolutionary function, and will provide further insight into probiotic-host interactions for beneficial microorganisms which produce S-layers.

# Identification and Functional Analysis of S-layer Associated Proteins in *Lactobacillus* Species

by
Brant Robert Johnson

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Microbiology

Raleigh, North Carolina

2016

APPROVED BY:

| | |
|---|---|
| _____ | _____ |
| Dr. Todd R. Klaenhammer<br>Committee Chair | Dr. Rodolphe Barrangou |
| _____ | _____ |
| Dr. Amy M. Grunden | Dr. Robert M. Kelly |

## DEDICATION

To my wife, Kiersten, and my daughter, Genevieve.

I love you both more than I can describe.

## BIOGRAPHY

Brant Johnson was born in Phoenix, AZ, to parents Mike and Tami Johnson on January 4, 1990. He was raised in Greensboro, NC along with his twin brother Michael and older brother Cody, and graduated from Western Guilford High School in 2008. He enrolled at North Carolina State University, where he studied Biology and Viola Performance. After graduating with a B.S. in Biological Sciences in 2011, Brant continued his education at North Carolina State University, joining the Microbiology Ph.D. program under the advisement of Dr. Todd R. Klaenhammer. During his tenure in the Klaenhammer lab, Brant was awarded the 2014 Lactic Acid Bacteria Industrial Platform International Science Award at the FEMS $11^{th}$ International Symposium on Lactic Acid Bacteria and the $10^{th}$ Annual North Carolina State University Graduate Research Symposium poster award. In his spare time, he was the principal violist of the Raleigh Civic Symphony and the Raleigh Civic Chamber Orchestra. Brant lives in Raleigh, NC with his wife Kiersten, who received her PhD in Psychology at NC State, his daughter, Eve Marie (born July 26, 2015), and his Golden Retriever, Link.

## ACKNOWLEDGEMENTS

I would like to take the opportunity to offer my sincerest thanks to all of those who, through their love, friendship, and tireless support, have guided me throughout my tenure as a PhD student in the Klaenhammer lab. I recognize that these words are wholly insufficient to truly express my heartfelt gratitude, but I will try my best.

First and foremost, I would like to thank Dr. Todd Klaenhammer for supporting, advising, and investing in me over these last four years. Your passion for scientific research, insatiable curiosity, and unflinching selflessness all created the ideal atmosphere for my growth as a scientist. You saw something in me that I never saw in myself, and your steadfast encouragement brought me through the anxieties connected with pursuing an advanced degree. Beyond your professional guidance, I am most grateful for your friendship.

I would also like to thank my committee members, Dr. Rodolphe Barrangou, Dr. Amy Grunden, and Dr. Robert Kelly, for their guidance and suggestions. I am especially grateful for Dr. Barrangou for our numerous one-on-one meetings and your invaluable perspective on varied topics.

Through my stay in the Klaenhammer lab, I have worked with some of the best thinkers and researchers in the world. These people have all contributed to my success, through their guidance, support, and most importantly, their friendship. I am thankful for all those whose friendship made significant and meaningful contributions to my development as a scientist, including Dr. Grace Douglas, Rosemary (Roseala) Sanozky-Dawes, Dr. Sarah O'Flaherty, Dr. Yong Jun Goh, Evelyn Durmaz, Kurt Selle, Emma Call, Janice Lee, Katheryne Daughtry, Emily and Mark Stout, Dr. Emily Henriksen, Jeff Hymes, Alexandra

Briner, Mia Theilmann, Dr. Joakim Andersen, Courtney Klotz, Katelyn Brandt, Cassie Canez, Candalyn and Michael Rade, Evan Lowder, and Kevin Abbas. I am particularly grateful to Roseala, Sarah, and Jun for their unwavering friendship and willingness to guide me when I was confused and to help me up when I was down.

I am indebted to my parents, Mike and Tami Johnson, who have supported me in every step of my life. Thank you for instilling in me my Christian faith, which I rely on every day. Thank you, Mom, for always reminding me to laugh, smile, and find the humor in every situation. And thank you, Dad, for pushing me and reminding me to "finish strong." I love you both! To my brothers, Cody and Michael; I have no two greater friends in the world than you. It is refreshing to know that no matter what I do, I can always count on you guys to make fun of me and make me laugh.

Last, but not least, I am thankful for my wife, Kiersten Johnson. You are my rock, and without you I would certainly be lost in this world. My life forever changed when I first saw your face in August of 2004. Thank you for believing in me and supporting me, no matter what. I am so thankful that we have been able to grow and share in our journey through graduate school. Most importantly, thank you for our daughter, Genevieve Marie Johnson. You are the most amazing mother, and you two inspire me every day to be the best version of myself. Though I don't know what the future holds for us, I know that through it all, our family will grow in love and faith. I love you dearly; there is no one else I'd rather journey with.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

# CHAPTER 1

# Literature Review

## Impact of genomics on the field of probiotic research: Historical perspectives to modern paradigms

Published in 2014: Antonie van Leeuwenhoek 80th Anniversary Issue 106 (1): 141-156

## 1.1 Abstract

For thousands of years, humans have safely consumed microorganisms through fermented foods. Many of these bacteria are considered probiotics, which act through diverse mechanisms to confer a health benefit to the host. However, it was not until the availability of next generation sequencing and the era of genomics that mechanisms of probiotic efficacy could be discovered. In this review, we explore the history of the probiotic concept and the current standard of integrated genomic techniques to discern the complex, beneficial relationships between probiotic microbes and their hosts.

## 1.2 Introduction

### 1.2.1 History of probiotic bacteria and the probiotic concept

A multitude of autochthonous (naturally occurring) commensal bacterial species inhabit the mucosal surfaces of the gastro-intestinal tract (GIT), as well as those of the nose, mouth and vagina. It has long been held that the consumption of allochthonous (transient) beneficial bacteria, either through food products or supplements, has a positive influence on general health and well-being of the host via commensal interactions with the GIT immune system and resident microbiota. These beneficial microorganisms, known as probiotics, are defined by the World Health Organization as "live microorganisms, which when administered in adequate amounts, confer a health benefit upon the host" (FAO/WHO, 2002). Over the past four decades, there has been substantial research in the field of probiotics and, more specifically, into the mechanism of probiotic action within the host. However, the probiotic concept is not novel to the 20$^{th}$ and 21$^{st}$ centuries.

For millennia, humans have consumed microorganisms via fermented foods, which served to prevent putrefaction as well as increase sensory aspects in the food. Some of the first fermentations were likely the result of serendipitous contaminations in favorable environments resulting in soured milk products such as kefir, leben, koumiss, yogurt and sour cream – products that are still consumed worldwide (1). Furthermore, through the continued practice of milk souring along with back slopping techniques, humans inadvertently aided in the domestication of certain microorganisms to diverse food environments over time (2). Not only were these products safe to consume, fermented dairy foods were culturally significant,

as evidenced by their mention in the Bible and early sacred Hindu texts, as well as therapeutically consumed (1, 3-4)

In the late 19th century, French biochemist Louis Pasteur premiered significant discoveries leading to a greater scientific understanding of fermentation (Figure 1.1). Upon studying wine and beer fermentations, Pasteur demonstrated that fermentation reactions are carried out by microorganisms. Furthermore, he established that the growth of these microbes is not a product of spontaneous generation, as was the prevailing scientific and cultural consensus, but is instead due to biogenesis, which posits that all living things come only from other living things. On the foundation of Pasteur's research, Russian Nobel laureate, Élie Metchnikoff first popularized the concept of probiotics around the turn of the 20th century. In his book, *The Prolongation of Life: Optimistic Studies*, Metchnikoff (5) proposed that putrefaction in the intestines correlated with shortened life expectancy. Reconciling long-held observations involving lactic acid food fermentations with microbial feeding studies in animals and humans, Metchnikoff proposed that lactic acid-producing microorganisms may act as anti-putrefactive agents in the gastrointestinal tract when consumed. In fact, he hypothesized that by transforming the "wild population of the intestine into a cultured population… the pathological symptoms may be removed from old age, and… in all probability, the duration of the life of man may be considerably increased" (5). His theory was bolstered upon observing a higher prevalence of centenarians in Bulgaria, a region known for the consumption of soured milk. Michel Cohendy, a colleague at the Pasteur Institute, provided experimental data to support Metchnikoff's hypothesis. In two feeding trials of human subjects, Cohendy found that the Bulgarian bacillus (now known as

*Lactobacillus delbrueckii* subsp. *bulgaricus*) was recoverable from feces; reduced the prevalence of putrefactive toxins; and aided in the treatment of colitis following transplantation to the large intestine (6, 7). The aforementioned studies on *L. bulgaricus* enthralled the health-conscious society of Europe in the early 20$^{th}$ century and soon the Pasteur Institute of Paris began selling the *Lactobacillus* under the label of "Le Ferment" (3, 4).

Despite the promising observations made by Metchnikoff and colleagues at the genesis of the probiotic concept, there was still meager scientific evidence suggesting any definitive probiotic strains or their purported effector mechanisms. In fact, Leo Rettger and coworkers at Yale University found that *L. bulgaricus* could not survive gastric passage to colonize the small intestine (8). This study called into question which strain(s) may have been present in the original therapeutic administration studies performed by Cohendy, and subsequently sold as "Le Ferment." Instead, *Lactobacillus acidophilus* was touted to be a more suitable candidate for therapeutic applications because of its ability to survive gastric passage and transform the intestinal flora in conditions of lactose and dextrin supplementation (9). It is based on these seminal studies that the foundation of therapeutic treatment with *L. acidophilus* originated. However, even rigorous studies such as these were limited by the techniques and technologies of their time. *L. acidophilus* could not be distinguished from other aciduric commensal lactobacilli, such as *Lactobacillus gasseri*, until electrophoretic DNA-DNA hybridization studies on *Lactobacillus* lactate dehydrogenase enzymes were performed in the 1970s (10, 11). Therefore, it is unknown whether the cultures

administered during these studies were indeed pure *L. acidophilus*, or mixed culture with *L. acidophilus*, *L. gasseri* and other aciduric lactobacilli.

After examining the burgeoning experimental evidence of probiotic bacteria, a Japanese physician named Minoru Shirota sought to isolate a human-derived strain of *Lactobacillus* for therapeutic application. And thus, in 1930, Shirota selected a species of *Lactobacillus* (now known as *Lactobacillus casei* Shirota) from human feces that could survive passage through the GIT (4). From this culture, Shirota developed and commercialized one of the first fermented milk products, Yakult (4). Not only was this a major advancement for the commercial dairy industry, but one of the first products to deliver a pure, defined strain-cultured product. Yakult remains a staple product in Japanese, Korean, Australian and European markets. Since then, there has been a massive expansion of the functional food market, especially in fermented dairy products containing probiotic bacteria (12). In fact, a recent global market analysis on probiotics revealed a 7% annual growth during the 2012 fiscal year, with a forecast of $48 billion in earnings within the next five years (Global Industry Analysis Report, 2012). Furthermore, probiotics are expanding from functional food markets to pharmaceutical, therapeutic markets. This market increase correlates to the advancements of the scientific and regulatory aspects of probiotic mechanisms and delivery (13). Considering that there are still a great number of scientific questions to explore concerning probiotic activities and interactions in the GIT, there remains a bright future for the field of probiotic research and the market thereof.

### 1.2.2 Modern use of probiotic bacteria

Despite the long, storied history of probiotic discovery and therapeutic application, resounding clinical and experimental evidence for the use of probiotic bacteria has only recently come to a head (Table 1). One prominent example is the use of probiotics to treat functional gastrointestinal disorders (FGID). For many FGID, such as irritable bowel syndrome (IBS), there are few pharmacological treatment options due to low efficacy and serious side effects (14). Furthermore, IBS is quite common and is thought to be caused by changes in the GI microbiome (15). Recently, a systematic review of successful clinical interventions using probiotics to treat various FGID has been compiled as a reference for clinicians to make evidence-based treatment decisions (16). This systematic analysis reflects a notable caveat that must be made in probiotic research; namely, that probiotic activities are strain-specific (16, 17). Because evidence clearly suggests not only the efficacy of probiotic therapy, but also the importance of understanding each strain, the paradigm of probiotic research is rightfully shifting towards understanding the mechanistic action of each specific strain.

Among the most studied species of probiotic bacteria are those from the genera *Lactobacillus* and *Bifidobacteria* (Table 2). The genus *Lactobacillus* is comprised of a diverse clade of Gram-positive, anaerobic/microaerophilic, non-sporulating, low G+C content lactic acid bacteria (LAB) belonging to the phylum *Firmicutes* (18). Biochemically, they are strictly fermentative; sugar fermentations result in either the sole production of lactic acid, or the production of lactic acid in conjunction with smaller amounts of carbon dioxide and ethanol/acetic acid (19, 18). Lactobacilli inhabit diverse ecological niches including the

GIT of humans and animals, as well as vegetable, plant and dairy food environments. While *Lactobacillus* species are not dominant members of the colonic microbiota, many are probiotic because of their ability to survive in the less-diverse small intestine. Members of the genus *Bifidobacterium*, of the phylum *Actinobacteria,* are Gram-positive, non-motile, anaerobic bacteria, with low levels of genomic and phylogenetic diversity (20). They were originally isolated from the feces of breast-fed infants and nearly 50 species isolated from the GIT of humans, animals, and insects have since been classified (20). In fact, bifidobacteria are among the most prominent commensal bacteria found in the human colon and dominate the developing microbiome in breast-fed infants (21, 22).

Since the resolution of the first bacterial genome sequence (*Haemophilus influenzae*), an exponential advancement in sequencing processing, genome assembly and annotation technologies, at increasingly economical pricing, has yielded well over a thousand publicly available genomes (23, 24). Notably, many of these genomes are derived from lactic acid bacteria used as probiotics or starter cultures for food fermentations (25, 26). Access to these data has revolutionized the molecular view of probiotic bacteria, as well as the way research questions related to probiotic mechanisms are formulated. Specifically, advancements in genomic tools including functional genomics, transcriptomics, proteomics and secretomics, have hastened research deciphering the interactions between probiotics and the GIT (Figure 1.2). These techniques are being used to bridge the mechanistic gap between what has been seen clinically and anecdotally for hundreds of years.

### 1.3 Characterizing probiotic mechanisms using genomic tools

Referencing the genome sequences of probiotic bacteria, the mechanism and interaction of probiotics with the host GIT are being discovered through the integration of functional genomic techniques. Within this context, there are three points of focus relating to probiotic action: i) survival through gastrointestinal transit and adhesion to intestinal epithelia; ii) competitive exclusion and antimicrobial activity; and iii) modulation of the host GIT immune system (Figure 1.3).

#### 1.3.1 Survival in and adhesion to the GIT

One of the most essential qualities of a probiotic microorganism is the ability to survive the varied environments of the GIT (Figure 1.3A). The probiotic must be able to adapt to acidic gastric juices and bile in the small intestine. Like many aciduric bacteria, the lipid membranes of lactobacilli exposed to acid and bile are altered in order to increase survival. The lipid membrane of *L. casei* demonstrated a marked increase of mono-unsaturated fatty acids in response to acidification (27). Similarly, the lipid membrane of *Lactobacillus reuteri* exposed to bile salts and cholesterol increased the number of mono-unsaturated fatty acids compared to saturated fatty acids (28). Considering these observations, a recent study using the probiotic *Lactobacillus rhamnosus* GG found that an exogenous oleic acid [C18:1 (cis-9)] source significantly increased acid survival by incorporating the oleic acid into the membrane, which is reduced to stearic acid (C18:0) in the acidified environment (29). Aside from the biochemical changes to the lipid membranes, the *Lactobacillus* species have global transcriptional responses to these stressors, usually

through two-component regulatory systems (2CRS; 30). Numerous transcriptomic analyses have been used in lactobacilli to identify differentially expressed genes, such as those corresponding to 2CRS, surface proteins and proton efflux systems, in response to gastric acid stress (31, 32) and bile stress (33, 34). Bacteria quickly sense and respond to changing environmental conditions via 2CRS through the sensing domains of a transmembrane histidine protein kinase (HPK). Upon receiving the environmental signal, the HPK is activated to autophosphorylate a specific histidine residue which is transferred to the regulatory domain of the response regulator (RR), a DNA-binding transcriptional regulator. Therefore, 2CRS can be predicted from bacterial genome sequence annotations based on the presence of putative HPK and RR in close proximity to one another (35, 36). In *L. acidophilus* NCFM, a gene (*lba1524*) encoding a functional HPK was knocked out, resulting in a mutant with increased sensitivity to acid stress compared to the parent strain. Furthermore, transcriptomic analysis via DNA microarray comparing the *lba1524* mutant to wild-type demonstrated an impact on 80 genes. Notably, one upregulated gene in the HPK mutant was the LuxS homolog of the autoinducer-2 quorum sensing compound, important for survival in gastric juices and adhesion to intestinal epithelial cell lines (37, 38).

The response of lactobacilli to bile salts has also been measured through microarray analysis. In *Lactobacillus plantarum* a DNA-microarray was performed after exposure to porcine bile, resulting in the identification of bile response genes encoding stress response proteins, cell envelope proteins and an $F_0F_1$ ATPase (33). A similar transcriptomic profiling of *L. acidophilus* revealed multiple genes involved in bile tolerance, including a 2CRS and multi-drug resistance (MDR) transporter efflux pumps (34). Mutants with insertionally

inactivated genes for the bile inducible 2CRS HPK and RR were more sensitive to bile compared to parent strains, confirming their role in bile tolerance (34). A recent comparative proteomic analysis on bile sensitive and bile tolerant strains of *L. plantarum* corroborated these transcriptomic data and elucidated potential biomarkers for the selection of bile tolerant probiotic strains (39). Additionally, the role of efflux pumps and MDR transporters in probiotic bile tolerance are beginning to be recognized. Functional genomic analyses of MDR transporters in probiotic strains of *L. reuterii* and *L. acidophilus* demonstrated roles in bile tolerance (40, 41). Furthermore, a MDR transporter gene in *Bifidobacterium longum*, *betA* (bile efflux transporter), was recently identified through *in silico* genome analysis and functionally characterized (42). Heterologous expression of *betA* in *Escherichia coli* conferred bile tolerance through active efflux of bile salts.

Survivability and enhancement of beneficial microbes in the GIT can be accomplished by providing selectively utilizable carbohydrates, called prebiotics (43, 44). These carbohydrates, including β-galactooligosaccharide (GOS), lactulose, fructo-oligosaccharide and inulin, are resistant to gastric acidity, hydrolysis and gastrointestinal absorption (45). As growth substrates, prebiotic carbohydrates are preferentially metabolized by species of health-promoting bacteria. Recently, differential transcriptomic and functional genomic analyses have demonstrated the capabilities of the probiotic bacteria *L. acidophilus* NCFM (46) and *Bifidobacterium lactis* B1-04 (44) to utilize prebiotic oligosaccharides. With these data, novel symbiotic formulations of corresponding prebiotics for *L. acidophilus* and *B. lactis* can be created to aid in the survival and probiotic effectiveness in the host small intestines and colon, respectively. In a similar vein, there is compelling evidence to suggest

glycogen metabolism is a colonization factor for probiotic LAB. Glycogen is a large molecular mass, soluble α-1,4-linked glucose polymer with numerous α-1,6-linked branches. It has multiple physiological functions in various bacteria and has been theorized to function as a carbon capacitor for the regulation of energy flux (47). Recent work by Goh and Klaenhammer (48) demonstrated the functionality of a putative glycogen metabolism operon found in the genome sequence. Remarkably, through a series of chromosomal deletions and phenotypic assays, glycogen metabolism was found to regulate growth maintenance, bile tolerance and complex carbohydrate utilization in *L. acidophilus* (48).

Beyond surviving gastrointestinal transit, a second key factor for probiotic activity is through adhesion to intestinal epithelia of the GIT. Preliminary *in vitro* studies using Caco-2 human intestinal epithelial cell lines revealed multiple probiotic lactobacilli with adhesive capabilities (49, 50). Notably, there has also been work demonstrating the adhesiveness of *Bifidobacterium* spp. to human intestinal mucus (51). However, access to genome sequence data, paired with integrated genomic techniques, elucidated mediators of probiotic adhesion. The majority of these factors are secreted or attached to the cell wall in a sortase-dependent manner, in order to interface with the intestinal epithelia (reviewed by: 30, 52). In a study using *L. plantarum* WCFS1, two of these sortase-dependent proteins (SDP) were found to be induced in the murine GIT (53). Mutational analysis of one of these genes (*lp_2940*) resulted in a 100- to 1000-fold decrease in persistence capacity of the *L. plantarum* lp_2940 knockout mutant in a mouse model. In *L. acidophilus* NCFM, *in silico* genome screening lead to the selection of five putative adhesion cell surface proteins, including a fibronectin binding protein (FbpA), s-layer protein (SlpA), mucin-binding protein (Mub) and two R28

homologues involved in streptococcal adhesion (54). Through mutational analysis, FbpA, Mub, and SlpA were all found to contribute to adhesion to Caco-2 epithelial cell lines. Similarly, a stress response protein and an aggregation-promoting factor (both cell surface proteins) were found in later studies to contribute to adherence to Caco-2 cells (55, 56). In *Lactobacillus crispatus* JCM5810, the S-layer protein (CbsA) contains domains that bind to laminin and collagens (57). Genome screening and secretome analysis of *Lactobacillus salivarius* UCC118 led to the identification of three SDPs with mucus-binding domains. A sortase-deficient strain was created, resulting in significantly reduced adherence to Caco-2 and HT-29 cell lines *in vitro* (58). Notably, genomic analysis between two strains of *L. rhamnosus* revealed the presence of a genomic island in *L. rhamnosus* GG that contained three secreted, sortase-dependent pilins encoded by *spaCBA* (59). Immunoblotting and immunogold electron microscopy confirmed the formation of cell wall-bound pili (Figure 1.4). Furthermore, mutational analysis of the *spaC* gene abolished the adherence capability of *L. rhamnosus* GG to human intestinal mucus, implicating the role of these unique pili structures in adherence and retention in the GIT. Since this initial report, a type IVb tight adherence (Tad) pilus-encoding gene cluster has been identified in *Bifidobacterium breve* UCC2003 (60; Figure 1.4). Mutational analysis demonstrated that the Tad gene cluster was essential for colonization in a murine model. Collectively, these data suggest that there are multiple cell surface factors which contribute to probiotic adherence to human intestinal epithelia.

### 1.3.2 Competitive exclusion and antimicrobial activity

Another health-promoting aspect of probiotic bacteria is the prevention of pathogenic infection (Figure 1.3B). When probiotic lactobacilli are ingested, they temporarily coat the mucosal layer and epithelia of the small intestine (see above) leading to both physical and chemical barriers against harmful bacteria (61). Initial studies demonstrated that lactobacilli inhibited adherence of Gram-negative uropathogens when uroepithelial cells were pre-incubated with whole, viable *Lactobacillus* (62). Furthermore, *in vivo* mice models demonstrated that *L. casei* GR1 was capable of preventing urinary tract infections from *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonus aeruginosa* (63). In both cases, the mechanism of pathogenic antagonism was due to the ability of lactobacilli to adhere to the urogenital epithelia, thus preventing infection through competitive exclusion of the pathogen. These studies and others suggested that similar competitive exclusion could be possible in the human GIT using probiotic lactobacilli and bifidobacteria. In fact, numerous studies have demonstrated the *in vitro* inhibition of numerous gastrointestinal pathogens through competitive exclusion of probiotic lactobacilli and bifidobacteria using intestinal cell lines (reviewed by: 61).

In addition to competitive exclusion of pathogens, probiotic bacteria produce numerous chemical antimicrobials which may prevent pathogenic infection. These include: hydrogen peroxide (64, 65), lactic acid (66), biosurfactants (67), immunomodulatory products (68) and bacteriocins (69). Bacteriocins are bacterially derived antimicrobial peptides that are active against other bacteria, but to which the producing bacterium is immune (70). Lactic acid bacteria produce numerous broad-spectrum bacteriocins which are

divided into three main classes: class I bacteriocins (lantibiotics; 71), small peptides possessing lanthionine residues; class II bacteriocins, which are heat-stable and do not contain lanthionine residues; and bacteriolysins, which are large, heat-labile murein hydrolases (70; Figure 1.5). Historically, scientists have sought to characterize the genetics and biochemistry of bacteriocins produced by LAB, in part due to their safety implications in the dairy fermentation industries (72, 73). In fact, one of the most industrially relevant bacteriocins is nisin, a lantibiotic produced by *Lactococcus lactis* (74). Nisin has two modes of bacteriocidal activity (Figure 1.5). First, it can bind lipid II, the main transporter of peptidoglycan subunits, disrupting cell wall synthesis (75). Nisin also targets lipid II as a docking mechanism for pore formation, leading to rapid cell death due to disruption of the proton motive force (76). Notably, Gram-positive bacteriocins generally have a narrow range of toxicity, as they are primarily lethal to closely related bacterial species such as *Staphylococcus*, *Listeria* and other LAB (61). Most research involving LAB-associated bacteriocins has been *in vitro*. However, a landmark study by Corr et al. (77) demonstrated that a bacteriocin produced by *L. salivarius* UCC118 caused *in vivo* protection in mice challenged with the food-borne pathogen *Listeria monocytogenes*. Using a functional genomics-based mutational analysis, generating a stable *L. salivarius* UCC118 strain deficient in bacteriocin production, undoubtedly established the role of this bacteriocin in protection against *L. monocytogenes* infection.

### 1.3.3 Probiotic modulation of the gastrointestinal mucosal immune system

Perhaps one of the most important aspects of probiotic bacteria is the ability to modulate the host GIT mucosal immune system locally and systemically (Figure 1.3C). The interaction between the probiotic microbe with the resident microbiota, gastrointestinal epithelia and gut immune cells to produce an immunomodulatory response is quite complex, and has been reviewed exhaustively (78-83). Probiotic microbes modulate mucosal immunity through the interaction of proteinacious microorganism-associated molecular patterns (MAMPs) with pattern recognition receptors (PRRs) on antigen-presenting cells (APCs), such as dendritic cells and macrophages. Upon exposure to MAMPs, the PRRs (including NOD-like receptors, Toll-like receptors, and C-type lectin receptors) activate nuclear factor (NF)-κB and mitogen-activated protein kinase signaling cascades, which modulate the expression of cytokine and chemokine genes. The most common MAMPs from probiotic microorganisms are lipoteichoic acids (LTA), peptidoglycan and S-layer proteins (81). Multiple studies have explored the immunomodulatory effect of these MAMPs using functional genomic techniques. In a seminal study, the probiotics *L. casei* and *L. reuteri* were found to induce Il-10 producing regulatory T-cells through the modulation of the DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN; 84). Targeting of DC-SIGN by probiotic bacteria is potentially an important factor for treatment of inflammatory conditions via the production of anti-inflammatory IL-10. The S-layer protein (SlpA) of *L. acidophilus* NCFM was found to bind DC-SIGN, which regulate immature DC and T-cell functionality (Konstantinov et al., 2008). Using *L. plantarum* NCIMB8826, cell wall composition was examined for immunomodulatory effects by creating a mutant (*dlt*$^{-}$) which produced modified teichoic

acids with less D-alanine than the parent strain (86). The mutant demonstrated a significant reduction in production of proinflammatory cytokines compared to wild type, along with a simultaneous increase in anti-inflammatory IL-10. Furthermore, the *dlt*⁻mutant was more protective in an *in vivo* murine colitis model than the parent strain (86). An LTA-deficient strain of *L. acidophilus* NCFM, created by a clean deletion of the *lba0447* phosphoglycerol transferase, was able to abate induced colonic-inflammation in a colitis mouse model through the down regulation of pro-inflammatory IL-12 and TNF-α and the up regulation of anti-inflammatory IL-10 (87). Additionally, this same mutant reduced colonic polyposis in a colon cancer mouse model, through the normalization of pathogenic immune responses (88).

Like many probiotic effectors, most MAMPs are found on the cell surface of Gram-positive microbes. Recently, the genomes and proteomes of several lactobacilli were bioinformatically screened to create a secretome database cataloging the various extracellular proteins in LAB (89, 90). Consequently, using *in silico* genome analysis and by reference to the LAB secretome, a putative MAMP can be selected and validated through mutagenesis (81). Indeed, a recent study of *L. acidophilus* used a proteomic-based method to identify S-layer associated proteins (SLAPs) *in situ* (91). After extraction, the SLAPs were identified through mass spectrometry and referenced to the LAB secretome. Mutational analysis of one SLAP (LBA1029), revealed an immunomodulatory phenotype using *in vitro* bacterial-DC co-incubation assays, suggesting the potential of multiple unknown MAMPs associated with the S-layer of *L. acidophilus* NCFM. Researchers are also trying to understand the complex dynamic of host-microbe crosstalk by using whole transcriptome profiling of human intestinal epithelia upon exposure to probiotics. In one study, transcriptomes were obtained

from the mucosa of the proximal small intestines of healthy volunteers exposed to probiotic *L. acidophilus, L. casei,* and *L. rhamnosus* (92). The transcriptional networks induced by each probiotic were unique to each strain and remarkably similar to response profiles obtained from bioactive components and drug treatments. *In vitro* transcriptome profiling of Caco-2 intestinal epithelial cell lines exposed to *L. acidophilus* NCFM corroborated these data (93). Similarly, *B. bifidum* PRL2010 transcriptome analyses with both *in vitro* human cell lines and *in vivo* murine models demonstrated the capacity for strain PRL2010 to modulate host innate immunity (94).

### 1.4 Conclusions and Future Directions

While the paradigm of discovery based genomics in probiotic LAB has uncovered vital aspects of probiotic mechanisms, it has also revealed the complexity of these interactions with the resident microbiota and the mucosal immune system. But with this challenge has come great opportunity. For example, probiotic bacteria are now being explored as suitable models for vaccine/drug delivery, due to their close association with host immunity and immunomodulatory action (95-97). Furthermore, recent discoveries are also demonstrating that the roles of probiotic bacteria and the resident microbiota extend far beyond gastrointestinal health. Specifically, studies on the bi-directional crosstalk between the GIT and the brain (the gut-brain axis) are revealing the neurochemical importance of gut homeostasis (98). Along with these advancements, it is important that human clinical trials continue with experimental designs that are well-controlled and well-defined, reflecting the great progress that has been made in the field of probiotic and GIT microbiome research

(reviewed by: 17). With more than a century passing since Metchnikoff's observations, keen experimental design using integrated genomics has led to a clearer definition of probiotic bacteria, as well as a model for continued discovery.

## 1.4 Acknowledgements

Research on the functional genomics of probiotic microbes in the Klaenhammer group is partially supported by the North Carolina Agricultural Foundation and Danisco/Dupont Nutrition and Health. The authors wish to thank Dr. Sarah O'Flaherty for helpful discussion on figure design.

## 1.5 References


1. **Hosono, A**. 1992. Fermented milk in the orient in *Functions of Fermented Milk: Challenges for the Health Sciences*, 61-78, Elsevier Science Publishers Ltd, Barking

2. **Douglas GL, Klaenhammer TR**. 2010. Genomic evolution of domesticated microorganisms. Annu Rev Food Sci Technol **1**:397-414.

3. **Bibel, DJ**. 1988. Elie Metchnikoff's bacillus of long life. ASM News **54**:661-665.

4. **Shortt, C**. 1999. The probiotic century:historical and current perspectives. Trends Food Sci Technol **10**:411-417

5. **Metchnikoff, E**. 1907 The prolongation of life: optomistic studies. William Heinemann, London.

6. **Cohendy, M**. 1906. Déscription d'un ferment lactique puissant capable de s'acclimater dans l'intestin d'homme. Comptes redus des séances et mémoirs de la société de biologie **60**:558-560.

7. **Cohendy, M**. 1906. Essai de traitement de l'enterite muco-membraneuse aigue par l'acclimation d'un ferment lactique dans le gros intestine. Comptes redus des séances et mémoirs de la société de biologie **60**:363.

8. **Rettger LF**. 1915. The Influence of Milk Feeding on Mortality and Growth, and on the Character of the Intestinal Flora. J Exp Med **21**:365-388

9. **Rettger LF, Cheplin HA**. 1921. A treatise on the transformation of the intestinal flora, with special reference to implantation of *Bacillus acidophilus*. Yale University press, New Haven, CT.

10. **Gasser F**. 1970. Electrophoretic characterization of lactic dehydrogenases in the genus *Lactobacillus*. Microbiology **62**:223-239

11. **Gasser F, Doudoroff M, Contopoulos R**. 1970. Purification and properties of NAD-dependent lactic dehydrogenases of different species of *lactobacillus*. Microbiology **62**:241-250

12. **Sanders ME, Huis in't Veld J**. 1999. Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labeling issues. Antonie van Leeuwenhoek **76**:293-315

13. **Foligne B, Daniel C, Pot B**. 2013. Probiotics from research to market: the possibilities, risks and challenges. Curr Opin Microbiol **16**:284-292.

14. **Shen YH, Nahas R**. 2009. Complementary and alternative medicine for treatment of irritable bowel syndrome. Can Fam Physician **55**:143-148

15. **Porter CK, Gormley R, Tribble DR, Cash BD, Riddle MS**. 2011. The Incidence and gastrointestinal infectious risk of functional gastrointestinal disorders in a healthy US adult population. Am J Gastroenterol **106**:130-138.

16. **Hungin AP, Mulligan C, Pot B, Whorwell P, Agreus L, Fracasso P, Lionis C, Mendive J, Philippart de Foy JM, Rubin G, Winchester C, de Wit N, European Society for Primary Care G**. 2013. Systematic review: probiotics in the management of lower gastrointestinal symptoms in clinical practice -- an evidence-based international guide. Aliment Pharmacol Ther **38**:864-886.

17. **Sanders ME, Guarner F, Guerrant R, Holt PR, Quigley EM, Sartor RB, Sherman PM, Mayer EA**. 2013. An update on the use and investigation of probiotics in health and disease. Gut **62**:787-796.

18. **Pot B, Ludwig W, Kersters K, Schleifer KH**. 1994. Taxonomy of lactic acid bacteria. In *Bacteriocins of lactic acid bacteria*, pp 13-90. Chapman and Hall, Glasgow.

19. **Hammes WP, Vogel RF**. 1995. The genus *Lactobacillus*. In *The genera of lactic acid bacteria*, pp 19-54. Blackie academic and profesional, Glasgow.

20. **Ventura M, Canchaya C, Del Casale A, Dellaglio F, Neviani E, Fitzgerald GF, van Sinderen D**. 2006. Analysis of *bifidobacterial* evolution using a multilocus approach. Int J Syst Evol Microbiol **56**:2783-2792.

21. **Turroni F, Ribbera A, Foroni E, van Sinderen D, Ventura M**. 2008. Human gut microbiota and bifidobacteria: from composition to functionality. Antonie van Leeuwenhoek **94**:35-50.

22. **Favier CF, Vaughan EE, De Vos WM, Akkermans AD**. 2002. Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol **68**:219-226

23. **Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM, et al**. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269**:496-512

24. **Lagesen K, Ussery DW, Wassenaar TM**. 2010. Genome update: the 1000th genome--a cautionary tale. Microbiology **156**:603-608.

25. **Klaenhammer T, Altermann E, Arigoni F, Bolotin A, Breidt F, Broadbent J, Cano R, Chaillou S, Deutscher J, Gasson M, van de Guchte M, Guzzo J, Hartke A, Hawkins T, Hols P, Hutkins R, Kleerebezem M, Kok J, Kuipers O, Lubbers M, Maguin E, McKay L, Mills D, Nauta A, Overbeek R, Pel H, Pridmore D, Saier M, van Sinderen D, Sorokin A, Steele J, O'Sullivan D, de Vos W, Weimer B, Zagorec M, Siezen R**. 2002. Discovering lactic acid bacteria by genomics. Antonie van Leeuwenhoek **82**:29-58

26. **Lukjancenko O, Ussery DW, Wassenaar TM**. 2012. Comparative genomics of *Bifidobacterium*, *Lactobacillus* and related probiotic genera. Microb Ecol **63**:651-673.

27. **Fozo EM, Kajfasz JK, Quivey RG**. 2004. Low pH-induced membrane fatty acid alterations in oral bacteria. FEMS Microbiol Lett **238**:291-295.

28. **Taranto MP, Fernandez Murga ML, Lorca G, de Valdez GF**. 2003. Bile salts and cholesterol induce changes in the lipid cell membrane of *Lactobacillus reuteri*. J Appl Microbiol **95**: 86-91.

29. **Corcoran BM, Stanton C, Fitzgerald GF, Ross RP**. 2007. Growth of probiotic lactobacilli in the pressence of oleic acid enhances subsequent survival in gastric juice. Microbiology **153**:291-299.

30. **Lebeer S, Vanderleyden J, De Keersmaecker SC**. 2008. Genes and molecules of lactobacilli supporting probiotic action. Microbiol Mol Biol Rev **72**:728-764

31. **Azcarate-Peril MA, McAuliffe O, Altermann E, Lick S, Russell WM, Klaenhammer TR**. 2005. Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in *Lactobacillus acidophilus*. Appl Environ Microbiol **71**:5794-5804.

32. **Pieterse B, Leer RJ, Schuren FHJ, van der Werf MJ**. 2005. Unravelling the multiple effects of lactic acid stress on *Lactobacillus plantarum* by transcription profiling. Microbiology **151**:3881-3894.

33. **Bron PA, Molenaar D, de Vos WM, Kleerezebem M**. 2006. DNA micro-array-based identification of bile-responsive genes in *Lactobacillus plantarum*. J Appl Microbiol **100**:728-738.

34. **Pfeiler EA, Azcarate-Peril MA, Klaenhammer TR**. 2007. Characterization of a novel bile-inducible operon encoding a two-component regulatory system in

*Lactobacillus acidophilus*. J Bacteriol **189**:4624-4634.

35. **Altermann E, Russell WM, Azcarate-Peril MA, Barrangou R, Buck BL, McAuliffe O, Souther N, Dobson A, Duong T, Callanan M, Lick S, Hamrick A, Cano R, Klaenhammer TR**. 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. Proc Natl Acad Sci USA **102**:3906-3912.

36. **Morita H, Toh H, Oshima K, Murakami M, Taylor TD, Igimi S, Hattori M.** 2009. Complete genome sequence of the probiotic *Lactobacillus rhamnosus* ATCC 53103. J Bacteriol **191**:7630-7631.

37. **Lebeer S, Claes IJ, Verhoeven TL, Shen C, Lambrichts I, Ceuppens JL, Vanderleyden J, De Keersmaecker SC**. 2008. Impact of *lux*S and suppressor mutations on the gastrointestinal transit of *Lactobacillus rhamnosus* GG. Appl Environ Microbiol **74**:4711-4718.

38. **Buck BL, Azcarate-Peril MA, Klaenhammer TR**. 2009. Role of autoinducer-2 on the adhesion ability of *Lactobacillus acidophilus*. J Appl Microbiol **107**:269-279.

39. **Hamon E, Horvatovich P, Izquierdo E, Bringel F, Marchioni E, Aoude-Werner D, Ennahar S**. 2011. Comparative proteomic analysis of *Lactobacillus plantarum* for the identification of key proteins in bile tolerance. BMC Microbiol **11**:63

40. **Whitehead K, Versalovic J, Roos S, Britton RA**. 2008. Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. Appl Environ Microbiol **74**:1812-1819.

41. **Pfeiler EA, Klaenhammer TR**. 2009. Role of transporter proteins in bile tolerance of *Lactobacillus acidophilus*. Appl Environ Microbiol **75**:6013-6016.

42. **Gueimonde M, Garrigues C, van Sinderen D, de los Reyes-Gavilan CG, Margolles A**. 2009. Bile-inducible efflux transporter from *Bifidobacterium longum* NCC2705, conferring bile resistance. J Bacteriol **75**:3153-3160

43. **Roberfroid M**. 2007. Prebiotics: the concept revisited. J Nutr **137**:830S-837S

44. **Andersen JM, Barrangou R, Abou Hachem M, Lahtinen SJ, Goh YJ, Svensson B, Klaenhammer TR**. 2013. Transcriptional analysis of oligosaccharide utilization by *Bifidobacterium lactis* Bl-04. BMC Genomics **14**:312.

45. **Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, Wolvers D, Watzl B, Szajewska H, Stahl B, Guarner F, Respondek F, Whelan K, Coxam V, Davicco MJ, Leotoing L, Wittrant Y, Delzenne NM, Cani PD, Neyrinck**

**AM, Meheust A**. 2010. Prebiotic effects: metabolic and health benefits. Br J Nutr **104**:S1-63.

46. **Andersen JM, Barrangou R, Hachem MA, Lahtinen SJ, Goh YJ, Svensson B, Klaenhammer TR**. 2012. Transcriptional analysis of prebiotic uptake and catabolism by *Lactobacillus acidophilus* NCFM. PLoS One 7 **9**:e44409.

47. **Belanger AE, Hatfull GF**. 1999. Exponential-phase glycogen recycling is essential for growth of *Mycobacterium smegmatis*. J Bacteriol **181**:6670-6678

48. **Goh YJ, Klaenhammer TR**. 2013. A functional glycogen biosynthesis pathway in *Lactobacillus acidophilus*: expression and analysis of the glg operon. Mol Microbiol **89**:1187-1200.

49. **Chauviere G, Coconnier MH, Kerneis S, Fourniat J, Servin AL**. 1992. Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. Microbiology **138**:1689-1696

50. **Tuomola EM, Salminen SJ**. 1998. Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. Int J Food Microbiol **41**:45-51

51. **He F, Ouwehan AC, Hashimoto H, Isolauri E, Benno Y, Salminen S**. 2001. Adhesion of *Bifidobacterium* spp. to human intestinal mucus. Microbiol Immunol **45**:259-262

52. **Velez MP, De Keersmaecker SC, Vanderleyden J**. 2007. Adherence factors of *Lactobacillus* in the human gastrointestinal tract. FEMS Microbiol Lett **276**:140-148.

53. **Bron PA, Grangette C, Mercenier A, de Vos WM, Kleerebezem M**. 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. J Bacteriol **186**:5721-5729.

54. **Buck BL, Altermann E, Svingerud T, Klaenhammer TR**. 2005. Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol **71**:8344-8351.

55. **O'Flaherty SJ, Klaenhammer TR**. 2010. Functional and phenotypic characterization of a protein from *Lactobacillus acidophilus* involved in cell morphology, stress tolerance and adherence to intestinal cells. Microbiology **156**:3360-3367.

56. **Goh YJ, Klaenhammer TR**. 2010. Functional roles of aggregation-promoting-like factor in stress tolerance and adherence of *Lactobacillus acidophilus* NCFM. Appl

Environ Microbiol **76**:5005-5012.

57. **Antikainen J, Anton L, Sillanpaa J, and Korhonen TK**. 2002. Domains in the S-layer protein CbsA of *Lactobacillus crispatus* involved in adherence to collagens, laminin and lipoteichoic acids and in self-assembly. Mol Microbiol **46**:381-394.

58. **van Pijkeren JP, Canchaya C, Ryan KA, Li Y, Claesson MJ, Sheil B, Steidler L, O'Mahony L, Fitzgerald GF, van Sinderen D, O'Toole PW**. 2006. Comparative and functional analysis of sortase-dependent proteins in the predicted secretome of *Lactobacillus salivarius* UCC118. Appl Environ Microbiol **72**:4143-4153.

59. **Kankainen M, Paulin L, Tynkkynen S, von Ossowski I, Reunanen J, Partanen P, Satokari R, Vesterlund S, Hendrickx AP, Lebeer S, De Keersmaecker SC, Vanderleyden J, Hamalainen T, Laukkanen S, Salovuori N, Ritari J, Alatalo E, Korpela R, Mattila-Sandholm T, Lassig A, Hatakka K, Kinnunen KT, Karjalainen H, Saxelin M, Laakso K, Surakka A, Palva A, Salusjarvi T, Auvinen P, de Vos WM**. 2009. Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human- mucus binding protein. Proc Natl Acad Sci USA **106**:17193-17198.

60. **O'Connell Motherway M, Zomer A, Leahy SC, Reunanen J, Bottacini F, Claesson MJ, O'Brien F, Flynn K, Casey PG, Moreno Munoz JA, Kearney B, Houston AM, O'Mahony C, Higgins DG, Shanahan F, Palva A, de Vos WM, Fitzgerald GF, Ventura M, O'Toole PW, van Sinderen, D**. 2011. Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pilli as an essential and conserved host-colonization factor. Proc Natl Acad Sci USA **108**:11217-11222.

61. **Servin AL**. 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol Rev **28**:405-440.

62. **Chan RC, Reid G, Irvin RT, Bruce AW, Costerton JW**. 1985. Competitive exclusion of uropathogens from human uroepithelial cells by *Lactobacillus* whole cells and cell wall fragments. Infect Immun **47**:84-89

63. **Reid G, Chan RC, Bruce AW, Costerton JW**. 1985. Prevention of urinary tract infection in rats with an indigenous *Lactobacillus casei* strain. Infect Immun **49**:320-324

64. **St Amant DC, Valentin-Bon IE, Jerse AE**. 2002. Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* species that are commonly isolated from the female genital tract. Infect Immun **70**:7169-7171

65. **Pridmore RD, Pittet AC, Praplan F, Cavadini C**. 2008. Hydrogen peroxide production by *Lactobacillus johnsonii* NCC 533 and its role in anti-*Salmonella* activity. FEMS Microbiol Lett **283**:210-215.

66. **Fayol-Messaoudi D, Berger CN, Coconnier-Polter MH, Lievin-Le Moal V, Servin AL**. 2005. pH-, Lactic acid-, and non-lactic acid-dependent activities of probiotic Lactobacilli against *Salmonella enterica* serovar *Typhimurium*. Appl Environ Microbiol **71**:6008-6013.

67. **Velraeds MM, van der Mei HC, Reid G, Busscher HJ**. 1996. Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. Appl Environ Microbiol **62**:1958-1963

68. **Ryan KA, O'Hara AM, van Pijkeren JP, Douillard FP, O'Toole PW**. 2009. *Lactobacillus salivarius* modulates cytokine induction and virulence factor gene expression in *Helicobacter pylori*. J Med Microbiol **58**:996-1005.

69. **Dobson A, Cotter PD, Ross RP, Hill C**. 2012. Bacteriocin production: a probiotic trait? Appl Environ Microbiol **78**:1-6.

70. **Cotter PD, Hill C, Ross RP**. 2005. Bacteriocins: developing innate immunity for food. Nat Rev Microbiol **3**:777-788.

71. **Schnell N, Entian KD, Schneider U, Gotz F, Zahner H, Kellner R, Jung G**. 1988. Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulfide-rings. Nature **333**:276-278.

72. **Klaenhammer TR**. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev **12**:39-85

73. **Nes IF, Bao Diep D, Sigve Havarstein L, Bente Brurberg M, Eijsink V, Holo H**. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie van Leeuwenhoek **70**:113-128.

74. **Delves-Broughton J, Blackburn P, Evans RJ, Hugenholtz J**. 1996. Applications of the bacteriocin, nisin. Antonie van Leeuwenhoek **69**:193-202

75. **Breukink E, Wiedemann I, van Kraaij C, Kuipers OP, Sahl HG, de Kruijff B**. 1999. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science **286**:2361-2364.

76. **Wiedemann I, Breukink E, van Kraaij C, Kuipers OP, Bierbaum G, de Kruijff B, Sahl HG**. 2001. Specific binding of nisin to the peptidoglycan precursor lipid II

combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J Biol Chem **276**:1772-1779.

77. **Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG**. 2007. Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. Proc Natl Acad Sci USA **104**:7617-7621.

78. **Lebeer S, Vanderleyden J, De Keersmaecker SC**. 2010. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol **8**:171-184.

79. **O'Flaherty SJ, Klaenhammer TR**. 2010. The role and potential of probiotic bacteria in the gut, and the communication between gut microflora and the gut/host. Int Dairy J **20**:262-268.

80. **Reid G, Younes JA, Van der Mei HC, Gloor GB, Knight R, Busscher HJ**. 2011. Microbiota restoration: natural and supplemented recovery of human microbial communities. Nat Rev Microbiol **9**:27-38.

81. **Bron PA, van Baarlen P, Kleerebezem M**. 2012. Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. Nat Rev Microbiol **10**:66-78.

82. **Klaenhammer TR, Kleerebezem M, Kopp MV, Rescigno M**. 2012. The impact of probiotics and prebiotics on the immune system. Nat Rev Immunol **12**:728-734.

83. **Selle K, Klaenhammer TR**. 2013. Genomic and phenotypic evidence for probiotic influences of *Lactobacillus gasseri* on human health. FEMS Microbiol Rev **37**:915-935.

84. **Smits HH, Engering A, van der Kleij D, de Jong EC, Schipper K, van Capel TMM, Zaat BAJ, Yazdanbakhsh M, Wierenga EA, van Kooyk Y, Kapsenberg ML**. 2005. Selective probiotic bacteria induce IL-10-producing regulatory T cells *in vitro* by modulating dendritic cell functiont through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. J Allergy Clin Immunol **115**:1260-1267

85. **Konstantinov SR, Smidt H, de Vos WM, Bruijns SC, Singh SK, Valence F, Molle D, Lortal S, Altermann E, Klaenhammer TR, van Kooyk Y**. 2008. S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. Proc Natl Acad Sci USA **105**:19474-19479.

86. **Grangette C, Nutten S, Palumbo E, Morath S, Hermann C, Dewulf J, Pot B, Hartung T, Hols P, Mercenier A**. 2005. Enhanced antiinflammatory capacity of

*Lactobacillus plantarum* mutant synthesizing modified teichoic acids. Proc Natl Acad Sci USA **102**:10321-10326.

87. **Mohamadzadeh M, Pfeiler EA, Brown JB, Zadeh M, Gramarossa M, Managlia E, Bere P, Sarraj B, Khan MW, Pakanati KC, Ansari MJ, O'Flaherty S, Barrett T, Klaenhammer TR**. 2011. Regulation of induced colonic inflammation by *Lactobacillus acidophilus* deficient in lipoteichoic acid. Proc Natl Acad Sci USA **108**:4623-4630.

88. **Khazaie K, Zadeh M, Khan MW, Bere P, Gounari F, Dennis K, Blatner NR, Owen JL, Klaenhammer TR, Mohamadzadeh M**. 2012. Abating colon cancer polyposis by *Lactobacillus acidophilus* deficient in lipoteichoic acid. Proc Natl Acad Sci USA **109**:10462-10467.

89. **Kleerebezem M, Hols P, Bernard E, Rolain T, Zhou M, Siezen RJ, Bron PA**. 2010. The extracellular biology of the lactobacilli. FEMS Microbiol Rev **34**:199-230.

90. **Zhou M, Theunissen D, Wels M, Siezen RJ**. 2010. LAB-Secretome: a genome-scale comparative analysis of the predicted extracellular and surface-associated proteins of Lactic Acid Bacteria. BMC Genomics **11**:651.

91. **Johnson B, Selle K, O'Flaherty S, Goh YJ, Klaenhammer TR**. 2013. Identification of extracellular surface-layer associated proteins in *Lactobacillus acidophilus* NCFM. Microbiology **159**:2269-2282.

92. **van Baarlen P, Troost F, van der Meer C, Hooiveld G, Boekschoten M, Brummer RJ, Kleerebezem M**. 2011. Human mucosal in vivo transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways. Proc Natl Acad Sci USA **108**:4562-4569.

93. **O'Flaherty S, Klaenhammer TR**. 2012. Influence of exposure time on gene expression by human intestinal epithelial cells exposed to *Lactobacillus acidophilus*. Appl Environ Microbiol **78**:5028-5032.

94. **Turroni F, Taverniti V, Ruas-Madiedo P, Duranti S, Guglielmetti S, Lugli GA, Gioiosa L, Palanza P, Margolles A, van Sinderen D, Ventura M**. 2014. *Bifidobacterium bifidum* PRL2010 Modulates the Host Innate Immune Response. Appl Environ Microbiol **80**:730-740.

95. **Kajikawa A, Nordone SK, Zhang L, Stoeker LL, LaVoy AS, Klaenhammer TR, Dean GA**. 2011. Dissimilar properties of two recombinant *Lactobacillus acidophilus* strains displaying *Salmonella* FliC with different anchoring motifs. Appl Environ Microbiol **77**:6587-6596.

96. **Stoeker L, Nordone S, Gunderson S, Zhang L, Kajikawa A, LaVoy A, Miller M, Klaenhammer TR, Dean GA**. 2011. Assessment of *Lactobacillus gasseri* as a candidate oral vaccine vector. Clinical and vaccine immunology Clin Vaccine Immunol **18**:1834-1844.

97. **Kajikawa A, Zhang L, Long J, Nordone S, Stoeker L, LaVoy A, Bumgardner S, Klaenhammer T, Dean G**. 2012. Construction and immunological evaluation of dual cell surface display of HIV-1 gag and *Salmonella enterica* serovar *Typhimurium* FliC in *Lactobacillus acidophilus* for vaccine delivery. Clin Vaccine Immunol **19**:1374-1381.

98. **Bercik P, Collins SM, Verdu EF**. 2012. Microbes and the gut-brain axis. Neurogastroenterol Motil **24**:405-413.

99. **Corr SC, O'Neill LA**. 2009. *Listeria monocytogenes* infection in the face of innate immunity. Cell Microbiol **11**:703-709.

100. **Mattila-Sandholm T, Blum S, Collins JK, Crittenden R, de Vos W, Dunne C, Fondén R, Grenov G, Isolauri E, Kiely B, Marteau P, Morelli L, Ouwehand A, Reniero R, Saarela M, Salminen S, Saxelin M, Schiffrin E, Shanahan F, Vaughan E & von Wright A**. 1999. Probiotics: towards demonstrating efficacy. Trends Food Sci Technol **10**:393-399.

101. **Lonnermark E, Friman V, Lapas G, Sandberg T, Berggren A, Alderberth I**. 2010. Intake of *Lactobacillus plantarum* reduces certain gastrointestinal symptoms during treatment with antibiotics. J Clin Gastroenterol **44**:106-112.

102. **Gao XW, Mubasher M, Fang CY, Reifer C, Miller LE**. 2010. Dose-response efficacy of a proprietary probiotic formula of *Lactobacillus acidophilus* CL1285 and *Lactobacillus casei* LBC80R for antibiotic-associated diarrhea and *Clostridium difficile*-associated diarrhea prophylaxis in adult patients. Am J Gastroenterol **105**:1636-1641.

103. **Plummer S, Weaver MA, Harris JC, Dee P, Hunter J**. 2004. *Clostridium difficile* pilot study: effects of probiotic supplementation on the incidence of *C. difficile* diarrhoea. Int Microbiol **7**:59-62

104. **Ringel-Kulka T, Palsson OS, Maier D, Carroll I, Galanko JA, Leyer G, Ringel Y**. 2011. Probiotic bacteria *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* Bi-07 versus placebo for the symptoms of bloating in patients with functional bowel disorders: a double-blind study. J Clin Gastroenterol **45**:518-525.

105. **Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, Dubuquoy C, Merour E, Geboes K, Chamaillard M, Ouwehand A, Leyer G, Carcano D, Colombel JF, Ardid D, Desreumaux P**. 2007. *Lactobacillus acidophilus* modulates intestinal pain and induces opioid and cannabinoid receptors. Nat Med **13**:35-37.

106. **Leyer GJ, Li S, Mubasher ME, Reifer C, Ouwehand AC**. 2009. Probiotic effects on cold and influenza-like symptom incidence and duration in children. Pediatrics **124**:e172-179.

107. **Lee YK, Puong KY, Ouwehand AC, Salminen S**. 2003. Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. J Med Microbiol **52**:925-930

108. **Ushiyama A, Tanaka K, Aiba Y, Shiba T, Takagi A, Mine T, Koga Y**. 2003. *Lactobacillus gasseri* OLL2716 as a probiotic in clarithromycin-resistant *Helicobacter pylori* infection. J Gastroenterol Hepatol **18**:986-991

109. **Fujimura S, Watanabe A, Kimura K, Kaji M**. 2012. Probiotic mechanism of *Lactobacillus gasseri* OLL2716 strain against *Helicobacter pylori*. J Clin Microbiol **50**:1134-1136.

110. **Deshpande G, Rao S, Patole S, Bulsara M**. 2010. Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. Pediatrics **125**:921-930.

111. **Hao Q, Lu Z, Dong BR, Huang CQ, Wu T.** 2011. Probiotics for preventing acute upper respiratory tract infections. Cochrane Database Syst Rev **9**:CD006895.

112. **van Baarlen P, Troost FJ, van Hemert S, van der Meer C, de Vos WM, de Groot PJ, Hooiveld GJ, Brummer RJ, Kleerebezem M**. 2009. Differential NF-κB pathways induction by *Lactobacillus plantarum* in the duodenum of healthy humans correlating with immune tolerance. Proc Natl Acad Sci USA **106**:2371-2376.

113. **Rafter J, Bennett M, Caderni G, Clune Y, Hughes R, Karlsson PC, Klinder A, O'Riordan M, O'Sullivan GC, Pool-Zobel B, Rechkemmer G, Roller M, Rowland I, Salvadori M, Thijs H, Van Loo J, Watzl B, Collins JK**. 2007. Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. Am J Clin Nutr **85**:488-496

114. **Engelbrektson A, Korzenik JR, Pittler A, Sanders ME, Klaenhammer TR, Leyer G, Kitts CL**. 2009. Probiotics to minimize the disruption of faecal microbiota in healthy subjects undergoing antibiotic therapy. J Med Microbiol **58**:663-670.

115. **Mennigen R, Bruewer M**. 2009. Effect of probiotics on intestinal barrier function. Ann NY Acad Sci 1165:183-189.

116. **Takeda K, Okumura K**. 2007. Effects of a fermented milk drink containing *Lactobacillus casei* strain Shirota on the human NK-cell activity. J Nutr **137**:791S-793S

117. **Viljanen M, Kuitunen M, Haahtela T, Juntunen-Backman K, Korpela R, Savilahti E**. 2005. Probiotic effects on faecal inflammatory markers and on faecal IgA in food allergic atopic eczema/dermatitis syndrome infants. Pediatr Allergy Immunol **16**:65-71.

118. **Ataie-Jafari A, Larijani B, Alavi Majd H, Tahbaz F**. 2009. Cholesterol-lowering effect of probiotic yogurt in comparison with ordinary yogurt in mildly to moderately hypercholesterolemic subjects. Ann Nutr Metab **54**:22-27.

119. **Macfarlane GT, Blackett KL, Nakayama T, Steed H, Macfarlane S**. 2009. The gut microbiota in inflammatory bowel disease. Curr Pharm Des **15**:1528-1536

120. **Wells JM, Mercenier A**. 2008. Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. Nat Rev Microbiol **6**:349-362.

121. **Maze A, Boel G, Zuniga M, Bourand A, Loux V, Yebra MJ, Monedero V, Correia K, Jacques N, Beaufils S, Poncet S, Joyet P, Milohanic E, Casaregola S, Auffray Y, Perez-Martinez G, Gibrat JF, Zagorec M, Francke C, Hartke A, Deutscher J**. 2010. Complete genome sequence of the probiotic *Lactobacillus casei* strain BL23. J Bacteriol **192**:2647-2648.

122. **Pridmore RD, Berger B, Desiere F, Vilanova D, Barretto C, Pittet AC, Zwahlen MC, Rouvet M, Altermann E, Barrangou R, Mollet B, Mercenier A, Klaenhammer T, Arigoni F, Schell MA**. 2004. The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. Proc Natl Acad Sci USA **101**:2512-2517

123. **Zhang ZY, Liu C, Zhu YZ, Zhong Y, Zhu YQ, Zheng HJ, Zhao GP, Wang SY, Guo XK**. 2009. Complete genome sequence of *Lactobacillus plantarum* JDM1. J Bacteriol **191**:5020-5021.

124. **Claesson MJ, Li Y, Leahy S, Canchaya C, van Pijkeren JP, Cerdeno-Tarraga AM, Parkhill J, Flynn S, O'Sullivan GC, Collins JK, Higgins D, Shanahan F, Fitzgerald GF, van Sinderen D, O'Toole PW**. 2006. Multireplicon genome architecture of

*Lactobacillus salivarius*. Proc Natl Acad Sci USA **103**:6718-6723.

125. **van de Guchte M, Penaud S, Grimaldi C, Barbe V, Bryson K, Nicolas P, Robert C, Oztas S, Mangenot S, Couloux A, Loux V, Dervyn R, Bossy R, Bolotin A, Batto JM, Walunas T, Gibrat JF, Bessieres P, Weissenbach J, Ehrlich SD, Maguin E**. 2006. The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing evolution. Proc Natl Acad Sci USA **103**:9274-9279.

126. **Barrangou R, Briczinski EP, Traeger LL, Loquasto JR, Richards M, Horvath P, Coute-Monvoisin AC, Leyer G, Rendulic S, Steele JL, Broadbent JR, Oberg T, Dudley EG, Schuster S, Romero DA, Roberts RF**. 2009. Comparison of the complete genome sequences of *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and Bl-04. J Bacteriol **191**:4144-4151.

127. **Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen MC, Desiere F, Bork P, Delley M, Pridmore RD, Arigoni F**. 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci USA **99**:14422-14427.

**Table 1.1** Roles and benefits of probiotic bacteria in the GIT

| Probiotic role/benefit | Reference |
|---|---|
| Protection against infection | (99) |
| Symptom relief from irritable bowel syndrome | (16) |
| Lactose digestion for lactose-intolerant individuals | (100) |
| Lowered incidence of diarrhea | (101) |
| Lowered risk of antibiotic-associated diarrhea | (102) |
| Lowered risk of *C. dificile*-associated diarrhea | (102, 103) |
| Reduction in intestinal bloating | (104) |
| Abdominal pain analgesic (via μ-opiod and cannabinoid receptors) | (105) |
| Lowered levels of cold and influenza-like symptoms in children | (106) |
| Antimicrobial activity | (68) |
| Competitive exclusion of pathogens | (107) |
| Inhibition of *H. pylori* growth | (108, 109) |
| Reduced incidence of necrotizing enterocolitis | (110) |
| Prevention of upper respiratory infections | (111) |
| Immune tolerance | (112) |
| Reduction in colorectal cancer biomarkers | (113) |
| Return to pre-antibiotic baseline flora | (114) |
| Epithelial barrier function | (115) |
| Increased natural killer cell activity | (116) |
| Increased humoral immunity via secretion of IgA | (117) |
| Lowered blood cholesterol levels | (118) |
| Reduction in irritable bowel disease symptoms | (119) |
| Delivery of therapeutics | (120) |

Modified from O'Flaherty and Klaenhammer (79)

**Table 1.2** Common probiotic *Lactobacillus* sp. and *Bifidobacterium* sp.

| Probiotic (strain designation) | Genome sequence reference (accession number) |
|---|---|
| **Lactobacillus** | |
| *L. acidophilus* (NCFM, La-1) | 35 (NC_006814.3) |
| *L. casei* (BL23) | 121 (NC_010999.1) |
| *L. johnsonii* (NCC 533) | 122 (NC_ 005632.1) |
| *L. plantarum* (JDM1) | 123 (NC_012984.1) |
| *L. reuteri* (SD2112) | (NC_015697.1) |
| *L. rhamnosus* (GG) | 59 (NC_013198.1) |
| *L. salivarius* (UCC118) | 124 (NC_007929.1) |
| *L. bulgaricus* (ATCC 11842) | 125 (NC_008054.1) |
| **Bifidobacteria** | |
| *B. animalis* subsp. *lactis* (B1-04) | 126 (NC_012814.1) |
| *B. breve* (UCC2003) | 60 (NC_020517.1) |
| *B. longum* (NCC 2705) | 127 (NC_004307.2) |

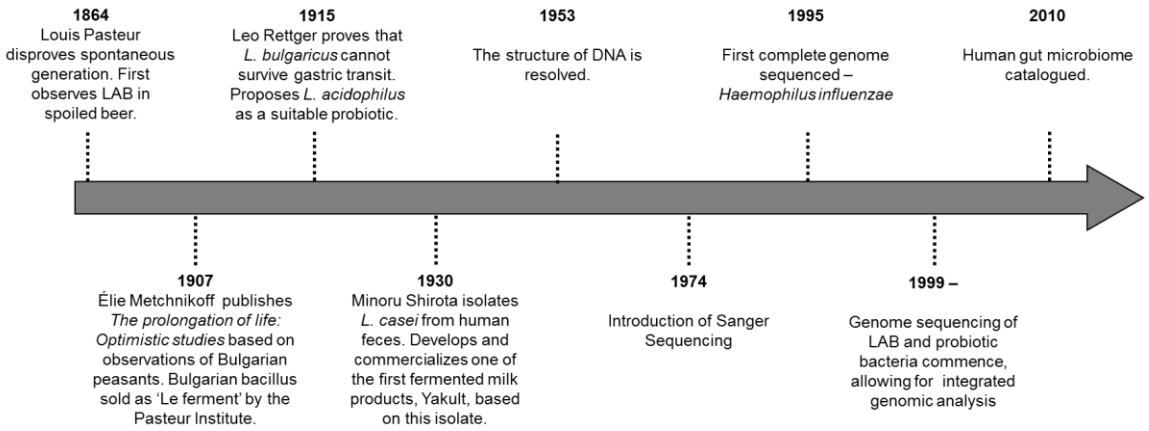


**Figure 1.1.** Seminal milestones contributing to the functional characterization of probiotic lactic acid bacteria.

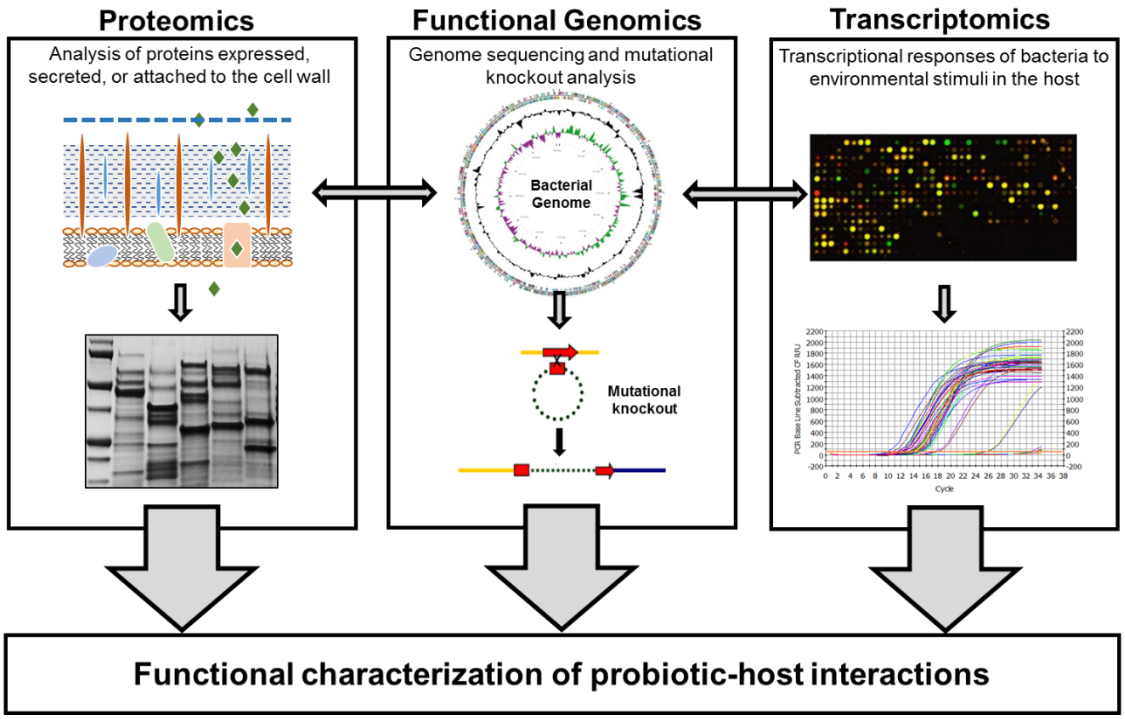


**Figure 1.2.** With the advent of next generation sequencing, integrated genomic techniques including proteomics, transcriptomics and functional genomics have collectively characterized the mechanism of probiotic host-interactions. These analyses rely on access to annoted sequence data from whole genome sequencing. Genetic systems for deletions and mutational knockouts allow for phenotyping specific genetic loci. Proteomic approaches involve the characterization of proteins expressed, secreted, and/or attached to the cell wall. In this way, proteins are isolated, characterized by mass spectrometry, and mapped back to the proteome and corresponding genome for functional analysis  Finally, transcriptomic profiling using DNA microarrays, RNA sequencing, and RT-qPCR can measure the transcriptional responses of both bacteria and host cells in response to one another, via measurement of mRNA.

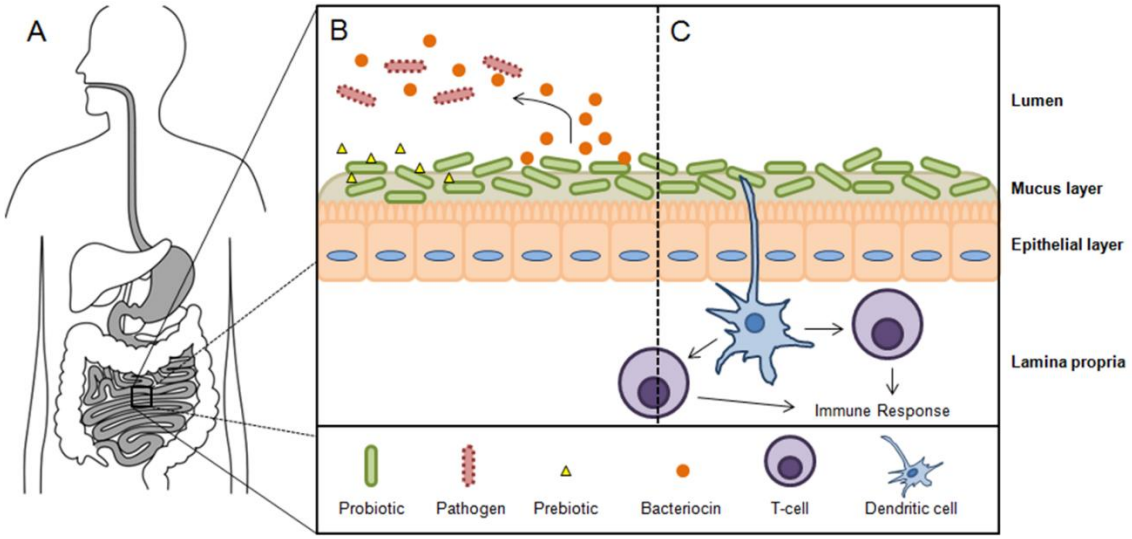


**Figure 1.3.** (A) Probiotic microbes delivered orally must survive varying environments encountered through gastrointestinal transit, including acidic gastric juices (pH ~2) in the stomach, and bile in the small intestines. (B) At the intestinal epithelia, probiotics have been reported to adhere in high numbers, leading to competive exclusion of pathogens. The growth of certain probiotics can be stimulated by the pressence of complex prebiotic oligossacharides. Additionally, some probiotics produce bacteriocins and other antimicrobial agents which may antagonize pathogens in the lumen. (C) Probiotics bound in the mucus and epithelial layers are proximal to dendritic cells of the mucosal immune system, leading to immunomodulation.

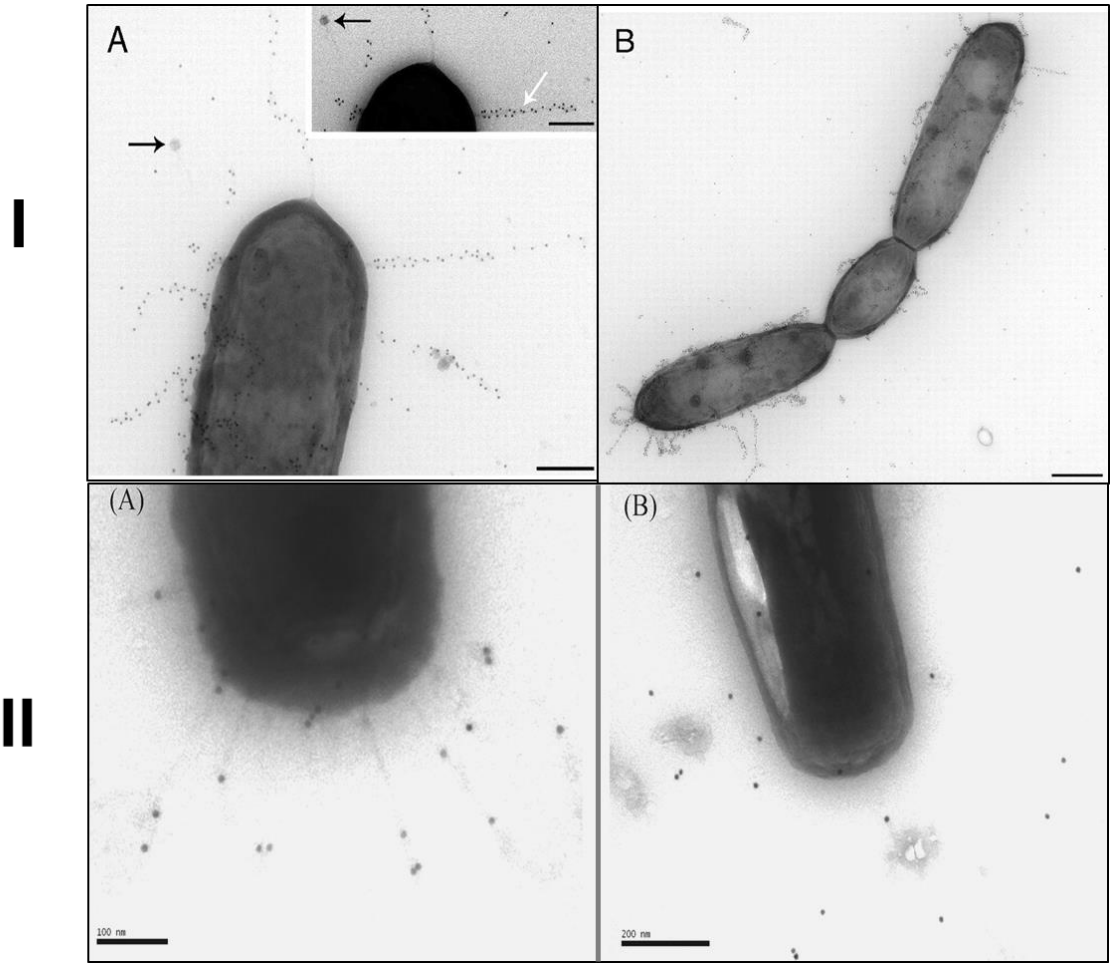


**Figure 1.4.** Identification of pili structures in *Lactobacillus rhamnosus* GG (I) and *Bifidobacterium breves* UCC2003 (II). Images were obtained using transmission electron microscopy on negatively stained, immunogold-labeled anti-*SpaC* pili in *L. rhamnosus* (I) and anti-$Flp_{2003}$ pili in *B. breves* (II). Reprinted with permission from Kankainen et al. (2009), copyright © 2009 National Academy of Sciences, U.S.A; and O'Connell Motherway et al. (2011), copyright © 2011 National Academy of Sciences, U.S.A.

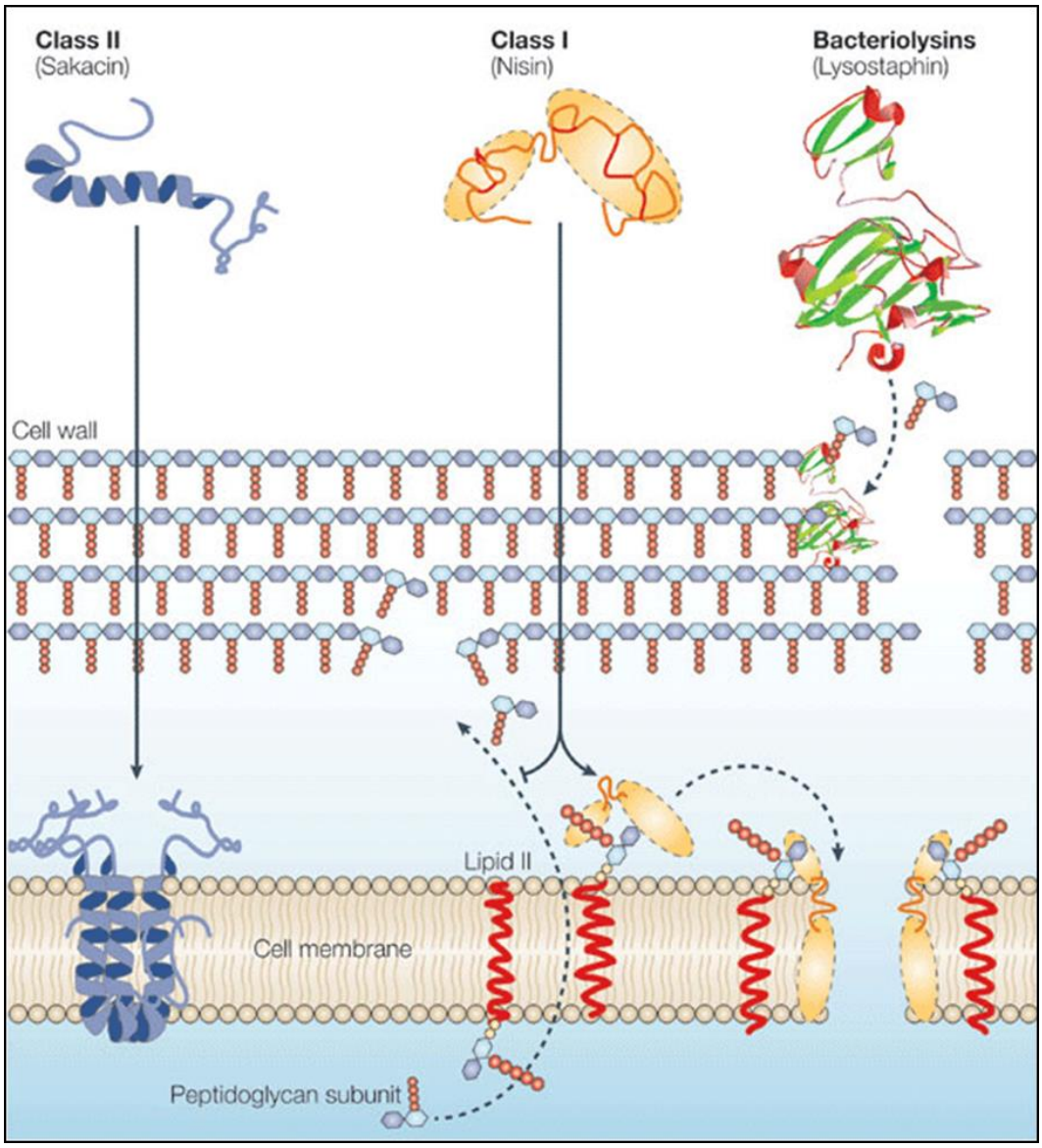


**Figure 1.5.** Bacteriocins produced by LAB can are grouped into three classes based on structure and function: class I (lantibiotics), class II, and bacteriolysins. Class I lantibiotics, such as nisin, can have two modes of action. First, they bind lipid II to prevent peptidoglycan subunit transport, disrupting peptidoglycan synthesis and cell division. Second, they dock at lipid II to create pores in the cytoplasmic membrane of the bacteria. Class II bacteriocins, such as sakacin, often contain amphiphilic helical structures which can insert insert into the cell membrane, leading to cell lysis. Bacteriolysins, such as lyostaphin, are large hydrolases which directly degrade the peptidoglycan cell wall. Reprinted with permission from Cotter et al. (2005), copyright © 2005 Nature Publishing Group.

# CHAPTER 2

## Identification of extracellular surface-layer associated proteins in *Lactobacillus acidophilus* NCFM

Published in 2013: Microbiology 159 (11): 2269-2282

## 2.1 Abstract

Bacterial surface (S-) layers are crystalline arrays of self-assembling, proteinaceous subunits called S-layer proteins (Slps), with molecular masses ranging from 40 to 200 kDa. The S-layer-forming bacterium *Lactobacillus acidophilus* NCFM expresses three major Slps: SlpA (46 kDa), SlpB (47 kDa) and SlpX (51 kDa). SlpA has a demonstrated role in adhesion to Caco-2 intestinal epithelial cells *in vitro*, and has been shown to modulate dendritic cell (DC) and T-cell functionalities with murine DCs. In this study, a modification of a standard lithium chloride S-layer extraction revealed 37 proteins were solubilized from the S-layer wash fraction. Of these, 30 have predicted cleavage sites for secretion, 24 are predicted to be extracellular, six are lipid-anchored, three have N-terminal hydrophobic membrane spanning regions and four are intracellular, potentially moonlighting proteins. Some of these proteins, designated S-layer associated proteins (SLAPs), may be loosely associated with or embedded within the bacterial S-layer complex. *Lba-1029*, a putative SLAP gene, was deleted from the chromosome of *L. acidophilus*. Phenotypic characterization of the deletion mutant demonstrated that the SLAP LBA1029 contributes to a pro-inflammatory TNF-α response from murine DCs. This study identified extracellular proteins and putative SLAPs of *L. acidophilus* NCFM using LC-MS/MS. SLAPs appear to impart important surface display features and immunological properties to microbes that are coated by S-layers.

## 2.2 Introduction

Bacterial surface (S-) layers are crystalline arrays of self-assembling, proteinaceous subunits called S-layer proteins (Slps), with molecular masses ranging from 40 to 200 kDa (1). Present as the outermost component of the cell wall, S-layers are found in many species of eubacteria and archaebacteria, and in varying environments. S-layer lattices are 5-25 nm thick and form oblique (p1, p2), square (p4), or hexagonal (p3, p6) symmetries, as observed by freeze-etched electron microscopy (2). Further structural observations have revealed that S-layers are highly porous in nature, with pores occupying up to 70% of the cell surface (3). Previous work comparing amino acid sequences from different S-layer forming bacteria reveal that most S-layers are high in hydrophobic and acidic amino acids (4, 5). Furthermore, the isoelectric points (pI) of many Slps are low, mostly found in the weakly acidic pH range (1). These sequence analyses have also revealed the presence of S-layer homologous (SLH) motifs on the N-terminal section of many Slps (1), which are responsible for tethering the S-layer to the secondary cell wall polysaccharide (6, 7).

Lactic acid bacteria of the genus *Lactobacillus* are a diverse group of gram-positive, anaerobic/microaerophilic, nonsporulating, low G+C content bacteria belonging to the *Firmicutes* phylum (8). Biochemically, they are strictly fermentative; sugar fermentations result in either the sole production of lactic acid, or production of lactic acid in conjunction with smaller amounts of carbon dioxide and ethanol/acetic acid (8, 9). There are several species of *Lactobacillus* that form S-layers, including mucosal-associated species (e.g. *L. acidophilus*, *L. crispatus*, *L. amylovorus*, and *L. gallinarum*) and dairy fermentation-associated species (e.g *L. helveticus* and *L. kefiranofaciens*) (10, 11). Compared to S-layers

of other gram-positive bacteria, those from *Lactobacillus* are biochemically unique. Specifically, S-layers from *Lactobacillus* do not possess SLH domains (12). Furthermore, their Slps are among the smallest known (25 kDa – 71 kDa) and are highly basic with calculated pI values ranging from 9.35 to 10.4 (10).

*L. acidophilus* NCFM is a widely used probiotic microbe, found in both fermented dairy products and dietary supplements (13). Utilizing a completely sequenced and annotated genome (14), *L. acidophilus* NCFM has been the subject of many investigations seeking to understand the mechanisms of probiotic functionality. *L. acidophilus* NCFM forms an S-layer composed principally of SlpA, with auxiliary components SlpB and SlpX (14, 15). Given its proximity to the cell surface, the S-layer is one of the first bacterial components to interact with the gastrointestinal surface of the human host. SlpA of *L. acidophilus* NCFM has demonstrated important roles in adhesion to the Caco-2 intestinal cell line (16) and has been shown to modulate dendritic cell (DC) and T-cell functionality in the murine gut (17). Taken together, these studies highlight the potential role of S-layers in probiotic activities.

Transmission electron microscopy images of *L. acidophilus* reveal a cell envelope that is abundant with S-layer components. In fact, $\sim 5 \times 10^5$ Slp subunits are required to generate the S-layer of rod-shaped cells, such as *L. acidophilus* (5). Slps have been extracted from Gram-positive bacterial cell surfaces via treatment with high concentrations of salts (e.g. guanidine hydrochloride or lithium chloride [LiCl]), which disrupt hydrogen bonding between the S-layer and the secondary cell wall polysaccharide (1). Specifically, LiCl treatments at 5 M and 1 M concentrations have been used to isolate the Slps from many *Lactobacillus* species (15, 18-23). Despite the dramatic presence of S-layer and the highly

expressed Slp subunits, it is notable that research on exoproteins associated with the S-layer is scarce. Although proteins associated with the S-layer have been observed in *L. acidophilus* (22), there has been no work identifying these proteins using mass spectrometry.

In order to identify these proteins in *L. acidophilus*, a LiCl S-layer extraction protocol noted above (15, 21) was modified to isolate proteins associated with the S-layer, while mostly excluding the major Slps. After protein identification through liquid chromatography-tandem mass spectrometry (LC-MS/MS), 37 proteins were identified that may be associated with or embedded within the S-layer. Many of these proteins, designated as Surface-Layer Associated Proteins (SLAPs, Figure 2.1), have unknown function and offer potential in advancing our understanding on the probiotic mechanism, cell envelope biology, and immunomodulation in *L. acidophilus.* One of the predicted SLAPs, encoded by *lba-1029* was deleted from the chromosome of *L. acidophilus* NCFM using a *upp*-based counterselective gene replacement system (15). This study aimed to (i) develop a modified method for extracting SLAPs; (ii) identify the SLAPs found in *L. acidophilus* NCFM; and (iii) functionally characterize the SLAP, LBA1029.

## 2.3 Materials and Methods

### 2.3.1 Bacterial strains and growth conditions

Bacterial strains, plasmids, and primers used in this study are reported in Table 1. *L. acidophilus* strains were propagated in de Man Rogosa Sharpe (MRS) broth (Difco Laboratories, Inc., Detroit, MI) under aerobic conditions, statically or on MRS solid medium containing 1.5% (wt/vol) agar (Difco) under anaerobic conditions at 37°C, and at 42°C where indicated. Recombinant strains were selected with 2 µg erythromycin $ml^{-1}$ (em) (Sigma-Aldrich, St. Louis, MO) and/or 5 µg chloramphenicol $ml^{-1}$ (cm) (Sigma-Aldrich). *Escherichia coli* strains were grown in brain heart infusion (Difco) medium at 37°C with shaking for aeration. *E. coli* EC101 was grown in the presence of 40 µg kanamycin $ml^{-1}$ (Kn) (Sigma-Aldrich) while NCK1911 and transformants were grown with 40 µg Kn $ml^{-1}$ and 150 µg em $ml^{-1}$. Counterselection of plasmid-free excision recombinants was performed using 5-fluorouracil supplemented glucose semi-defined media, as previously described (15).

### 2.3.2 LiCl extraction of S-layer associated proteins

The extraction protocol for SLAPs was modified from a standard LiCl S-layer extraction protocol for *L. acidophilus* (15, 21). Bacterial cells were grown in 200 ml MRS to stationary phase (16 h), centrifuged at 2,236 × *g* for 10 minutes (4°C), and washed twice with 25 ml cold phosphate-buffered saline (PBS, Gibco), pH 7.4. Cells were agitated for 15 min at 4°C following the addition of 5 M LiCl (Fisher Scientific, Pittsburg, PA). Supernatants, containing S-layer proteins and SLAPs, were harvested via centrifugation at 8,994 × *g* for 10 min (4°C) and transferred to an 6-8,000 kDa Spectra/Por molecular porous membrane

(Spectrum Laboratories, Inc, Rancho Dominguez, CA) and dialyzed against cold distilled water for 24 h, changing the water every 2 h for the first 8 h. The dialyzed precipitate was harvested via centrifugation at 20,000 × *g* for 30 min and agitated for a second time for 15 min with 1 M LiCl at 4$^{o}$C in order to disassociate the SLAPs from the S-layer proteins, which are insoluble in 1 M LiCl. Next, the suspension was centrifuged at 20,000 × *g* for 10 minutes and the supernatants, containing the SLAPs, were once again transferred to the 6-8,000 kDa Spectra/Por molecular porous membrane and dialyzed against cold distilled water for 24 hours. Finally, the precipitate was harvested via centrifugation at 20,000 × *g* for 30 minutes and pellets were resuspended in 10% sodium dodecyl sulfate (SDS, wt/vol, Fisher). Proteins were quantified via bicinchoninic acid assay kit (Thermo Scientific, Waltham, MA) and visualized on SDS polyacrylamide gel electrophoresis (SDS-PAGE) using precast 4-20% Precise Tris-HEPES protein gels (Thermo Scientific). The gels were stained using AcquaStain (Bulldog Bio, Inc, Portsmouth, NH) according to the instructions of the manufacturer.

### 2.3.3 Protein Identification

The SLAP proteins were electrophoresed for approximately 7 min in the resolving gel of SDS-PAGE and excised using a sterile blade. The protein gel was submitted to the Genome Center Proteomics Core at the University of California, Davis, for Mass Spectrometry (LC-MS/MS) based protein identification. Briefly, proteins were reduced and alkylated according to previously described procedures (24), and digested with sequencing grade tryspin according to the manufacturer's instructions (Promega, Madison, WI).

Peptides were dried down in a vacuum concentrator after digestion and then resolubilized in 2% acetonitrile/ 0.1% trifluoroacetic acid. Digested peptides were analyzed by LC-MS/MS on a Thermo Scientific Q Exactive Orbitrap Mass spectrometer in conjunction with a Paradigm MG4 HPLC and CTC Pal auto sampler (Michrom Bio Resources, Auburn, CA). The digested peptides were loaded onto a Michrom C18 trap and desalted before they were separated using a Michrom 200 μm x 150 mm Magic $C_{18}$AQ reverse phase column. A flow rate of 2 μL $min^{-1}$ was used. Peptides were eluted using a 120 min gradient with 2% acetonitrile to 35% acetonitrile over 94 min, 35% acetonitrile to 80% acetonitrile for 10 min, 80% acetonitrile for 2 min, and then a decrease from 80% to 5% acetonitrile in 1 min. A MS survey scan was obtained for the m/z range of 300-1600 nm. MS/MS spectra were acquired using a top 15 method, where the top 15 ions in the MS spectra were subjected to HCD (High Energy Collisional Dissociation). An isolation mass window of 2.0 m/z was for the precursor ion selection, and normalized collision energy of 27% was used for fragmentation. A 5 sec duration was used for the dynamic exclusion.

### 2.3.4 Criteria for protein identification

Scaffold (version Scaffold_3.6.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. X! Tandem identifications required scores of greater than 1.2 with a mass accuracy of 5 ppm. Protein identifications were accepted if they contained at least 2 identified peptides. Using the parameters above, the False Discovery Rate was calculated to be 1.1% on the protein level

and 0% on the peptide level (25). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. For this study, only proteins with unique spectral counts of ≥ 10 were considered significant.

### 2.3.5 Molecular techniques

Genomic DNA from *L. acidophilus* strains were isolated using a Zymo Research Fungal/Bacterial DNA MiniPrep kit (Zymo Research Corporation, Irvice, CA). Plasmid DNA from *E. coli* was isolated using QIAprep Spin Miniprep Kit (Qiagen, Inc., Valencia, CA). Restriction enzyme digestions and ligations were performed using Roche restriction enzymes (Roche Diagnostics, Indianapolis, IN) and T4 DNA Ligase (New England Biolabs, Beverly, MA), respectively. PCR primers were designed based on the genomic sequence data and synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Inc., Coralville, IA). PCRs were carried out in Bio-Rad MyCycler thermocyclers (Bio-Rad Laboratories, Inc., Berkley, CA) using Choice-*Taq* Blue DNA polymerase (Denville Scientific, Inc., Metuchen, NJ) for screening of recombinants and *PfuUltra* II fusion HS DNA polymerase (Agilent Technologies, Santa Clara, CA) for cloning purposes. PCR amplicons were analyzed on 0.8% agarose gels and purified using QIAquick Gel Extraction kits (Qiagen). DNA sequencing was performed by Davis Sequencing, Inc (Davis, CA). *E. coli* EC101 cells were made competent using a rubidium chloride competent cell protocol (26). *L. acidophilus* cells were prepared for electrotransformation using a modified penicillin treatment protocol (15, 27, 28).

### 2.3.6 Sequence analysis

Identified protein sequences were compared against the nonredundant protein database using BlastP (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Further protein alignments were performed comparing the deduced protein sequence to those from all of the protein databases of the following organisms: *L. helveticus*, *L. crispatus*, *L. amylovorus*, *L. gasseri*¸ *L. johnsonii*, and *L. delbrueckii* subsp. *bulgaricus*. All BLAST analyses were performed with adjusted algorithms to display only alignments with an E-value of $\leq 1 \times 10^{-6}$. Rho-independent transcriptional terminators were predicted by TransTermHP (29).

### 2.3.7 Construction of a *L. acidophilus* Δ*lba1029* mutant

The *upp*-based counterselection gene replacement method, described previously (15), was used as a strategy for creating an internal deletion of 1,155 bp in *lba-1029* of NCK1909, a *upp*-deficient background strain of *L. acidophilus* NCFM. Using splicing by overlap extension PCR (SOE-PCR) (30), the 2 kb flanking regions of the deletion target were spliced with *Bam*HI restricted site added on the upstream end and *Sac*I on the downstream end. This construct was digested with BamHI and SacI then ligated into the polylinker of the similarly digested integration plasmid pTRK935 and transformed into competent *E. coli* EC101. The resulted recombinant plasmid, pTRK1067, was transformed into *L. acidophilus* NCK1909 harboring the helper plasmid, pTRK669 (NCK1910). Single crossover integrants were screened as described previously (15). Colonies with the Δ*lba-1029* genotype were screened among the double recombinants recovered on 5-fluorouracil glucose semi-defined media agar plates. Deletion of *lba-1029* was confirmed by PCR and sequencing.

### 2.3.8 Caco-2 intestinal epithelial cells adherence assay

For cell adherence assays, the Caco-2 intestinal epithelial cell line (ATCC HTB-37 American Type Culture Collection, Rockville, MD), was used. Cell culture media and reagents were purchased from Gibco (Gibco-Invitrogen Corp., Carlsbad, CA) and the assay protocol was performed as described previously (15). Caco-2 cultures were grown at 37°C in a 95% air-5% $CO_2$ atmosphere. A minimal essential medium (MEM) supplemented with 1 mM sodium pyruvate, 20% (v/v) heat-inactivated fetal bovine serum (FBS), 0.1 mM nonessential amino acids, penicillin G (100 mg $ml^{-1}$), streptomycin sulfate (100 mg $ml^{-1}$), and amphotericin (0.25 mg $ml^{-1}$) was used. Monolayers for the adhesion assay were prepared in 12-well tissue culture plates by seeding approximately $6.5 \times 10^4$ cells per well in 2 ml of cell culture medium. The culture medium was replaced every 2 days, while the monolayers were used for the assay two weeks postconfluence. On the day of the assay, monolayers were washed twice with 1 ml PBS before adding 1 ml of MEM without antibiotics and incubating at 37ºC in a 5% $CO_2$ incubator prior to adding bacterial cells. Overnight bacterial cultures (10 ml) were pelleted via centrifugation (3,166 × *g*, 10 min) at room temperature, washed, and resuspended in PBS to a final concentration of ~ 1 X $10^8$ CFU/ml. Next, 1 ml of the bacterial suspension was added to each well of the cell monolayer in triplicates and incubated at 37ºC in a 5% $CO_2$ atmosphere for 1 h. Following incubation, monolayers were washed five times with 1 ml PBS and treated with 1 ml of 0.05% Triton X-100 (wt/vol). After 10 min at 37 ºC, cell monolayers were disrupted via pipetting and transferred to microcentrifuge tubes. Finally, microbial cells from the monolayer were diluted and enumerated after plating onto MRS agar.

### 2.3.9 Bile tolerance assays

Cells were tested for bile tolerance in two separate assays. First, cells were inoculated into a 96-well plate containing 200 µl per well of MRS, MRS + 0.3% oxgall (wt/vol), or MRS + 0.5% oxgall in triplicates. Growth curves were monitored over 24 h by measuring the absorbance ($OD_{600}$) using a FLUOStar Optima microtiter plate reader (BMG Labtech, Cary, NC). Secondly, cells were measured in planktonic growth in 10 ml of each of the three media above. At each time point, $OD_{600}$ was measured and cells were plated for CFU $ml^{-1}$ enumeration on MRS agar.

### 2.3.10 Bacterial-DC co-incubation and cytokine measurement

An *in vitro* DC co-incubation assay was performed based on the modification of previous protocols (31, 32). Bone marrow-derived Balb/c murine immature DCs (iDCs) were acquired (Astarte-Biologics LLC, Redmond, WA) and preserved in liquid nitrogen. On the day of the assay, iDCs were thawed in a 37ºC water bath and transferred to a 50 ml conical tube containing 100 µg DNase I $ml^{-1}$ (Stem Cell technologies Inc., Vancouver, Canada) in order to prevent clumping. Rosewell Park Memorial Institute (RPMI)-1640 medium with 10% FBS was added to the DCs which were subsequently centrifuged in a swing arm rotor (200 × *g*) at room temperature for 10 min. An aliquot of cells was removed for enumeration of live cells using Trypan Blue (Sigma) and the Invitrogen Countess (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Viable cells were then diluted to a final concentration of $1 \times 10^6$ cells $ml^{-1}$ in the RPMI-1640 + 10% FBS and aliquoted (100 µl per well) into round bottom polypropylene 96 well plates and held in 5% $CO_2$ at 37ºC. Bacterial

strains grown to stationary phase (16 h) were harvested by centrifugation, washed, resuspended in PBS, and then standardized to ~ $1 \times 10^8$ CFU $ml^{-1}$. A portion of these aliquots was set aside for dilution and enumerative plating of on MRS agar. The standardized cell suspension was centrifuged and resuspended in RPMI-1640 + 10% FBS and $1 \times 10^6$ cells were combined with $1 \times 10^5$ viable iDCs in each well, resulting in a final bacterial to DC ratio of 10:1. The bacterial cells and iDCs were co-incubated for 24 h in 5% $CO_2$ at 37ºC, after which the suspension was centrifuged and the supernatant was harvested and stored at -80ºC for cytokine analysis.

Cytokine measurements for tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, and IL-12 were quantified using Single-Analyte ELISArray kits (Qiagen), according to the manufacturer's instructions. Following cytokine quantification, the cytokine expression data were compared between the parent and mutant strains using univariate analysis of variance.

## 2.4 Results

### 2.4.1 Identification of extracellular and putative S-layer associated proteins (SLAPs) from modified LiCl extraction

Previous work characterizing the S-layer of lactobacilli used 5 M LiCl salt in order to extract S-layer proteins efficiently with less lethality than other denaturing salts, such as guanidine hydrochloride (15, 18-23). In order to investigate the potential presence of SLAPs, a LiCl S-layer extraction protocol (15, 21) was modified. Cells were treated with 5 M LiCl, solubilizing all non-covalently bound Slp and proteins associated with the S-layer, and the proteins were extracted via dialysis. This fraction was then treated with 1 M LiCl in order to separate the Slp from the proteins associated with the S-layer. These supernatants, containing only the proteins associated with the S-layer, were centrifuged, dialyzed, and electrophoresed on SDS-PAGE (Figure 2.2, lane 1). This subset of proteins may be associated with or embedded within the S-layer of *L. acidophilus* NCFM.

Proteins from the modified LiCl extraction protocol were identified using LC-MS/MS following trypsin digestion. After database searching, the identified proteins were presented using Scaffold proteome software. Proteomic data were processed by removing human contaminants (e.g. keratin), falsely identified mammalian proteins, and any protein with a unique spectral count $\leq 10$, leaving 37 proteins of interest with molecular masses ranging from 10 to 78 kDa (Table 2). The newly identified SLAPs were ordered from the highest to lowest unique spectral counts. Interestingly, GRAVY values predicted that all proteins of interest were hydrophilic in nature. Secretion of proteins in Gram-positive bacteria is mediated through the Sec translocase system (33). All proteins processed by the Sec

translocase contain an N-terminal signal peptide sequence which, after translocation, is targeted by one of two signal peptidases (SPases). Type-I SPases recognize an AxAA cleavage site (34), while Type-II SPases recognize an L-x-x-C, or lipobox, cleavage site (35). Using SignalP 4.1 (36), 30 out of the 37 identified proteins have predicted cleavage sites either through the Type-I SPase or Type-II SPase pathway.

Referencing LocateP and the LAB Secretome Database (37, 38), proteins were sorted by their predicted subcellular locations. Twenty-four of the 37 proteins in the fraction are predicted to be extracellular with predicted Type-I SPase-mediated N-terminal cleavage sites (Table 2). Notably, 9 of these extracellular proteins are predicted, via hidden Markov models, to remain N-terminally associated with the cell membrane despite predicted cleavage sites. While these so-called "sec-attached" proteins have been proteomically described in *Bacillus subtilis* (39), there has been no work demonstrating the existence of such proteins in *Lactobacillus* species to date. In fact, the exact method in which such proteins avoid processing through SPase activity has not been elucidated (39, 40). Six of the remaining 13 proteins are predicted to be lipid-anchored proteins, mediated through Type-II SPase activity; three are proteins predicted to be N-terminally anchored to the cell membrane due to the lack of predicted cleavage sites; and 4 are predicted to be intracellular or non-classically secreted proteins (Table 2).

In order to deduce which of the proteins in the LiCl extracted fraction may be SLAPs, we compared the protein sequences against the deduced proteomes of closely related S-layer forming (*L. helveticus*, *L. crispatus*, and *L. amylovorus*) and non-S-layer forming (*L. gasseri*, *L. johnsonii*, and *L. delbrueckii* subsp. *bulgaricus*) lactobacilli using BlastP (Table 3). The

majority of the extracellular proteins (18/24), including those described as potentially sec-attached, demonstrate high levels of sequence identity to the corresponding proteins in S-layer forming lactobacilli, but with either weak sequence similarity or no orthologs in the non-S-layer forming lactobacilli. Whereas, the lipid-anchored proteins and the intracellular proteins demonstrate equally high sequence identity in both the S-layer forming and non-S-layer forming species of *Lactobacillus* examined. There were 8 proteins with highly inferred homology in the S-layer forming species with no hits in all three non-S-layer forming species. Furthermore, 19 proteins demonstrate a highly inferred homology in the S-layer forming species with no hit in at least two of the non-S-layer forming species. We propose that these 17 proteins (excluding SlpA and SlpX) are candidate SLAPs, given their absence in non-Slayer forming *Lactobacillus* species that are closely related to *L. acidophilus*. While both SlpX and SlpA were found to be present in the SLAP fraction, they appeared at a significantly lower concentration compared to the other proteins (Table 2). For reference of comparison, the standard LiCl extraction protocol was performed along with the modification presented in this study (Figure 2.2B). It is clear that the method presented in this study recovered more proteins than the standard method. Of the 37 proteins identified, 21 are annotated as putative or uncharacterized proteins of unknown function. These include proteins with putative fibronectin-binding domains, putative surface proteins, bacterial Ig-like domain proteins, putative surface exclusion proteins, uncharacterized ABC transporters, a 78 kDa putative serine protease, and a putative surface layer protein (LBA1029).

### 2.4.2 Deletion of *lba1029* from the chromosome of *L. acidophilus* NCFM

LBA1029 was observed to be a prevalent protein in the LiCl fraction. Examining annotated sequence data from *L. acidophilus* NCFM, we found that *lba-1029* encodes an uncharacterized protein, annotated as a putative surface layer protein. Notably, this protein was selected for functional analysis because of its apparent singularity to *L. acidophilus* compared to closely related S-layer- and non-S-layer- forming lactobacilli (Table 3). In fact, the deduced protein sequence of LBA1029 demonstrated low sequence identity to proteins in the dairy starter culture, *L. helveticus* H10 (38% sequence identity) and the vaginal commensal, *L. crispatus* ST1 (36 % sequence identity), both of which are S-layer forming members of the Group A acidophilus complex. This 385 amino acid-residue protein of unknown function has a predicted N-terminal signal peptide cleavage site between two alanine residues at positions 37 and 38. Furthermore, *lba-1029* is flanked by two hairpin terminators, suggesting monocistronic mRNA expression and control (Figure 2.3A).

In order to assess the roles these putative SLAPs may play in cell function and immunomodulatory interaction, a Δ*lba-1029* strain was created and phenotypically characterized. The *lba-1029* gene was deleted from the chromosome using a *upp*-based counterselection gene replacement system (Figure 2.3B). A colony containing the in-frame deletion of *lba-1029* (Δ*lba-1029*) was confirmed via sequencing and designated as NCK2258. When the SLAPs of NCK2258 were profiled (Figure 2.2 lane 2) and identified through LC-MS/MS, LBA1029 was not found in the fraction – further confirming the absence of *lba-1029*from the cell envelope of NCK2258.

### 2.4.3 Phenotypic characterization of Δ*lba1029* as pertaining to probiotic functionality

Comparative analysis between the NCK1909 (wild-type reference) and NCK2258 (Δ*lba-1029*) was employed in order to characterize the function of LBA1029. NCK2258 showed no difference in growth in MRS medium or cell morphology under the light microscope compared to NCK1909. Likewise, mutant cells settled to the bottom of tubes in a similar fashion to NCK1909 when grown in planktonic culture, suggesting no difference in the aggregative properties between the two strains.

Assays for simulated gastric and small intestinal juices, adhesion to a Caco-2 epithelial cell line, and bile tolerance were also performed. Survival through the gastrointestinal transit was evaluated *in vitro* through exposure to simulated gastric juice and simulated small intestinal juice over 1.5 h and 5 h, respectively. There was no significant difference in the survival rate between NCK2258 compared to the NCK1909 reference strain (Data not shown). The Caco-2 epithelial cell line were employed for *in vitro* analysis of bacterial adherence to intestinal epithelia. Compared to the reference strain, NCK2258 showed insignificant changes in the adherence to Caco-2 (Data not shown). Finally, NCK2258 and the NCK1909 reference were exposed to 0.3 % and 0.5 % bile (Oxgall) in order to assay bile tolerance, but no difference was observed between the strains (Data not shown).

### 2.4.4 LBA1029 contributes to a pro-inflammatory response through the induction of TNF- α.

Previous work on the S-layer of *L. acidophilus* NCFM demonstrated a role of SlpA in modulating the host immune system (17). In order to test the immunomodulatory action of the LBA1029 protein, a bacterial/murine DC co-incubation assay was performed. After co-incubation for 24 h, the cytokines TNF-α, IL-6, IL-10, and IL-12 were quantified using ELISA. Both TNF-α and IL-6 were measured as an indicator of a general pro-inflammatory response. IL-10, however, was measured as a marker for an anti-inflammatory response via the down regulation of $T_H1$ cell response. Conversely, IL-12 was measured as a pro-inflammatory response via the activation of $T_H1$ cells. Three independent assays were performed in duplicate for each cytokine.

For each co-incubation, bacterial cells were diluted and exposed to DC at a ratio of approximately 10:1. Notably, there was a significant decrease ($p=0.006$) in TNF-α production for DCs co-incubated withNCK2258 compared to the parental reference strain, NCK1909 (Figure 2.4A). There was no significant difference between DCs co-incubated with NCK2258 and the NCK1909 reference with regard to IL-6, IL-10, and IL-12 production (Figure 2.4B). Ultimately, NCK2258 demonstrated a 36% reduction in TNF-α induction (Figure 2.4B), suggesting that LBA1029 contributes to pro-inflammatory response via the induction of TNF-α.

## 2.5 Discussion

This study utilized a method modified from a standard LiCl S-layer extraction in order to isolate proteins associated with the primary S-layer fraction and identify extracellular proteins and putative SLAPs in *L. acidophilus* NCFM. In the present method, 37 proteins were identified and reported. One such protein, LBA1029, was eliminated via deletion of the gene from the chromosome. While the absence of LBA1029 did not seem to affect survival in simulated gastric juice, simulated small intestinal juice, bile tolerance, or affinity to Caco-2 epithelial cells, it did demonstrate important capacity for immunomodulation through murine DCs. The 36% reduction of TNF-α production by DCs co-incubated with the LBA1029 deficient strain compared to its parent strain suggests that the protein is pro-inflammatory through the TNF-α pathway.

Of the 37 reported proteins, 30 have predicted cleavage sites for secretion, of which 24 are predicted to be extracellular and 6 are lipid-anchored. Three of the remaining seven have an N-terminal transmembrane hydrophobic anchor (with no cleavage site) and the final 4 are intracellular, potentially non-classically secreted moonlighting proteins (Table 2). Both LocateP (37) and LAB secretome database (38) reported 9 of the 24 extracellular proteins as "sec-attached." These sec-attached proteins, discovered in *Bacillus subtilis* (39) are proteins that have predicted cleavage sites but avoid Sec-pathway SPase cleavage to remain N-terminally anchored to the cell membrane. The method in which these proteins avoid SPase activity for Sec processing has yet to be elucidated (39, 40). Notably, sec-attached proteins have not been proteomically characterized in any *Lactobacillus* species to date. The basis with which the LAB proteomes were analyzed for the presence of sec-attached proteins used

Hidden Markov modeling (HMM) comparing 63 cleaved proteins (extracellular) to 53 un-cleaved proteins (sec-attached) from *B. subtilis* and other *Bacillus* orthologs (37). While this HMM scoring model is useful for processing large proteomic data sets, such as those from the LAB secretome database, it is important to acknowledge the possibility of incorrect predictions. For example, SlpA was predicted to be sec-attached despite the fact that it has been visually and biochemically characterized as the self-assembling constituent of the *L. acidophilus* S-layer (12, 41, 42). Due to this incongruity and because these 9 predicted sec-attached proteins were isolated using LiCl, we consider these proteins to be extracellular.

Interestingly, 4 of the 37 proteins identified (Table 2) in the LiCl precipitate do not have predicted secretion pathways: among them, a diaminopimelate decarboxylase, 30S ribosomal protein S3, an uncharacterized protein, as well as a glyceraldehydes 3-phospate dehydrogenase (GAPDH). Because the cells were dialyzed at stationary phase, it is possible that limited cell lysis may have begun by the time cells were treated with LiCl, allowing small amounts of intracellular and membrane anchored proteins to be recovered non-specifically along with the S-layer. However, it is also possible that these intracellular proteins are non-classical, extracellular "moonlighting" proteins (43). GAPDH, for example, is a moonlighting protein which mediates microbe-host interactions in lactic acid bacteria (44-46). In fact, the GAPDH of *L. plantarum* LA 318 has demonstrated adherence capabilities to human colonic mucin (45).

Of particular interest in this study is the isolation and identification of possible SLAPs in the cell-surface proteome of *L. acidophilus* NCFM. It is worth noting that the *in vivo* localization or association of these SLAPs in relation to the S-layer has not been fully

demonstrated, and is based on co-extraction following LiCl treatment. In order to select candidate SLAPs from the fraction of secreted proteins identified through LiCl extraction, we aligned the deduced protein sequences to the deduced proteomes of closely related S-layer and non-S-layer forming *Lactobacillus* species (Table 3). The analysis revealed that the 24 extracellular proteins had high sequence identity to the corresponding orthologs in S-layer forming lactobacilli and either no or low sequence identity to the non-S-layer forming lactobacilli. In contrast, the lipid-anchored proteins, many of which are ABC sugar transporters, and the intracellular proteins shared inferred homology in both S-layer and non-S-layer forming *Lactobacillus* species. Based on this *in silico* analysis, the proteins that are most likely SLAPs are the 17 extracellular proteins with no hits in two or three of the non-S-layer forming lactobacilli. Further work will be required to characterize the specific localization of these SLAPs and the Slp subunits with which they interact. Furthermore, there should be a distinction between the organizational definitions of SLAPs in the context of this study and the SLAP Pfam domain (PF03217) designated for bacterial surface layer proteins (41).

To our knowledge, SLAPs have not been identified in any organism using the method of this study. However, in the S-layer forming pathogen, *Bacillus anthracis*, 22 *B. anthracis* S-layer proteins (BSLs) have been identified and characterized (47-50). While these proteins are described as S-layer associated in recent publications (49,5 0), these proteins were localized *in silico* based on N-terminal S-layer Homology (SLH) domains and were originally designated S-layer proteins. In contrast, the SLAPs observed in the present study were found to be constituents of the S-layer after translational expression and secretion. An

important distinction should therefore be made between the methodology of this study and the methodology for identifying BSLs of *B. anthracis.* Application of this study's methodology to *B. anthracis* may yield novel SLAPs that are not tethered to the S-layer through SLH domains.

The SLAPs in *L. acidophilus* NCFM offer potential in understanding cell envelope biology and function, as well as illuminating important factors pertaining to probiotic function. A cell division protein CdpA, which has previously been functionally characterized (51), was found to be a prevalent protein in the SLAP fraction. CdpA, which may be a SLAP due to its low sequence identity in the non-S-layer forming lactobacilli (Table 3), has important roles in cell-wall processing during growth and cell-cell separation. This finding substantiates the prediction that certain SLAPs, especially the putative cell division proteins, aminopeptidases, and penicillin-binding proteins, may play a role in cell growth,-turnover and cell envelope function. A great deal of work has already been done pertaining to probiotic functions in *L. acidophilus* NCFM. This includes work on adherence factors (16), prebiotic sugar utilization (52), and bacteriocin production (53). However, there are still many factors of probiotic mechanism that have not been fully elucidated. Because of their localization to the cell surface, the SLAPs identified in this study are candidate mediators of probiotic function. Furthermore, we have access to microarray data pertaining to acid tolerance (54), bile tolerance (55) and oligosaccharide utilization (52), which could offer insight into the role that these extracellular proteins may play in probiotic survival, persistence, and immunomodulation in the host gastrointestinal tract.

Understanding the role of intestinal microbiota in gut homeostasis has been regarded with greater interest given the prevalence of inflammatory bowel disease, such as ulcerative colitis, and Crohn's disease (56). Recent work regarding cell surface components of *L. acidophilus* NCFM, such as lipoteichoic acid (LTA) and SlpA have demonstrated regulation of colonic inflammation and T-cell functionality, respectively (17, 31). Given their localization to the outermost layer of the cell envelope, SLAPs are ideal candidates for studying the immunomodulatory interaction between *L. acidophilus* and the gut immune system. The findings of this study support this observation, given that LBA1029 exhibited an effect on immunomodulatory properties through the induction of TNF-α. Exploring the SLAPs of *L. acidophilus*, as well as those of other S-layer forming commensal bacteria, will be important in understanding the full context of interaction between gut epithelial cells, gut immune system, and intestinal microbiota.

Beyond *L. acidophilus*, this study highlights further potential in understanding S-layer and cell envelope function of other S-layer forming bacteria and archaebacteria. In certain S-layer forming pathogens, such as *B. anthracis* (47), *Rickettsia* species (57), *Aeromonas salmonicida* (58), *Campylobacter fetus* (59), and *Clostridium difficile* (60), S-layer and cell surface components are important pathogenicity factors. Studying potential SLAPs within the cell surface proteome in these and other S-layer forming pathogens may offer further insight into pathogenicity. The hyperthermophilic archaebacteria that form S-layers, such as *Methanococcus* and *Methanothermus* species, are of certain biotechnological interest. In particular, the hexagonal S-layers of these archaea have been regarded for their potential uses in nanotechnology because of their heat stability and the ability to self-assemble (61).

Examining potential SLAPs in these and other extremophile archaebacteria could explicate cell envelope function, as well as potentiate the discovery of important thermostable proteins for use in biotechnological application.

Overall, the characterization of SLAPs significantly expands the opportunities to understand the probiotic activities and immunomodulatory actions of *L. acidophilus* NCFM. Likewise, SLAPs may be of particular interest in other members of the *Lactobacillus* genus. Comparing the SLAPs of *L. acidophilus* to the proteomes of other S-layer forming lactobacilli, such as the dairy starter *L. helveticus* and the vaginal commensal *L. crispatus*, could grant key ecological and evolutionary insights. Novel proteinases or proteases could be identified as SLAPs in the cell surface proteome of *L. helveticus* that may be important in the cheese ripening process. Moreover, SLAPs could be important factors of adherence and retention for *L. crispatus* in the vaginal mucosa. Ultimately, the proteins identified in this study are potentially novel extracellular proteins, some of which may be associated with the bacterial S-layer. These proteins afford the possibility to functionally characterize bacterial S-layers and will provide important insights into the architecture and physiology of bacterial cell surfaces.

## 2.6 Acknowledgements

This work was supported in part by funding from the North Carolina Agriculture Foundation (Raleigh, NC) and DuPont Nutrition & Health (Madison, WI). Our thanks are extended to Dr. Brett Phiney and Michelle Salemi at the University of California Davis Proteomics Core Facility for LC-MS/MS services. We are grateful to Rosemary Sanozky-Dawes for technical assistance in preparing Caco-2 epithelial cells. Finally, we thank Dr. Dahlia Nielsen for insightful statistical discussion and Kiersten Johnson for assistance with statistical analyses using SPSS.

## 2.7 References


1. **Sara M, Sleytr UB**. 2000. S-layer proteins. J Bacteriol **182**:859-862.

2. **Sleytr UB, Sara M, Pum D, Schuster B**. 2001. Characterization and use of crystalline bacterial cell surface layers. Prog Surf Sci **68**:231-278.

3. **Sleytr UB, Beveridge TJ**. 1999. Bacterial S-layers. Trends Microbiol **7**:235-260.

4. **Sara M, Sleytr UB**. 1996. Crystalline bacterial cell surface layers (S-layer): From cell structure to biomimetics. Prog Biophys Mol Bio **65**:83-111.

5. **Sleytr UB, Messner P**. 1983. Crystalline surface-layers on bacteria. Annu Rev Microbiol **37**:311-339.

6. **Brechtel E, Bahl H**. 1999. In *Thermoanaerobacterium thermosulfurigenes* EM1 S-layer homology domains do not attach to peptidoglycan. J Bacteriol **181**:5017-5023.

7. **Chaevaux S, Matuschek M, Beguin P**. 1999. Distinct affinity of binding sites for S-layer homologous domains in *Clostridium thermocellum* and *Bacillus anthracis* cell envelopes. J Bacteriol **181**:2455-2458.

8. **Pot B, Ludwig W, Kersters K, Schleifer KH**. 1994. Taxonomy of lactic acid bacteria. In *Bacteriocins of Lactic Acid Bacteria*, pp. 13-90. Edited by L. de Vuyst & E. J. Vandamme. Glasgow, U.K.: Chapman & Hall.

9. **Hammes WP, Vogel RF**. 1995. The genus *Lactobacillus*. In *The Genera of Lactic Acid Bacteria*, pp. 19-54. Edited by B. J. B. Wood & W. H. Holzapfel. Glasgow, U.K.: Blackie Academic & Professional.

10. **Avall-Jaaskelainen S, Palva A**. 2005. *Lactobacillus* surface layers and their applications. FEMS Microbiol Rev **29**:511-529.

11. **Hynonen U, Plava A**. 2013. *Lactobacillus* surface layer proteins: structure function and applications. Appl Microbiol Biotechnol **97**:5225-5244.

12. **Boot HJ, Pouwels PH**. 1996. Expression, secretion and antigenic variation of bacterial S-layer proteins. Mol Microbiol **21**:1117-1123.

13. **Sanders ME, Klaenhammer TR**. 2001. The scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic. J Dairy Sci **84**:319-331.

14. **Altermann E, Russell WM, Azcarate-Peril MA, Barrangou R, Buck BL, McAuliffe O, Souther N, Dobson A, Duong T, Callanan M, Lick S, Hamrick A, Cano R, Klaenhammer TR**. 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. Proc Natl Acad Sci USA **102**:3906–3912.

15. **Goh YJ, Azcarate-Peril MA, O'Flaherty S, Durmaz E, Valence F, Jardin J, Lortal S, Klaenhammer TR**. 2010. Development and application of a *upp*-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol **75**:3093-3105.

16. **Buck BL, Altermann E, Svingerud T, Klaenhammer TR**. 2005. Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol **71**:8344-8351.

17. **Konstantinov SR, Smidt H, de Vos WM, Bruijns SCM, Singh SK, Valence F, Molle D, Lortal S, Altermann E, Klaenhammer TR, van Kooyk Y**. 2008. S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. Proc Natl Acad Sci USA **105**:19474–19479.

18. **Ashida N, Yanagihara S, Shinoda T, Yamamoto N**. 2011. Characterization of adhesive molecules with affinity to Caco-2 cells in *Lactobacillus acidophilus* by proteome analysis. J Biosci Bioeng **112**:333-337.

19. **Beganovic J, Frece J, Kos B, Pavunc AL, Habjanic K, Sislpvoc J**. 2011. Functionality of the S-layer protein from the probiotic strain *Lactobacillus helveticus* M92. Antonie van Leewuenhoek **100**:43-53.

20. **Frece J, Kos B, Svetec IK, Zgaga Z, Mrsa V, Suskovic J**. 2004. Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. J Appl Microbiol **98**:285-292.

21. **Lortal S, Vanheijenoort J, Gruber K, Sleytr UB**. 1992. S-layer of *Lactobacillus helveticus* ATCC12046; isolation, chemical characterization and reformation after extraction with lithium chloride. Microbiology **138**:611-618.

22. **Smit E, Oling F, Demel R, Martinez B, Pouwels PH**. 2001. The S-layer protein of *Lactobacillus acidophilus* ATCC 4356: Identification and characterization of domains responsible for S-protein assembly and cell wall binding. J Mol Biol **305**:245-257.

23. **Taverniti V, Stuknyte M, Minuzzo M, Arioli S, De Noni I, Scabiosi C, Martinez Cordova S, Junttila I, Hamalainen S, Turpeinen H, Mora D, Karp M, Pesu M, Guglielmetti S**. 2013. S-layer protein mediates the stimulatory effect of *Lactobacillus helveticus* MIMLh5 on innate immunity. Appl Environ Microbiol **79**:1221-1231.

24. **Shevchenko A, Wilm M, Vorm O, Mann M**. 1996. Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. Anal Chem **68**:850-858.

25. **Tabb DL, Ma ZQ, Martin DB, Ham AJL, Chamber MC**. 2008. DirecTag: accurate sequence tags from peptide MS/MS through statistical scoring. J Proteome Res **7**:3838-3846.

26. **Hanahan D**. 1983. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol **166**:557-580.

27. **Walker DC, Aoyama K, Klaenhammer TR**. 1996. Electrotransformation of *Lactobacillus acidophilus* group A1. FEMS Microbiol Lett **138**: 233-237.

28. **Wei MQ, Rush CM, Norman JM, Hafner LM, Epping RJ, Timms P**. 1995. An improved method for the transformation of *Lactobacillus* strains using electroporation. J Microbiol Methods **21**: 97-109.

29. **Kingsford CL, Ayanbule K, Salzberg SL**. 2007. Rapid, accurate, computational discovery of Rho-independent transcription terminators illuminates their relationship to DNA uptake. Genome Biol **8**:R22.

30. **Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR**. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77**:61-68.

31. **Mohamadzadeh M, Pfeiler EA, Brown JB, Zadeh M, Gramorossa M, Managlia E, Bere P, Sarraj B, Khan MW, Chaitanya Pakanati K, Javeed Ansari M, O'Flaherty S, Barrett T, Klaenhammer TR**. 2011. Regulation of induced colonic inflammation by *Lactobacillus acidophilus* deficient in lipoteichoic acid. P Natl Acad Sci USA **108**:4623-4630.

32. **Stoeker L, Dordone S, Gunderson S, Zhang L, Kajikawa A, LaVoy A, Miller M, Klaenhammer TR, Dean GA**. 2011. Assessment of *Lactobacillus gasseri* as a candidate oral vaccine vector. Clin Vaccine Immunol **18**:1834-1844.

33. **Driessen AJM, Nouwen N**. 2008. Protein translocation across the bacterial cytoplasmic membrane. Annu Rev Biochem **77**:643-667.

34. **Van Roosmalen ML, Geukens N, Jongbloed JDH, Tjalsma H, Dubois JYF, Bron S, van Dijl JM, Anne J**. 2004. Type I signal peptidases of Gram-positive bacteria. Bba-Mol Cell Res **1694**:279-297.

35. **Sutcliffe IC, Harrington DJ**. 2002. Pattern searches for the identification of putative lipoprotein genes in Gram-positive bacterial genomes. Microbiology **148**:2065-2077.

36. **Petersen TN, Brunak S, von Heijne G, Nielsen H**. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods **8**:785–786.

37. **Zhou M, Boekhorst J, Francke C, Siezen RJ**. 2008. LocateP: Genome-scale subcellular-location predictor for bacterial proteins. BMC Bioinformatics **9**.

38. **Zhou M, Theunissen D, Wels M, Siezen RJ**. 2010. LAB-Secretome: a genome-scale comparative analysis of the predicted extracellular and surface-associated proteins of Lactic Acid Bacteria. BMC Genomics **11**:651.

39. **Tjalsma H, van Dijl JM**. 2005. Proteomics-based consensus prediction of protein retention in a bacterial membrane. Proteomics **5**:4472-4482.

40. **Dreisbach A, van Dijl JM, Buist G**. 2011. The cell surface proteome of *Staphylococcus aureus*. Proteomics **11**:3154-3168.

41. **Boot HJ, Kolen CP, Pouwels PH**. 1995. Identification, cloning, and nucleotide sequence of a silent S-layer protein gene of *Lactobacillus acidophilus* ATCC 4356 which has extensive similarity with the S-layer protein gene of this species. J Bacteriol **177**:7222-7230.

42. **Boot HJ, Kolen CP, Pot B, Kersters K, Pouwels PH**. 1996. The presence of two S-layer protein encoding genes is conserved among species related to *Lactobacillus acidophilus*. Microbiology **142**: 2375-2385.

43. **Jeffery CJ**. 1999. Moonlighting proteins. Trends Biochem Sci **24**: 8-11.

44. **Katakura Y, Sano R, Hashimoto T, Ninomiya K, Shioya S**. 2010. Lactic acid bacteria display on the cell surface cytosolic proteins that recognize yeast mannan. Appl Microbiol Biotechnol **86**:319-326.

45. **Kinoshita H, Uchida H, Kawai Y, Wakahara N, Matsuo H, Watanabe M, Kitazawa H, Ohnuma S, Miura K, Horil A, Saito T**. 2008. Cell surface *Lactobacillus plantarum* LA318 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) adheres to human colonic mucin. J Appl Microbiol **104**:1667-1674.

46. **Sanchez B, Schmitter JM, Urdaci MC**. 2009. Identification of novel proteins secreted by *Lactobacillus plantarum* that bind to mucin and fibronectin. J Mol Microb Biotech **17**:158-162.

47. **Kern JW, Schneewind O**. 2010. BslA, the S-layer adhesion of *B. anthracis*, is a virulence factor for anthrax pathogenesis. Mol Microbiol **75**:324-332.

48. **Kern JW, Schneewind O**. 2008. BslA a pXO1-encoded adhesion of *Bacillus anthracis*. Mol Microbiol **68**:504-515.

49. **Kern VJ, Kern JW, Theriot JA, Schneewind O, Missiakas D**. 2012. Surface-Layer (S-Layer) proteins Sap and EA1 govern the binding of the S-Layer Associated protein BslO at the cell septa of *Bacillus anthracis*. J Bacteriol **194**:3833-3840.

50. **Lundenberg JM, Nguyen-Mau SM, Richter GS, Wang YT, Dworkin J, Missiakas DM, Schneewind O**. 2013. *Bacillus anthracis* aceytyltransferases PatA1 and PatA2 modify the secondary cell wall polysaccharide and affect the assembly of S-layer protein. J Bacteriol **195**:977-989.

51. **Altermann E, Buck BL, Cano R, Klaenhammer TR**. 2004. Identification and phenotypic characterization of the cell division protein CdpA. Gene **342**:189-197.

52. **Barrangou R, Altermann E, Hutkins R, Cano R, Klaenhammer TR**. 2003. Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by *Lactobacillus acidophilus*. Proc Natl Acad Sci USA **100**:8957-8962.

53. **Dobson AE, Sanozky-Dawes RB, Klaenhammer TR**. 2007. Identification of an operon and inducing peptide involved in the production of lactacin B by *Lactobacillus acidophilus*. J Appl Microbiol **103**:1766-1778.

54. **Azcarate-Peril MA, Altermann E, Hoover-Fitzula RL, Cano RJ, Klaenhammer TR**. 2004. Identification and inactivation of genetic loci involved with *Lactobacillus acidophilus* acid tolerance. Appl Environ Microbiol **70**:5315-5322.

55. **Pfeiler EA, Azcarate-Peril MA, Klaenhammer TR**. 2007. Characterization of a novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus acidophilus*. J Bacteriol **189**:4624-4634.

56. **MacDonald TT, Monteleone G**. 2005. Immunity, inflammation, and allergy in the gut. Science **307**:1920-1925.

57. **Walker DV, Valbuena GA, Olano JP**. 2006. Pathogenic mechanisms of diseases caused by *Rickettsia* Ann New York Acad Sci **990**:1-11.

58. **Kay WW, Phipps BM, Ishiguro EE. Olafson RW, Trust TJ**. 1984. Surface layer virulence A-proteins from *Aeromonas salmonicida* strains. Can J Biochem Cell Biol

**62**:1064-1071.

59. **Blaser MJ, Smith PF, Repine JE, Joiner KA**. 1988. Pathogenesis of *Campylobacter fetus* infections: failure of encapsulated *Campylobacter fetus* to bind C3b explains serum and phagocytosis resistance. J Clin Invest **81**:1434-1444.

60. **Balabi E, Calabi F, Phillips AD, Fairweather NF**. 2002. Binding of *Clostridium difficile* surface layer proteins to gastrointestinal tissues. Infect Immun **70**:5770-5778.

61. **Sleytr UB, Sara M**. 1997. Bacterial and archeael S-layer proteins: structure-function relationships and their biotechnological applications. Trends Biotechnol **15**:20-26.

**Table 2.1** Strains, plasmids, and primers used in this study.

| Strain, plasmid, or primer | Genotype or characteristics | Reference |
|---|---|---|
| ***L. acidophilus* strains** | | |
| NCFM (NCK56) | Human intestinal isolate | (41) |
| NCK1909 | NCFM with chromosomal deletion of *upp*; background host for *upp*-based counterselective gene replacement system | (10) |
| NCK2258 | NCK1909 with chromosomal deletion of *lba-1029* | This study |
| ***E. coli* strains** | | |
| EC101 | $RepA^+$, $Km^r$, pWV01 integrated into chromosome | (42) |
| NCK1910 | Host for harboring pTRK669, $Cm^r$ | |
| NCK1911 | Host for harboring pTRK935, $Kn^r$, $Em^r$ | (10) |
| NCK2257 | EC101: harboring pTRK1067, $Kn^r$, $Em^r$ | This study |
| **Plasmids** | | |
| pTRK669 | Ori (pWV01), $Cm^r$, $RepA^+$ thermosensitive | (43) |
| pTRK935 | pORI *upp*-based counterselective integration vector | (10) |
| pTRK1067 | pTRK935 with flanking regions of *lba-1029* cloned into *Bam*HI and *Sac*I restriction sites. | This study |
| **Primers †** | | |
| 1*Bam*HI-F | GTAATAGGATCCGCAGAAATTAAGCCCGTTGT | This study |
| 2R | TGCAATTGTAGCCAAAATTAGTG | This study |
| 3Soe | TAATTTTGGCTACAATTGCACACACTGCTGTTTACGATCCA | This study |
| 4*Sac*I-R | TAAAGTAGAGCTCATCTTGCCCAATCGTGTAAA | This study |
| 1029up | CTTAATTCACTGGCCAAATC | This study |
| 1029dw | TCTGCTGACTTCTCTTGAGG | This study |

† Restriction sites are underlined.

**Table 2.2** Proteins extracted through modified exposure to LiCl

| ORF | Protein Description | Predicted Molecular Mass | SPase target | % AA coverage | GRAVY score | Predicted SPase Cleavage Site | Unique Spectral Count |
|---|---|---|---|---|---|---|---|
| **Extracellular proteins** | | | | | | | |
| LBA0695 | Putative uncharacterized protein | 62 kDa | SPI | 75% (410/550) | -0.58 | VSA-AD (37-38) | 141 |
| LBA1029 | Putative surface layer protein | 43 kDa | SPI | 78% (300/385) | -0.41 | VQA-AT (37-38) | 95 |
| LBA0512 | SlpX | 54 kDa | SPI | 65% (324/499) | -0.58 | VQA-DT (30-31) | 68 |
| LBA1567 | Aminopeptidase | 57 kDa | SPI | 67% (339/505) | -0.59 | AQA-AA (27-28) | 66 |
| LBA0222 | Putative uncharacterized protein | 30 kDa | SPI | 54% (152/282) | -0.78 | AHA-KG (39-40) | 61 |
| LBA0191 | Putative fibronectin domain protein | 52 kDa | SPI | 71% (329/463) | -0.59 | VQA-GT (24-25) | 60 |
| LBA0864 | Putative uncharacterized protein | 55 kDa | SPI | 64% (316/497) | -0.52 | AQA-QH (25-26) | 52 |
| LBA1568 | Putative surface protein | 39 kDa | SPI | 66% (233/353) | -0.38 | ambiguous | 46 |
| LBA1539 | Putative uncharacterized protein | 19 kDa | SPI | 71% (122/171) | -0.29 | ANA-AS (28-29) | 41 |
| LBA1006 | Penicillin-binding protein | 41 kDa | SPI | 76% (276/364) | -0.46 | VHA-AY (26-27) | 36 |
| LBA0176 | N-acetylmuramidase | 45 kDa | SPI | 43% (176/409) | -0.68 | VSA-AT (38-39) | 21 |
| LBA0494 | Putative surface exclusion protein | 40 kDa | SPI | 47% (168/355) | -0.53 | VQA-AS (32-33) | 18 |
| LBA0177 | Autolysin, amidase | 41 kDa | SPI | 44% (160/364) | -0.57 | VQA-DS (30-31) | 18 |
| LBA0046 | Putative uncharacterized protein | 13 kDa | SPI | 53% (62/118) | -0.29 | TQA-AS (30-31) | 12 |
| LBA1079 | Putative cell surface protein | 23 kDa | SPI | 39% (79/202) | -0.38 | VNA-TT (29-30) | 12 |
| **Extracellular proteins (predicted to be sec-attached)** | | | | | | | |
| LBA1578 | Putative serine protease | 78 kDa | SPI | 84% (583/694) | -0.62 | VKA-AD (34-35) | 202 |
| LBA0169 | SlpA | 47 kDa | SPI | 63% (278/444) | -0.25 | VSA-AT (31-32) | 65 |
| LBA0858 | Penicillin-binding protein | 42 kDa | SPI | 67% (248/369) | -0.43 | VNA-KV (30-31) | 43 |
| LBA1426 | Putative uncharacterized protein | 28 kDa | SPI | 63% (159/252) | -0.38 | VQA-AT (34-35) | 39 |
| LBA1690 | Putative surface exclusion protein | 31 kDa | SPI | 74% (207/280) | -0.65 | NQE-DN (30-31) | 35 |
| LBA1207 | Putative enterolysin A | 24 kDa | SPI | 65% (139/213) | -0.42 | VSA-DT (30-31) | 26 |
| LBA1661 | Putative membrane protein | 20 kDa | SPI | 48% (86/180) | -0.45 | VQA-AT (37-38) | 25 |
| LBA1227 | Putative uncharacterized protein | 21 kDa | SPI | 60% (109/182) | -0.59 | VNA-ST (33-34) | 23 |
| LBA1225 | Putative uncharacterized protein | 57 kDa | SPI | 19% (95/501) | -0.51 | VLA-CS (27-28) | 10 |
| **Lipid-anchored proteins** | | | | | | | |
| LBA0197 | OppA - oligopeptide binding protein | 65 kDa | SPII | 63% (366/585) | -0.58 | ALA-AC (21-22) | 53 |
| LBA1641 | Glycerol-3-phosphate ABC transporter | 47 kDa | SPII | 68% (293/433) | -0.48 | SSS-SS (32-33) | 44 |
| LBA1588 | PrsA/PrtM - peptidylprolyl isomerase | 33 kDa | SPII | 64% (193/300) | -0.58 | STA-AS (33-34) | 38 |
| LBA0014 | Putative alkylphosphonate ABC transporter | 35 kDa | SPII | 60% (187/313) | -0.38 | TSA-SS (31-32) | 29 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| LBA0585 | Glycerol-3-phosphate ABC transporter | 48 kDa | SPII | 34% (147/432) | -0.47 | NSS-ST (31-32) | 16 |
| LBA1497 | Putative uncharacterized protein | 36 kDa | SPII | 37% (125/336) | -0.77 | SQG-NS (26-27) | 11 |
| **N-terminally anchored proteins (no predicted cleavage site)** | | | | | | | |
| LBA0223 | CdpA - cell separation protein | 64 kDa | SPI[§] | 62% (372/599) | -0.53 | - | 66 |
| LBA0805 | Penicillin-binding protein | 79 kDa | SPI[§] | 53% (379/720) | -0.37 | - | 49 |
| LBA1010 | Putative secreted protein | 45 kDa | SPI[§] | 29% (115/401) | -0.58 | - | 14 |
| **Intracellular or moonlighting proteins** | | | | | | | |
| LBA0851 | LysA - diaminopimelate decarboxylase | 35 kDa | - | 70% (226/323) | -0.22 | - | 32 |
| LBA0040 | Putative uncharacterized protein | 10 kDa | - | 74% (64/87) | -0.98 | - | 15 |
| LBA0297 | RpsC - 30S ribosomal protein | 25 kDa | - | 44% (99/224) | -0.54 | - | 14 |
| LBA0698 | Glyceraldehyde 3-P dehydrogenase | 36 kDa | - | 30% (102/338) | -0.09 | - | 10 |

[§] Proteins predicted to have a SPase Type-I target with unknown cleavage site

**Table 2.3** Homology search of potential SLAPs to proteins in S-layer forming lactobacilli

| | | S-layer forming lactobacilli | | | Non-S-layer forming lactobacilli | | |
|---|---|---|---|---|---|---|---|
| **ORF** | **Protein Description** | ***L. helveticus*** | ***L. crispatus*** | ***L. amylovorus*** | ***L. gasseri*** | ***L. johnsonii*** | ***L. delbr*** |
| **Extracellular proteins** | | | | | | | |
| LBA0695* | Putative uncharacterized protein | 434/543 (80%) | 429/543 (79%) | 455/543 (84%) | - | - | 167/482 |
| LBA1029* | Putative surface layer protein | 145/377 (38%) | 134/371 (36%) | 87/255 (34%) | - | - | - |
| LBA0512 | SlpX | 385/511 (75%) | 313/446 (70%) | 314/517 (54%) | - | - | 52/126 |
| LBA1567* | Aminopeptidase | 414/505 (82%) | 405/505 (80%) | 407/505 (81%) | - | - | - |
| LBA0222* | Putative uncharacterized protein | 112/129 (86%) | 190/285 (67%) | 110/126 (87%) | - | - | - |
| LBA0191* | Putative fibronectin domain protein | 379/464 (82%) | 367/464 (79%) | 134/387 (35%) | - | - | - |
| LBA0864* | Putative uncharacterized protein | 196/474 (41%) | 346/502 (69%) | 401/496 (81%) | - | - | 226/517 |
| LBA1568* | Putative surface protein | 252/325 (78%) | 252/325 (78%) | 252/325 (78%) | - | - | 42/133 |
| LBA1539* | Putative uncharacterized protein | 111/176 (63%) | 115/173 (66%) | 115/175 (66%) | - | - | - |
| LBA1006 | Penicillin-binding protein | 303/365 (83%) | 304/368 (83%) | 300/364 (82%) | 75/305 (25%) | 65/234 (28%) | 152/336 |
| LBA0176 | N-acetylmuramidase | 324/409 (79%) | 300/409 (73%) | 334/410 (81%) | 124/273 (45%) | 123/272 (45%) | 190/414 |
| LBA0494 | Putative surface exclusion protein | 252/356 (71%) | 238/355 (67%) | 241/357 (68%) | - | 26/79 (33%) | 113/257 |
| LBA0177* | Autolysin, amidase | 280/364 (77%) | 271/365 (74%) | 284/369 (77%) | - | - | 166/385 |
| LBA0046* | Putative uncharacterized protein | 81/101 (80%) | 92/118 (78%) | 90/118 (76%) | - | - | - |
| LBA1079 | Putative cell surface protein | 76/165 (46%) | 78/167 (47%) | 164/202 (81%) | 89/190 (47%) | - | 89/234 |
| **Extracellular proteins (predicted to be sec-attached)** | | | | | | | |
| LBA1578* | Putative serine protease | 135/438 (31%) | 133/481 (28%) | 130/420 (31%) | - | - | - |
| LBA0169 | SlpA | 323/446 (72%) | 250/454 (59%) | 239/488 (49%) | - | - | 53/131 |
| LBA0858 | Penicillin-binding protein | 265/366 (72%) | 260/369 (70%) | 275/368 (75%) | 77/276 (28%) | 78/313 (25%) | 159/338 |
| LBA1426* | Putative uncharacterized protein | 154/263 (59%) | 153/257 (60%) | 148/260 (57%) | - | - | - |
| LBA1690 | Putative surface exclusion protein | 82/90 (91%) | 230/280 (82%) | 232/282 (82%) | - | 82/310 (26%) | 103/283 |
| LBA1207 | Putative enterolysin A | 117/147 (80%) | 150/211 (71%) | 130/184 (71%) | 63/169 (78%) | 65/170 (38%) | 100/164 |
| LBA1661 | Putative membrane protein | 68/140 (49%) | 127/183 (69%) | 138/182 (76%) | 76/192 (40%) | 80/184 (43%) | - |
| LBA1227* | Putative uncharacterized protein | 99/140 (71%) | 137/182 (75%) | 70/96 (73%) | - | - | 48/157 |
| LBA1225* | Putative uncharacterized protein | 189/294 (64%) | 305/501 (61%) | 195/510 (38%) | - | - | 90/368 |
| **Lipid-anchored proteins** | | | | | | | |
| LBA0197* | OppA - oligopeptide binding protein | 476/541 (88%) | 484/539 (90%) | 490/543 (90%) | - | - | 332/543 |
| LBA1641 | Glycerol-3-phosphate ABC transporter | 374/433 (86%) | 390/433 (90%) | 403/434 (93%) | 310/433 (72%) | 309/433 (71%) | 357/433 |
| LBA1588 | PrsA/PrtM - peptidylprolyl isomerase | 276/300 (92%) | 278/296 (94%) | 284/296 (96%) | 214/296 (72%) | 212/296 (72%) | 167/298 |
| LBA0014 | Putative alkylphosphonate ABC transporter | 278/308 (90%) | 298/311 (96%) | 300/314 (96%) | 218/308 (71%) | 220/308 (71%) | 176/309 |
| LBA0585 | Glycerol-3-phosphate ABC transporter | 383/433 (94%) | 369/434 (85%) | 404/434 (93%) | 250/436 (57%)\ | 291/432 (67%) | 258/433 |
| LBA1497* | Putative uncharacterized protein | 246/338 (73%) | 250/341 (73%) | 253/337 (75%) | - | - | 90/368 |
| **N-terminally anchored proteins (no predicted cleavage site)** | | | | | | | |
| LBA0223* | CdpA - cell separation protein | 213/308 (69%) | 337/602 (56%) | 392/583 (67%) | - | - | 74/232 |
| LBA0805 | Penicillin-binding protein | 629/720 (87%) | 609/720 (85%) | 642/720 (89%) | 449/722 (62%) | 445/720 (62%) | 434/722 |
| LBA1010 | Putative secreted protein | 323/401 (81%) | 328/401 (82%) | 324/401 (81%) | 193/390 (49%) | 185/363 (51%) | 190/405 |
| **Intracellular or moonlighting proteins** | | | | | | | |
| LBA0851 | LysA - diaminopimelate decarboxylase | 353/432 (82%) | 336/432 (78%) | 366/432 (85%) | - | 332/431 (77%) | 282/431 |
| LBA0040 | Putative uncharacterized protein | 74/87 (85%) | - | 82/87 (94%) | 52/85 (61%) | - | 45/86 |
| LBA0297 | RpsC - 30S ribosomal protein | 220/224 (98%) | 221/224 (99%) | 219/224 (98%) | 192/223 (86%) | 193/223 (87%) | 189/224 |
| LBA0698 | Glyceraldehyde 3-P dehydrogenase | 296/338 (88%) | 299/338 (88%) | 298/338 (88%) | 314/338 (93%) | 314/338 (93%) | 317/338 |

For each protein alignment, highest % identity score was presented for the six *Lactobacillus* species listed above.

BLAST analyses were set to display alignments with E-values $\leq 1 \times 10\text{-}6$

| 1-25% | 26-50% | 51-75% | 76-100% |
|---|---|---|---|

*Candidate potential SLAPs

\- = no hits

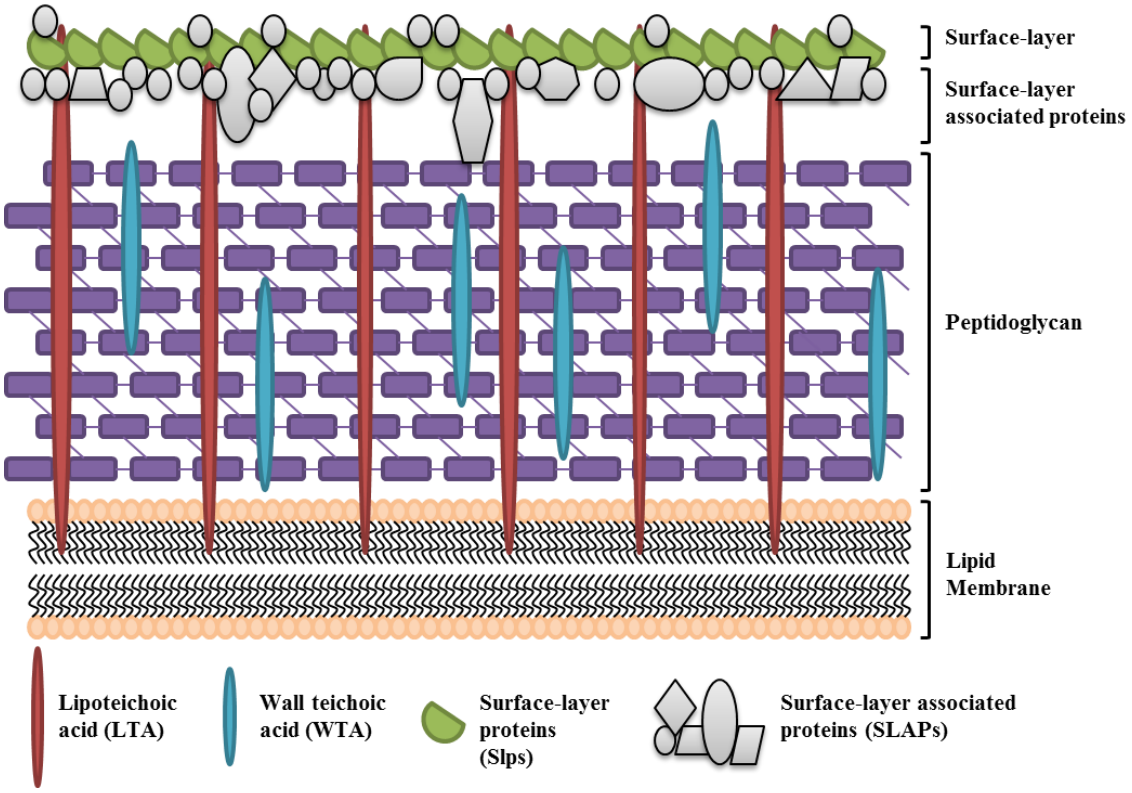


**Figure 2.1.** A proposed schematic for the localization SLAPs in *L. acidophilus* NCFM. The Gram-positive bacterial cell wall is comprised of a thick peptidoglycan (purple), stabilized by teichoic acids and tethered to the lipid membrane by lipoteichoic acid (LTA). The S-layer, composed of self-assembling S-layer proteins (green), is the outermost layer of the cell wall. SLAPs (grey) may be associated with this outermost S-layer.

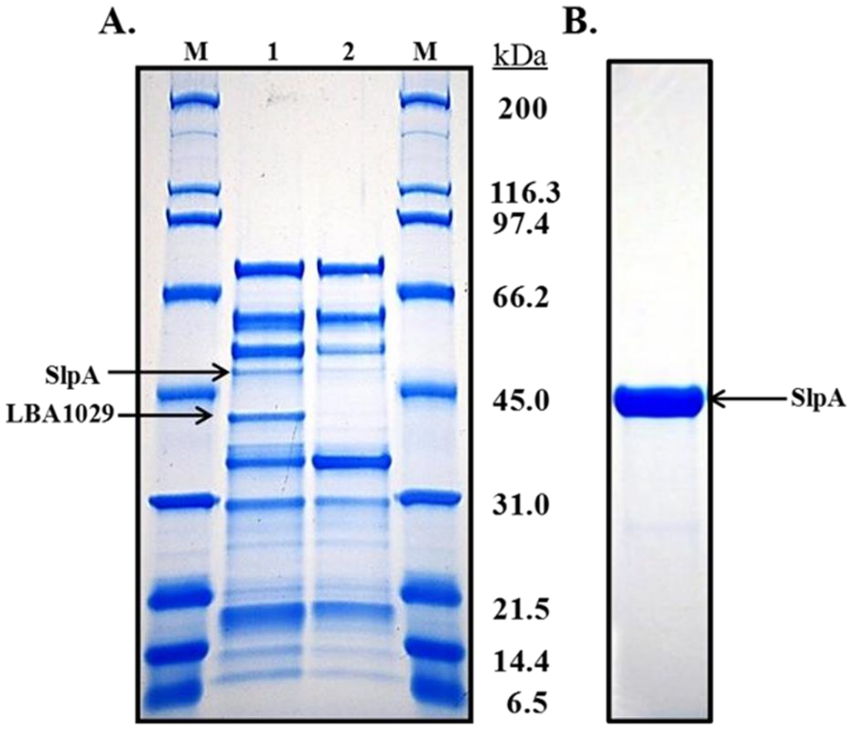


**Figure 2.2.** (A) Putative SLAPs of *L. acidophilus* NCFM (NCK56) and NCK2258. Proteins were extracted using a series of washes in LiCl followed by dialyses in molecular porous membranes at 4ºC. The relative molecular masses (M) are labeled. Lane 1: the SLAPs from *L. acidophilus* NCFM. Lane 2: SLAPs from NCK2258, demonstrating the absence of the 43 kDa LBA1029. (B) Pure SlpA from *L. acidophilus* NCFM. Note the absence of other potential SLAPs/extracellular proteins using the standard protocol.

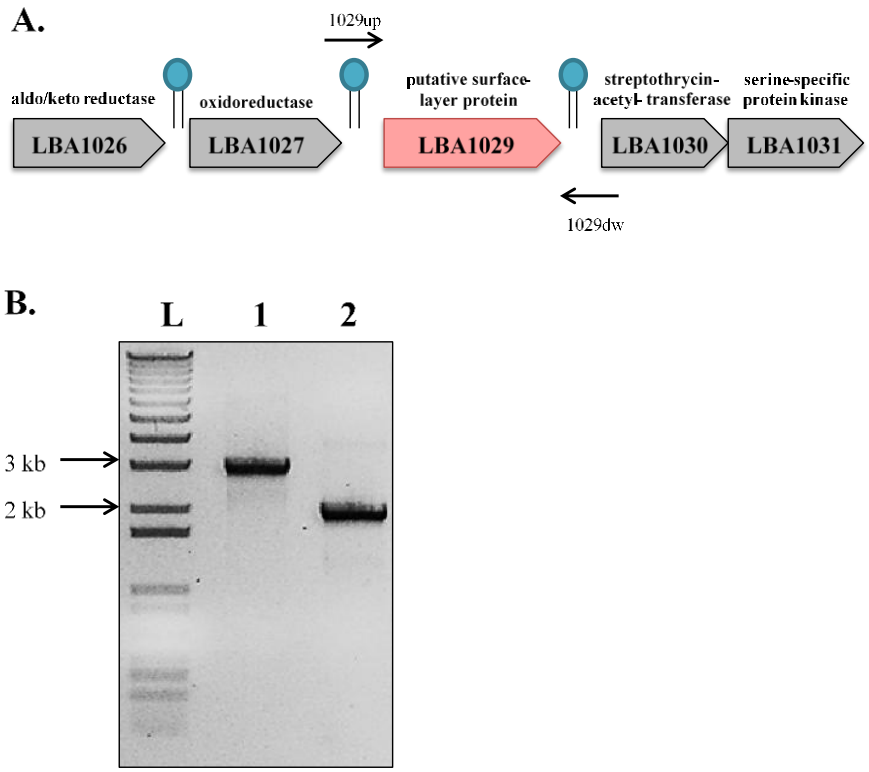


**Figure 2.3.** (A) The *lba-1029* open-reading frame in chromosomal context. It is flanked by genes encoding an aldo/keto reductase, an oxidoreductase, streptothrycin-acetyl transferase, and a serine-specific protein kinase. Note the predicted hairpin Rho-independent terminators flanking *lba-1029*. The forward and reverse primers used to confirm the deletion are indicated. (B) Gel electrophoresis using the forward and reverse primers noted in (A) on WT reference strain (lane 1) compared to NCK2258 (lane 2). The deletion of 1,155 bp from *lba-1029* in the chromosome of *L. acidophilus*, reflected in lane 2, was confirmed by sequencing. Colors were inverted for clarity.

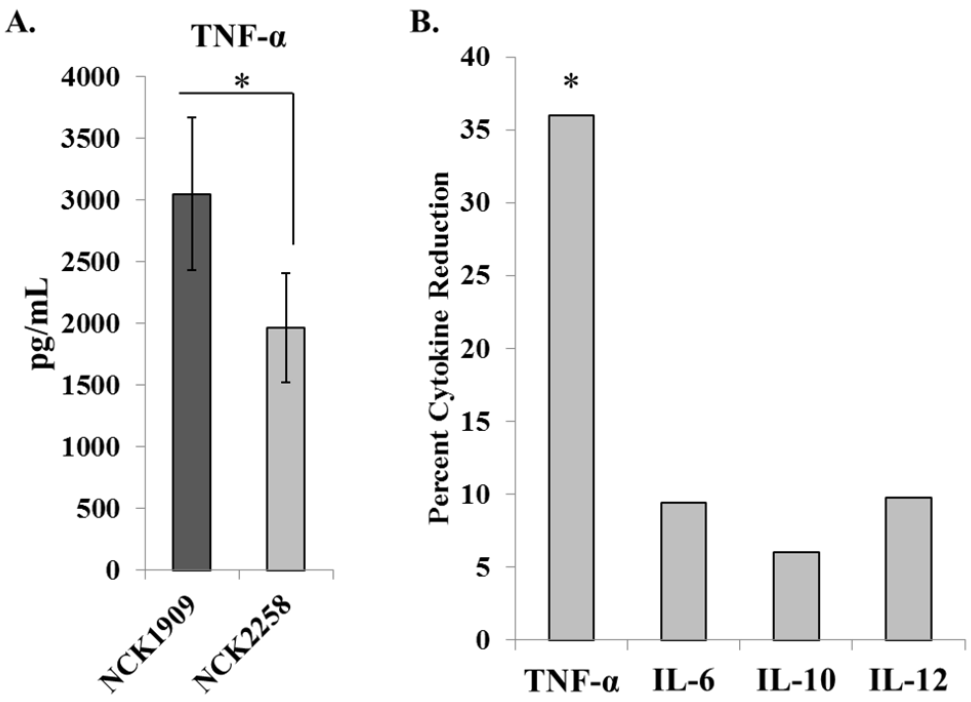


**Figure 2.4.** (A) Induction of TNF-α in murine DC after co-incubation with NCK1909 or NCK2258 as measured by ELISA. Three independent biological replicates were performed with each strain in duplicate, aiming for an approximate bacterial to DC ratio of 10:1. Using univariate ANOVA, the reduction in TNF-α induction between NCK1909 and NCK2258 was significant ($p = 0.006$). Error bars were determined by the standard deviation among the replicates. (B) Percent cytokine reduction of NCK2258 compared to NCK1909 for the cytokines TNF-α, IL-6, IL-10, and IL-12. The induction of IL-6, IL-10, and IL-12 was not significantly different between NCK2258 and NCK1909.

# CHAPTER 3

## Conserved S-layer associated proteins revealed by exoproteomic survey of S-layer forming lactobacilli

Published in 2016: Applied and Environmental Microbiology 82(1): 134-145

## 3.1 Abstract

The *Lactobacillus acidophilus* homology group are Gram-positive species that include *L. acidophilus, L. helveticus, L. crispatus, L. amylovorus, L. gallinarum, L. delbrueckii* subsp. *bulgaricus, L. gasseri*, and *L. johnsonii*. While these bacteria are closely related, they vary in ecological lifestyles ranging from dairy and food fermentations, to allochthonous probiotics, and autochthonous commensals of the host gastrointestinal tract. Bacterial cell surface components play a critical role in the molecular dialogue between bacteria and interaction signaling with the intestinal mucosa. Notably, the *L. acidophilus* complex are distinguished in two clades by the presence or absence of S-layers, which are semi-porous, crystalline arrays of self-assembling, proteinaceous subunits found as the outermost layer of the bacterial cell wall. In this study, S-layer associated proteins (SLAPs) in the exoproteomes of various S-layer forming *Lactobacillus* species were proteomically identified, genomically compared, and transcriptionally analyzed. Four gene regions, encoding six putative SLAPs, were conserved in the S-layer forming *Lactobacillus* species, but not identified in the extracts of the closely related progenitor, *L. delbrueckii* subsp. *bulgaricus*, which does not produce an S-layer. Therefore, the presence or absence of an S-layer has a clear impact on the exoproteomic composition of *Lactobacillus* species. This proteomic complexity and differences of the cell surface properties between S-layer and non-S-layer forming lactobacilli reveal the potential for SLAPs to mediate intimate probiotic interactions and signaling with the host intestinal mucosa.

## 3.2 Introduction

Bacterial cell surface proteins play a critical role in the molecular dialogue between bacteria and their interaction with the host. For beneficial microbes such as probiotics, these proteins mediate health-promoting functionalities through gastrointestinal adhesion, competitive exclusion of pathogens, enhancement of intestinal barrier function, and activation of gut mucosal immunity (1, 2). Probiotics are defined by the FAO/WHO as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (3). Some beneficial actions of these organisms are strain-specific and can be harnessed to treat or reduce the risk of multiple maladies, including: acute infectious diarrhea, irritable bowel syndrome, vaginal infections, ulcerative colitis, lactose maldigestion, and necrotizing enterocolitis (4). In fact, the efficacy of probiotic treatment depends largely on the varying cell surface components, which mediate this specificity (5). Therefore, characterizing effector cell surface ligands and their health-promoting interactions with the host is of increasing scientific and medical interest.

Some of the most prevalent and well-studied probiotics are lactobacilli, many of which are members of the *Lactobacillus acidophilus* homology group (6). The *L. acidophilus* group is a clade of homologous Gram-positive *Lactobacillus* species including *L. acidophilus*, *L. helveticus*, *L. crispatus*, *L. amylovorus*, *L. gallinarum*, *L. delbrueckii* subsp. *bulgaricus*, *L. gasseri*, and *L. johnsonii* (7-11). Although these bacteria are closely related phylogenetically, they have varied ecological lifestyles ranging from dairy and food fermentations, to allochthonous probiotics, and autochthonous commensals of the host gastrointestinal and urogenital tract. Biochemically, they are obligately homofermentative;

sugars are almost exclusively fermented (>85%) to lactate via the Embden-Meyerhof-Parnas pathway. Early taxonomic descriptions were based on the metabolic end products of fermentations, resulting in a seemingly indistinguishable group of microbes which were all called *L. acidophilus* (10). However, DNA-DNA hybridization studies revealed the heterogeneity in the group (11, 12). Since then, genome sequencing and comparative genomic analyses have clearly established and solidified the current description of the *L. acidophilus* group (13, 14). Notably, these closely related strains can be dichotomized based on their ability to create Surface (S-) layer protein arrays as the outermost constituent of the cell wall (15).

Bacterial S-layers are semi-porous, proteinaceous crystalline arrays comprised of self-assembling (glyco)protein subunits called S-layer proteins (SLPs; 15). They can be found in both Gram-positive and Gram-negative bacteria, as well as species of *Archaea*, but S-layers are not ubiquitous in all microorganisms. When present, S-layers form two-dimensional lattices on the outermost layer of the cell which are tethered through non-covalent interactions with the cell wall (15). S-layers from various species of the *L. acidophilus* homology group have been characterized for their roles in intestinal adhesion, competitive exclusion of pathogens, and immunomodulation of gastrointestinal mucosa. *In vitro* studies using intestinal epithelial cell lines suggest the S-layer as a major factor in intestinal adhesion for *L. acidophilus* (16, 17), *L. crispatus* (18 - 20), *L. helveticus* (21), and *L. amylovorus* (22). In fact, this adhesion has been shown to competitively exclude enteropathogenic bacteria by both *L. crispatus* (23) and *L. helveticus* (24, 25). Compelling studies have begun to reveal mechanisms of gastrointestinal immunomodulation. For example, SlpA, the primary

constituent of the S-layer in *L. acidophilus* NCFM, was found to bind to dendritic cell (DC) orthologous C-type lectin receptors (CLR), DC-specific ICAM-3-grabbing non-integrin (DC-SIGN; 26) and a specific intracellular adhesion molecule-3 grabbing non-integrin homolog-related 3 (SIGNR-3; 27). This SlpA-CLR interaction exerts regulatory signals which have been reported to mitigate inflammatory disease states and promote maintenance of healthy intestinal barrier function (27). Similar experiments are elucidating the roles of the S-layer in modulation of gastrointestinal immunity for *L. crispatus* (28), *L. helveticus* (29), and *L. amylovorus* (22).

The S-layer forming species of the *L. acidophilus* homology group form S-layers comprised of a dominant protein constituent, SlpA/Slp1 (~46 kDa), and minor constituents SlpB/Slp2 (~47 kDa) and SlpX (~51 kDa; 30). Recent evidence, however, suggests that the S-layer may not be as monomorphic as previously proposed. In *L. acidophilus* NCFM, proteomic analysis revealed the presence of 37 non-covalently bound extracellular S-layer associated proteins (SLAPs), 23 of which are putative/uncharacterized proteins of unknown function (31). In this study, the non-covalent exoproteomes of various S-layer and non-S-layer forming *Lactobacillus* strains were proteomically identified, genomically compared, and transcriptionally analyzed. These data reveal both the conservation and variability of SLAPs across lactobacilli and their potential to mediate intimate interactions with the intestinal mucosa.

## 3.3 Materials and Methods

### 3.3.1 Bacterial strains and growth conditions

Bacterial strains used in this study are reported in Table 1. *Lactobacillus* strains were propagated in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Inc., Detroit, MI) statically at 37°C under ambient atmospheric conditions.

### 3.3.2 Diversilab® analysis of strains

*L. crispatus* and *L. helveticus* strains were typed using the rep-PCR-based Diversilab® typing system (bioMérieux, Durham, NC). DNA from the *Lactobacillus* strains was extracted using the MoBio UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA) and quantified using a Nanodrop 1000 spectrophotometer (Thermo-Scientific, Waltham, MA). The DNA was then normalized to 20 ng $\mu l^{-1}$ with UltraPure distilled water (Invitrogen, Carlsbad, CA). Rep-PCR was performed in preparation for typing using the *Lactobacillus* Diversilab Kit (bioMérieux, Durham, NC). DNA amplification was performed in a BioRad MyCycler thermal cycler (BioRad, Hercules, CA), programmed for 2 min at 94°C (initial denaturation) and 35 cycles of 30 sec at 94°C (denaturation), 30 sec at 55°C (annealing), 90 sec at 70°C (extension), followed by a final extension cycle of 3 min at 70°C using Amplitaq® DNA polymerase from Applied Biosystems (Carlsbad, CA). The reaction was pipetted into the DiversiLab system chip along with the Diversilab DNA reagents and supplies (bioMérieux) according to the manufacturer's protocol. The chip samples were analyzed using the Diversilab software version 3.4 and similarity of strains was determined by comparing the resulting electropherogram/barcodes.

### 3.3.3 Extraction of extracellular, non-covalently bound cell surface proteins

Non-covalently bound cell surface proteins, including S-layer proteins and S-layer associated proteins were extracted from the *Lactobacillus* strains using LiCl denaturing salt, as described previously (31). Briefly, cells were grown in 200 ml MRS to stationary phase (16 h), centrifuged at 2,236 × *g* for 10 min (4 °C), and washed twice with 25 ml of cold PBS (Gibco), pH 7.4. Cells were agitated for 15 min at 4 °C following the addition of 5 M LiCl (Fisher Scientific). Supernatants, containing SLPs and SLAPs, were harvested via centrifugation at 8,994 × *g* for 10 min (4°C) and transferred to a 6,000–8,000 kDa Spectra/Por molecular porous membrane (Spectrum Laboratories) and dialyzed against cold distilled water for 24 h. The precipitate was harvested at 20,000 × *g* for 30 min and agitated for a second time with 1 M LiCl at 4 °C for 15 min to disassociate the SLAPs from the SLPs. The suspension was then centrifuged at 20,000 × *g* for 10 min and the SLAP supernatants were separated from the SLP pellet and transferred to the 6,000–8,000 kDa Spectra/Por molecular porous membrane and dialyzed against cold distilled water for 24 h. Finally, the precipitate was harvested via centrifugation at 20,000 × *g* for 30 min to pellet the SLAPs. Both SLP and SLAP pellets were resuspended in 10% (w/v) SDS (Fisher). Proteins were quantified via bicinchoninic acid assay kit (Thermo Scientific) and visualized via SDS-PAGE using precast 4–20% Precise Tris-HEPES protein gels (Thermo Scientific). The gels were stained using AcquaStain (Bulldog Bio) according to the instructions of the manufacturer. SLAP extractions were performed with two biological replicates for each strain and visualized through SDS-PAGE to confirm that the resultant banding patterns were reproducible.

### 3.3.4 Proteomic identification and analysis

SLAPs extracted from the various *Lactobacillus* species were identified using LC-MS/MS from the Genome Center Proteomics Core at the University of California, Davis, as described previously (31). Proteomic screenings were performed once per strain and used as a tool for selecting candidate SLAPs within each strain. Tandem mass spectra were extracted and charge state deconvoluted using MM File Conversion version 3. All MS/MS samples were analyzed using X! Tandem (The GPM, www.thegpm.org/; version TORNADO). UniProt searches were performed using proteome databases for the respective proteins isolated from *L. acidophilus* NCFM, *L. helveticus* CNRZ32, *L. crispatus* ST1, and *L. amylovorus* GRL1112. X! Tandem was searched with a fragment ion mass tolerance of 20 ppm and a parent ion tolerance of 20 ppm. The iodoacetamide derivative of cysteine was specified in X! Tandem as a fixed modification. Deamination of asparagine and glutamine, oxidation of methionine and tryptophan, sulfone of methionine, tryptophan oxidation to formylkynurenin of tryptophan and acetylation of the N terminus were specified in X! Tandem as variable modifications. Scaffold (version Scaffold_3.6.1, Proteome Software) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. X! Tandem identifications required scores of greater than 1.2 with a mass accuracy of 5 ppm. Protein identifications were accepted if they contained at least two identified peptides. Using the parameters above, the false discovery rate was calculated to be 1.1 % on the protein level and 0 % on the peptide level. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. For this study,

only proteins with unique spectral counts of greater than 20 were considered significant. For all analyses, total spectral counts were utilized as a semi-quantitative indicator of protein abundance (32). Two-way clustering of total spectral counts was performed using JMP Genomics (version 5, SAS). Protein domains were identified for analysis using the Pfam protein family database (33).

### 3.3.5 Genomic *in silico* analyses

Genomic analysis was performed on genomes curated from the genome library of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/genome/) including: *L. acidophilus* NCFM (NC_006814.3), *L. helveticus* CNRZ32 (NC_021744.1), *L. amylovorus* GRL1112 (NC_014724.1), *L. crispatus* ST1 (NC_014106.1), *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (NC_008054.1), and *L. casei* ATCC 334 (NC_008526.1). Identified genes were compared using the BLASTn and BLASTp features of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). SignalP 4.1 was used to predict the signal peptidase cleavage site of each identified protein (34). Genomes were uploaded to Geneious 8.0.5 (35) for comparative genomic and promoter analyses of identified SLAP genes. The genetic context of SLAP genes were examined using the chromosomal graphical interface in Geneious 8.0.5. *In silico* promoter elements were identified in the upstream intergenic regions of SLAP genes using PromoterWise (http://www.ebi.ac.uk/Tools/psa/promoterwise/). To identify conserved promoter elements between the various SLAP genes, genome-wide sequence motifs of the putative -10 and -35 regions were scanned against the four S-layer

forming genomes using Genious 8.0.5, with a variable spacer length of 16-23 nucleotides between the -10 and -35 regions.

### 3.3.6 RNA extraction, sequencing, and transcriptional analysis

Cells were grown to mid-log phase (8 hours) and flash frozen for RNA extraction and sequencing. RNA was extracted using the Zymo Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA) and analyzed for quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Library prep and RNA-sequencing were performed at the High-Throughput Sequencing and Genotyping Unit of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. For each sample, ribosomal RNA was removed with the Ribozero Bacteria Kit (Illumina, San Diego, CA) followed by library preparation with the TruSeq Stranded RNA Sample Prep Kit (Illumina, San Diego, CA). Single-read RNA-sequencing was performed using an Illumina HiSeq 2500 Ultra-High-Throughput Sequencing system (Illumina, San Diego, CA) with a read length of 180 nucleotides. Raw sequencing reads were quality assessed using FastQC Version 0.11.3 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and processed using Geneious 8.0.5 (35). Briefly, after adaptor sequences were trimmed, raw reads were quality trimmed to remove sequence reads with an error probability limit of 0.001 (Phred score of 30) and filtered to remove reads shorter than 20 nt. These quality trimmed and filtered sequences were then mapped to the reference genomes of the S-layer forming *Lactobacillus* using Bowtie 2 (36) with default settings within Geneious 8.0.5 (35). Sequencing depths were calculated to be 767, 730, 727, and 665 x coverage for NCK 56, NCK 776, NCK 777, and

NCK 938, respectively. Transcriptional analyses were based on the normalized transcripts per million (TPM) calculation within Geneious 8.0.5 (35).

## 3.4 Results

### 3.4.1 Proteomic identification of non-covalently bound extracellular proteins in S-layer and non-S-layer forming lactobacilli

Based on the previous identification of S-layer associated proteins (SLAPs) in *L. acidophilus* NCFM (31), we performed exoproteome screenings on multiple S-layer and non-S-layer forming strains of *Lactobacillus*. Thus, five S-layer and five non-S-layer forming *Lactobacillus* species were analyzed (Figure 3.1). Seventeen strains were tested in total, comprising twelve S-layer and five non-S-layer producing lactobacilli (Table 1). Notably, 15 of the strains are members of the closely related *L. acidophilus* homology group.

Electrophoresis of SLAP extractions revealed a surprisingly diverse array of protein banding patterns in the S-layer forming species, and a notable absence of proteins in the non-S-layer forming species (Figure 3.2). SLAP extractions were performed on two biological replicates and the SDS-PAGE banding patterns of the SLAPs extracted from each strain did not differ in the major banding patterns between replicates. Further, the LiCl extract of *L. acidophilus* demonstrated a similar banding profile to the SLAPs identified previously (28; Figure 3.2, lane 1). Proteins from the other S-layer forming strains, including *L. crispatus*, *L. amylovorus*, *L. gallinarum*, and *L. helveticus* were not only distinct from *L. acidophilus*, but also from one another. Moreover, there was also heterogeneity in the protein banding between varying strains within each species. In the five *L. helveticus* strains, there were distinctive differences between the various dairy isolates NCK936, NCK338, NCK230, NCK246, and NCK1088 (Figure 3.2A, lanes 2, and 6-9). The three *L. crispatus* strains were also discrete from one another (Figure 3.2A, lanes 3, 10, & 11). Rep-PCR-based Diversilab®

strain typing was performed on the five *L. helveticus* and three *L. crispatus* strains to examine genomic similaries (Figure 3.2B & C). The five *L. helveticus* strains clustered into two groups with >93% and >98% similarity, respectively (Figure 3.2B), and the *L. crispatus* strains were >85% similar (Figure 3.2C). Remarkably, the *L. helveticus* strains, NCK338 and NCK230, and NCK1088 and NCK936 were distinctly varied in terms of the identified extracellular proteins (Figure 3.2A), despite a >98% and > 95% similarity between the Rep-PCR typing patterns, respectively (Figure 3.2B). A similar trend was observed among the *L. crispatus* strains. Thus, there was no correlation between the genotype-clustering and the exoproteome profiles revealed by SDS-PAGE.

There were very few proteins isolated from the non-S-layer forming species of *Lactobacillus*, as observed in the gel lanes of the SDS-PAGE (Figure 3.2, lanes 13-17). *L. johnsonii* and *L. gasseri* of the *L. acidophilus* homology group exhibited no discernable proteins in the gel lanes (Figure 3.2, lanes 15 & 17). *L. delbrueckii* subsp. *bulgaricus*, the non-S-layer producing strain which is the most closely related and progenitor to the other S-layer forming members of the *L. acidophilus* homology group (Figure 3.1), showed only a small number of proteins isolated from the LiCl extract (Figure 3.2, lane 13). Distantly related *L. casei*, devoid of any S-layer, exhibited few proteins, as well (Figure 3.2, lane 14). To identify the electrophoresed proteins, lanes with visible proteins in the gel were sent for proteomic analysis (Table 1, bolded and underlined).

Of the twelve S-layer forming strains, seven were selected for proteomic identification including three *L. helveticus* strains, three *L. crispatus* strains, and one *L. amylovorus* (Table 1, underlined). Notably, *L. gallinarum* was not selected for analysis, as

there are no publically available genomes or proteomes published for this species. From the five non-S-layer forming species tested, only *L. delbrueckii* subsp. *bulgaricus* and *L. casei* were selected from proteomic screening, as they were the only non-S-layer forming species in which proteins were isolated from the SLAP extraction (Table 1, underlined). Proteins were identified from the LiCl extracts of the seven S-layer and two non-S-layer forming *Lactobacillus* species using liquid chromatography-tandem mass spectrometry. Two-way clustering was performed based on the total spectral counts of identified proteins, and visualized using a two-way clustering heat map (Figure 3.3A). The proteins identified in the two non-S-layer strains, *L. casei* and *L. delbrueckii* subsp. *bulgaricus*, are unambiguously distinct from the other seven S-layer-forming strains. Furthermore, almost all of the proteins identified in the non-S-layer strains were predicted intracellular proteins, likely presented extracellularly as the result of cell death occurring at stationary phase. With regard to the S-layer forming *Lactobacillus* species, there were three main groupings of proteins identified: SLAPs specific to *L. crispatus* (Figure 3.3B), SLAPs specific to *L. amylovorus* (Figure 3.3C), and SLAPs specific to *L. helveticus* (Figure 3.3D). Surprisingly, though each group had distinctive homologies, the same types of proteins were observed in each group. In fact, these proteins, including multiple putative uncharacterized proteins, cell surface proteases, and group 3 bacterial Ig-like domain proteins, were the same types of proteins identified as SLAPs in *L. acidophilus* NCFM. Notably, these putative SLAPs were not found in the non-S-layer producing strains analyzed, *L. casei* and *L. delbrueckii* subsp. *bulgaricus*.

### 3.4.2 Functional exoproteomic analysis of S-layer and non-S-layer forming lactobacilli

After proteomic identification, selected putative SLAPs and non-covalently bound extracellular proteins were functionally analyzed based on predicted protein domains. Four predominant protein domains were found consistently in the S-layer forming species tested (Figure 3.4A) including SLAP (PF03217), Big_3 (PF07523), SH3_8 (PF13457), and fn3 (PF00041). We propose that the SLAP (PF03217) domain, responsible for the non-covalent attachment of SLP and other extracellular proteins in lactobacilli, be re-designated as Non-Covalent Attachment Domains (NCAD). This domain designation prevents confusion with the abbreviation for S-layer associated proteins, SLAPs. Notably, the NCAD was the most abundant protein domain identified in the extracellular fractions tested (Figure 3.4A). Other domains associated with bacterial extracellular proteins, including Group 3 bacterial Ig-like domains (Big_3), SH3-like domains (SH3_8), and fibronectin type III domains (fn3) were found in the proteomic analysis of the S-layer forming species, while absent from the non-S-layer forming species (Figure 3.4A). Notably, only two NCAD domain-containing proteins were identified within the exoproteome of *L. delbrueckii* subsp. *bulgaricus*, while none of these domains were identified in the exoproteome of the non-S-layer forming *L. casei*.

Identified proteins were functionally categorized based on putative domains and placed into one of six groupings: extracellular fn3 domain proteins and extracellular BIg3/SH3_8 proteins (Figure 3.4B); putatively annotated SLPs and uncharacterized extracellular proteins (Figure 3.4C); and intracellular proteins and ribosomal proteins (Figure 4D). The distribution of the proteins within these functional groupings was plotted for each of the strains using the semi-quantitative total spectral counts identified through the LC-

MS/MS survey (Figure 3.4B-D). Group 3 bacterial Ig-like domain proteins, which contain the Big_3 and SH3_8 domains, were only found in the SLAP fractions of the S-layer forming lactobacilli (Figure 3.4B). Similarly, uncharacterized proteins putatively annotated as SLPs, as well as fibronectin binding proteins, were found solely in the S-layer forming species of *Lactobacillus* (Figure 3.4B, C). There was an increase in both the occurrence and abundance of NCAD domain-containing uncharacterized extracellular proteins in the SLAP fractions from the S-layer strains, compared to the non-S-layer strains (Figure 3.4C). Furthermore, there was an increase in the presence of intracellular proteins, including ribosomal proteins, in the non-S-layer strains (Figure 3.4D), as measured by total spectral counts. These data reveal a pattern of non-covalently bound proteins identified in S-layer species of *Lactobacillus*, when compared to non-S-layer forming lactobacilli.

### 3.4.3 Genomic characterization of genes corresponding to the extracellular S-layer associated proteins

The putative SLAPs identified in this study, along with the previously identified SLAPs of *L. acidophilus* NCFM, were curated to the genomes of *L. acidophilus* NCFM, *L. helveticus* CNRZ32, *L. amylovorus* GRL 1112, and *L. crispatus* ST1, respectively. By visualizing the corresponding genes on the four genomes, four conserved genetic regions comprising six genes were consistently observed (Figure 3.5). Two cell division related genes, including an *N*-acetylmuramidase and autolysin, are found in Region I (blue). Region II (yellow) is comprised of genes encoding fn3 domain-containing fibronectin binding proteins. Region III (green) also contains two cell division related genes, including the cell

division protein A (*cdpA*; 33). Finally, Region IV (red) includes genes encoding group 3 bacterial Ig-like proteins, which contain the domains Big_3 and SH3_8. The relative position of the four gene regions were conserved among the four genomes, with the exception of region II and III in *L. helveticus*, which were translocated to the minus strand leading away from the origin of replication (Figure 3.5).

In addition, the genetic context of each region was examined within the four strains. Notably, there was synteny observed between the four chromosomal regions of each organism (Figure 3.6). Although region I was the least syntenic overall, it is noteworthy that the *N*-acetylmuramidase and autolysin/amidase genes were positioned directly downstream of the genes encoding the primary S-layer protein, *SlpA*/*Slp1*. Conversely, region II exhibited increased conservation of genetic loci near the SLAP gene encoding a fibronectin binding protein, including genes for a high molecular weight glucan modifying protein, a tyrosine-tRNA synthetase, and an oligopeptide utilization gene cluster. Region III was also syntenic surrounding the putative SLAP genes, with genes encoding the *pur* operon repressor *purR* and the cell division gene, *glmU*. Lastly, region IV containing the gene encoding the putative SLAP with a group 3 bacterial Ig-like domain, was directly downstream of the endopeptidase, *clpP* and upstream of the glycolysis genes *gapA* and *pgk*.

### 3.4.4 RNA-sequencing and transcriptional analysis of the S-layer forming lactobacillus species

Whole transcriptome profiling through deep RNA sequencing (RNA-seq) was employed to examine the global expression of the putative SLAP gene regions in *L.*

*acidophilus*, *L. helveticus*, *L. crispatus*, and *L. amylovorus*. While expression was similar between the four strains in each gene region (Figure 3.7, bar graphs), the gene regions were themselves expressed at different levels (Figure 3.7, line graphs). Both regions I and II had expression between 100 and 500 TPM, while regions III and IV had expression above 1000 TPM (Figure 3.7). These data also confirmed the monocistronic expression of region IV and the predicted polycistronic expression of the *N*-acetylmuramidase and autolysin of region I. Conversely, the cell division genes in region III appeared to be monocistronically expressed. Surprisingly, the gene encoding a fibronectin-binding protein of region II was found to be polycistronically expressed along with a tyrosyl-tRNA synthetase, *tyrS*. Finally, *in silico* promoter identification and analysis suggested that the *N*-acetylmuramidase gene and the group 3 bacterial Ig-like domain gene were under the constitutive transcriptional control of a putative σ70 (rpoD)-like promoter with a TANAAT -10 region consensus motif and a NTGTNT -35 region consensus motif (Figure 3.8). This promoter was found upstream of numerous housekeeping genes, including *ftsA*, *ldhD*, *secA*, and *eno* (Figure 3.8).

### 3.5 Discussion

Previous work has shown that the S-layers are more complex than previously understood. SLAPs were first identified in *L. acidophilus* NCFM and were hypothesized to scaffold to the cell wall with the S-layer (31). Additionally, a recent proteomic cell-shaving study in the S-layer forming food bacterium *Propionibacterium freudenreichii* characterized varied cell surface proteins, including putative SLAPs, for their anti-inflammatory immunomodulatory capacity (37). In the present study, we demonstrate that the presence or absence of an S-layer has a clear and direct impact on the exoproteomic composition of *Lactobacillus* species (Figure 3.2). In S-layer forming species of the *L. acidophilus* homology group, numerous non-covalently bound proteins were identified which may be associated with the S-layer. In contrast, the few proteins which were isolated with LiCl treatment in the non-S-layer forming strains were mostly intracellular proteins. These observations substantiate the aforementioned studies, lending credence to the existence of SLAPs as an integral component of the complex S-layer.

There were four protein domains found consistently within the putative SLAPs: BIg_3 (PF07523), SH3_8 (PF13457), fn3 (PF00041), and NCAD (PF03217). NCAD domains are predicted to be responsible for the non-covalent attachment of S-layer proteins to the cell wall in *Lactobacillus* species (38). Notably, there are extracellular proteins within the annotated proteome of *L. delbrueckii* subsp *bulgaricus* which contain the NCAD domain. Similarly, the fn3 domain, an Ig-fold domain found in fibronectin binding proteins, was also within the predicted proteomes of the non-S-layer species *L. gasseri* and *L. johnsonii*. In both of these examples, the domains were ubiquitously identified in the non-covalently bound

extroproteome fractions of the S-layer forming strains, but were not apparent in the exoproteomes extracted from the non-S-layer forming strains. These observations suggest that the S-layer may be an important scaffold for extracellular proteins with NCAD domains.

From the numerous putative SLAPs, six were found to be conserved amongst the four S-layer forming strains, *L. acidophilus*, *L. crispatus*, *L. amylovorus*, and *L. helveticus* into four genomic regions. These four genomic regions include genes encoding cell division protein CdpA, an *N*-acetylmuramidase, an uncharacterized fibronectin binding protein, and an uncharacterized group 3 bacterial Ig-like domain protein. The cell division protein CdpA was first functionally described in *L. acidophilus* NCFM (39). Specifically, phenotypic analysis of a *cdpA*-knockout strain revealed a strain with increased chain length, aberrant cell morphology, decreased resistance to environmental stressors, and decreased adhesion to Caco-2 epithelial cells (39). The direct mechanism(s) regarding the function of CdpA and the aforementioned phenotypes was unclear, but thought to be a pleiotropic response to the modified cell wall structure. Notably, the results of the current study offer further insight into this mechanism. First, the protein has two of the NCAD domains, suggesting a localization to the cell wall along with the S-layer. Second, CdpA is one of the most prevalent SLAPs in the S-layer forming strains, but is not found in any non-S-layer forming *Lactobacillus* species. It is possible that CdpA may be a structural intermediary between the cell wall and the S-layer and other SLAPs during cell division. There is evidence for this through the original study in which the *cdpA*-deficient strain was treated with guanidine HCl, and the extracted extracellular SLAPs and Slp were reduced when compared to the parent strain (39). These

observations indicate that CdpA may be an important component of S-layer structure and function.

The conserved SLAP gene regions were organized into four regions which demonstrated remarkable conservation in genome position within the overall chromosome architectures (Figure 3.5). Strand location of genes on the bacterial chromosome is an important factor for codon usage, which correlates with gene expression (40 - 42). Moreover, genes of low G+C content Gram-positive bacteria illustrate a strand bias for the positive and negative leading strands diverging from the origin of replication (43, 44). The conserved SLAP genes reflect this bias, as they were all found on the leading strands of the positive and negative strands of the chromosomes (Figure 3.5).

Transcription of these genes, as measured by RNA sequencing, was similar among the four strains, albeit their rates of transcription were not uniform throughout all four gene regions (Figure 3.7). In fact, the genes encoding the *N*-acetylmuramidase and group 3 bacterial Ig-like domain protein appeared to be under the control of a putative σ70 (rpoD)-like promoter. The -10 region followed the TANAAT consensus described by Pribnow (45) while the -35 region followed a NTGTNT consensus. These motifs are similar to the σ70-like promoters of housekeeping genes identified in *L. plantarum* (46). Housekeeping genes such as *ftsA*, *ldhD*, *secA*, and *eno* were identified as genes under similar transcriptional control.

Taken together, the genomic architecture and transcription data suggest the conserved SLAPs found in the S-layer forming strains of *Lactobacillus* are housekeeping genes expressed at constitutive levels. Given their conservation, we conclude that they likely participate in various essential cell processes such as cell wall hydrolysis, maintenance of cell

shape, protein turnover, and cell adhesion. It is notable that genes encoding SLAPs with rudimentary function, such as *cdpA* and the *N*-acetylmuramidase, are absent in non-S-layer forming strains. There also remain the two uncharacterized proteins, the fibronectin-binding protein and the group 3 bacterial Ig-like domain proteins which have yet to be functionally characterized, and which are functionally associated with S-layer forming strains.

Given the extracellular localization of these proteins, the SLAPs identified in this study may have unexplored, potentially important roles in probiotic-host interactions and signaling. Among the conserved SLAPs explored, both the fibronectin-binding protein and the group 3 bacterial Ig-like domain protein have Ig-like folds within their respective amino acid tertiary structures which may be involved in cell-to-cell adhesion or cell-to-host adhesion. Furthermore, all of these proteins, regardless of their cellular function, are accessible for intimate interactions with the gut epithelium and mucosal immune system (31, 37). In this study, all proteomic and genomic comparisons made for *L. helveticus*, *L. crispatus*, and *L. amylvorus*, were made with only one respective genome for each species (*L. helveticus* CNRZ32, *L. amylovorus* GRL1112, *L. crispatus* ST1). A more complete picture could be made if the genomes of each strain tested were utilized as a proteomic and genomic reference.

Despite being prevalent among all bacterial types, there remains little known about the evolutionary function of S-layers. Here, we present the S-layer as a scaffold for numerous non-covalently attached secreted proteins. These S-layer associated proteins are conserved among S-layer forming species and absent in non-S-layer forming species. It is unambiguously clear that the non-covalent exoproteomes of the S-layer forming strains are

more diverse and dynamic than those of the non-S-layer forming strains. Understanding these exoproteins opens new avenues for the functional characterization of the S-layer, as well as the health-promoting mechanisms of probiotic-host signaling and cross-talk.

## 3.6 Acknowledgements

This work was supported through funding from the North Carolina Agriculture Foundation (Raleigh, NC) and DuPont Nutrition & Health (Madison, WI).

We thank Dr. Sarah O'Flaherty and Dr. Yong Jun Goh for insightful discussion and critical reading of the manuscript. We acknowledge Dr. Brett Phiney and Michelle Salemi at the University of California, Davis Proteomics Core Facility for LC-MS/MS services and Dr. Alvaro G. Hernandez at the University of Illinois at Urbana-Champaign Roy J Carver Biotechnology center for RNA-sequencing services.

## 3.7 References


1. **Lebeer S, Vanderleyden J, De Keersmaecker SCJ**. 2010. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol **8**:171–184.

2. **Johnson BR, Klaenhammer TR**. 2014. Impact of genomics on the field of probiotic research: historical perspectives to modern paradigms. Antonie Van Leeuwenhoek **106**:141–156.

3. **Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME**. 2014. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol **11**:506–514.

4. **Sanders ME, Guarner F, Guerrant R, Holt PR, Quigley EMM, Sartor RB, Sherman PM, Mayer EA**. 2013. An update on the use and investigation of probiotics in health and disease. Gut **62**:787–796.

5. **Bron PA, Tomita S, Mercenier A, Kleerebezem M**. 2013. Cell surface-associated compounds of probiotic lactobacilli sustain the strain-specificity dogma. Curr Opin Microbiol **16**:262–269.

6. **Klein G, Pack A, Bonaparte C, Reuter G**. 1998. Taxonomy and physiology of probiotic lactic acid bacteria. International Journal of Food Microbiology **41**:103–125.

7. **Schleifer KH, Ludwig W**. 1995. Phylogeny of the Genus *Lactobacillus* and Related Genera. Systematic and Applied Microbiology **18**:461–467.

8. **Hammes WP, Hertel C**. 2003. The genera *Lactobacillus* and *Carnobacterium*. In: The prokaryotes: an evolving electronic resource for the microbiological community, 3rd Addition. Springer-Verlag.

9. **Dellaglio F, Felis GE**. 2005. Taxonomy of *Lactobacilli* and *Bifidobacteria*. In: Probiotics and Prebiotics: Scientific Aspects, pp. 25–50. Edited by G.W. Tannock, Caister Academic Press.

10. **Hammes WP, Vogel RF**. 1995. The genus *Lactobacillus*, p. 19–54. *In* Wood, BJB, Holzapfel, WH (eds.), The Genera of Lactic Acid Bacteria. Springer US.

11. **Kandler O, Stetter K-O, Köhl R**. 1980. *Lactobacillus reuteri* sp. nov., a New Species of Heterofermentative Lactobacilli. Zentralblatt für Bakteriologie: I Abt Originale C:

Allgemeine, angewandte und ökologische Mikrobiologie **1**:264–269.

12. **Johnson JL, Phelps CF, Cummins CS, London J, Gasser F**. 1980. Taxonomy of the *Lactobacillus acidophilus* Group. Int J Syst Bacteriol **30**:53–68.

13. **Felis GE, Dellaglio F**. 2007. Taxonomy of *Lactobacilli* and *Bifidobacteria*. Curr Issues Intest Microbiol **8**:44–61.

14. **Berger B, Pridmore RD, Barretto C, Delmas-Julien F, Schreiber K, Arigoni F, Brüssow H**. 2007. Similarity and differences in the *Lactobacillus acidophilus* group identified by polyphasic analysis and comparative genomics. J Bacteriol **189**:1311–1321.

15. **Hynönen U, Palva A**. 2013. *Lactobacillus* surface layer proteins: structure, function and applications. Appl Microbiol Biotechnol **97**:5225–5243.

16. **Buck BL, Altermann E, Svingerud T, Klaenhammer TR**. 2005. Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol **71**:8344–8351.

17. **Frece J, Kos B, Svetec IK, Zgaga Z, Mrsa V, Susković J**. 2005. Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. J Appl Microbiol **98**:285–292.

18. **Sillanpää J, Martínez B, Antikainen J, Toba T, Kalkkinen N, Tankka S, Lounatmaa K, Keränen J, Höök M, Westerlund-Wikström B, Pouwels PH, Korhonen TK**. 2000. Characterization of the collagen-binding S-layer protein CbsA of *Lactobacillus crispatus*. J Bacteriol **182**:6440–6450.

19. **Antikainen J, Anton L, Sillanpää J, Korhonen TK**. 2002. Domains in the S-layer protein CbsA of *Lactobacillus crispatus* involved in adherence to collagens, laminin and lipoteichoic acids and in self-assembly. Mol Microbiol **46**:381–394.

20. **Sun Z, Kong J, Hu S, Kong W, Lu W, Liu W**. 2013. Characterization of a S-layer protein from *Lactobacillus crispatus* K313 and the domains responsible for binding to cell wall and adherence to collagen. Appl Microbiol Biotechnol **97**:1941–1952.

21. **Beganović J, Frece J, Kos B, Leboš Pavunc A, Habjanič K, Sušković J**. 2011. Functionality of the S-layer protein from the probiotic strain *Lactobacillus helveticus* M92. Antonie Van Leeuwenhoek **100**:43–53.

22. **Hynönen U, Kant R, Lähteinen T, Pietilä TE, Beganović J, Smidt H, Uroić K, Avall-Jääskeläinen S, Palva A**. 2014. Functional characterization of probiotic surface

layer protein-carrying *Lactobacillus amylovorus* strains. BMC Microbiol **14**:199.

23. **Chen X, Xu J, Shuai J, Chen J, Zhang Z, Fang W**. 2007. The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* O157:H7 and *Salmonella typhimurium*. Int J Food Microbiol **115**:307–312.

24. **Johnson-Henry KC, Hagen KE, Gordonpour M, Tompkins TA, Sherman PM**. 2007. Surface-layer protein extracts from *Lactobacillus helveticus* inhibit enterohaemorrhagic *Escherichia coli* O157:H7 adhesion to epithelial cells. Cell Microbiol **9**:356–367.

25. **Wine E, Gareau MG, Johnson-Henry K, Sherman PM**. 2009. Strain-specific probiotic (*Lactobacillus helveticus*) inhibition of *Campylobacter jejuni* invasion of human intestinal epithelial cells. FEMS Microbiol Lett **300**:146–152.

26. **Konstantinov SR, Smidt H, de Vos WM, Bruijns SCM, Singh SK, Valence F, Molle D, Lortal S, Altermann E, Klaenhammer TR, van Kooyk Y**. 2008. S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. Proc Natl Acad Sci USA **105**:19474–19479.

27. **Lightfoot YL, Selle K, Yang T, Goh YJ, Sahay B, Zadeh M, Owen JL, Colliou N, Li E, Johannssen T, Lepenies B, Klaenhammer TR, Mohamadzadeh M**. 2015. SIGNR3-dependent immune regulation by *Lactobacillus acidophilus* surface layer protein A in colitis. EMBO J **34**:881–895.

28. **Abramov V, Khlebnikov V, Kosarev I, Bairamova G, Vasilenko R, Suzina N, Machulin A, Sakulin V, Kulikova N, Vasilenko N, Karlyshev A, Uversky V, Chikindas ML, Melnikov V**. 2014. Probiotic properties of *Lactobacillus crispatus* 2,029: Homeostatic interaction with cervicovaginal epithelial cells and antagonistic activity to genitourinary pathogens. Probiotics Antimicrob Proteins **6**:165–176.

29. **Taverniti V, Stuknyte M, Minuzzo M, Arioli S, De Noni I, Scabiosi C, Cordova ZM, Junttila I, Hämäläinen S, Turpeinen H, Mora D, Karp M, Pesu M, Guglielmetti S**. 2013. S-layer protein mediates the stimulatory effect of *Lactobacillus helveticus* MIMLh5 on innate immunity. Appl Environ Microbiol **79**:1221–1231.

30. **Goh, YJ, Azcarate-Peril MA, O'Flaherty S, Durmaz E, Valence F, Jardin J, Lortal S, Klaenhammer TR.** 2009. Development and application of a *upp*-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. Appl. Environ. Microbiol. **75:**3093-3105.

31. **Johnson B, Selle K, O'Flaherty S, Goh YJ, Klaenhammer T**. 2013. Identification of extracellular surface-layer associated proteins in *Lactobacillus acidophilus* NCFM. Microbiology **159**:2269–2282.

32. **Liu H, Sadygov RG, Yates JR.** 2003 A model for random sampling and estimation of relative protein abundance in shotgun proteomics, *Anal Chem* 76: 4193–4201.

33. **Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, Heger A, Hetherington K, Holm L, Mistry J, Sonnhammer ELL, Tate J, Punta M**. 2014. Pfam: the protein families database. Nucleic Acids Res **42**:D222–230.

34. **Petersen TN, Brunak S, von Heijne G, Nielsen H**. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods **8**:785–786.

35. **Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A**. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics **28**:1647–1649.

36. **Langmead B, Salzberg SL**. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods **9**:357-359.

37. **Le Maréchal C, Peton V, Plé C, Vroland C, Jardin J, Briard-Bion V, Durant G, Chuat V, Loux V, Foligné B, Deutsch S-M, Falentin H, Jan G**. 2015. Surface proteins of *Propionibacterium freudenreichii* are involved in its anti-inflammatory properties. J Proteomics **113**:447–461.

38. **Boot HJ, Kolen CP, Pouwels PH**. 1995. Identification, cloning, and nucleotide sequence of a silent S-layer protein gene of *Lactobacillus acidophilus* ATCC 4356 which has extensive similarity with the S-layer protein gene of this species. J Bacteriol **177**:7222–7230.

39. **Altermann E, Buck LB, Cano R, Klaenhammer TR**. 2004. Identification and phenotypic characterization of the cell-division protein CdpA. Gene **342**:189–197.

40. **McInerney JO**. 1998. Replicational and transcriptional selection on codon usage in *Borrelia burgdorferi*. Proc Natl Acad Sci USA **95**:10698–10703.

41. **Lafay B, Lloyd AT, McLean MJ, Devine KM, Sharp PM, Wolfe KH**. 1999. Proteome composition and codon usage in spirochaetes: species-specific and DNA strand-specific mutational biases. Nucleic Acids Res **27**:1642–1649.

42. **Grocock RJ, Sharp PM**. 2002. Synonymous codon usage in *Pseudomonas aeruginosa* PA01. Gene **289**:131–139.

43. **Karlin S, Theriot J, Mrázek J**. 2004. Comparative analysis of gene expression among low G+C gram-positive genomes. Proc Natl Acad Sci USA **101**:6182–6187.

44. **Rocha EPC**. 2004. The replication-related organization of bacterial genomes. Microbiology **150**:1609–1627.

45. **Pribnow D**. 1975. Nucleotide sequence of an RNA polymerase binding site at an early T7 promoter. Proc Natl Acad Sci USA **72**:784–788.

46. **Todt TJ, Wels M, Bongers RS, Siezen RS, van Hijum SAFT, Kleerebezem M**. 2012. Genome-wide prediction and validation of sigma70 promoters in *Lactobacillus plantarum* WCFS1. PLoS One **7**:e45097.

47. **Altermann E, Russell WM, Azcarate-Peril MA, Barrangou R, Buck BL, McAuliffe O, Souther N, Dobson A, Duong T, Callanan M, Lick S, Hamrick A, Cano R, Klaenhammer TR**. 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. Proc Natl Acad Sci USA **102**:3906–3912.

48. **Joerger MC, Klaenhammer TR**. 1990. Cloning, expression, and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin J. J Bacteriol **172**:6339–6347.

49. **Joerger MC, Klaenhammer TR**. 1986. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J Bacteriol **167**:439–446.

50. **Barefoot SF, Klaenhammer TR**. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl Environ Microbiol **45**:1808–1815.

51. **Broadbent JR, Hughes JE, Welker DL, Tompkins TA, Steele JL**. 2013. Complete Genome Sequence for *Lactobacillus helveticus* CNRZ 32, an Industrial Cheese Starter and Cheese Flavor Adjunct. Genome Announc **1**.

52. **Ventura M, Callegari ML, Morelli L**. 1999. Surface layer variations affecting phage adsorption on seven *Lactobacillus helveticus* strains. Annali di Microbiologia ed Enzimologia (Italy).

53. **Cato EP, Moore WEC, Johnson JL**. 1983. Synonymy of Strains of "*Lactobacillus acidophilus*" Group A2 (Johnson et al. 1980) with the Type Strain of *Lactobacillus*

*crispatus* (Brygoo and Aladame 1953) Moore and Holdeman 1970. Int J Syst Bacteriol **33**:426–428.

54. **Ojala T, Kuparinen V, Koskinen JP, Alatalo E, Holm L, Auvinen P, Edelman S, Westerlund-Wikström B, Korhonen TK, Paulin L, Kankainen M**. 2010. Genome sequence of *Lactobacillus crispatus* ST1. J Bacteriol **192**:3547–3548.

55. **Kullen MJ, Sanozky-Dawes RB, Crowell DC, Klaenhammer TR.** 2000. Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. Journal of Applied Microbiology **89**:511–516.

56. **Nakamura LK**. 1981. *Lactobacillus amylovorus*, a new starch-hydrolyzing species from cattle waste-corn fermentations. Int J Syst Bacteriol **31**:56–63.

57. **Kant R, Paulin L, Alatalo E, Vos WM de, Palva A**. 2011. Genome sequence of *Lactobacillus amylovorus* GRL1112. J Bacteriol **193**:789–790.

58. **Fujisawa T, Benno Y, Yaeshima T, Mitsuoka T**. 1992. Taxonomic study of the *Lactobacillus acidophilus* group, with recognition of *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and synonymy of *Lactobacillus acidophilus* group A3 (Johnson et al. 1980) with the Type Strain of *Lactobacillus amylovorus* (Nakamura 1981). Int J Syst Bacteriol **42**:487–491.

59. **Azcarate-Peril MA, Altermann E, Goh YJ, Tallon R, Sanozky-Dawes RB, Pfeiler EA, O'Flaherty S, Buck BL, Dobson A, Duong T, Miller MJ, Barrangou R, Klaenhammer TR**. 2008. Analysis of the genome sequence of *Lactobacillus gasseri* ATCC 33323 reveals the molecular basis of an autochthonous intestinal organism. Appl Environ Microbiol **74**:4610–4625.

60. **Kandler O, Stetter K-O, Köhl R**. 1980. *Lactobacillus reuteri* sp. nov., a new species of heterofermentative lactobacilli. Zentralblatt für Bakteriologie: I Abt Originale C: Allgemeine, angewandte und ökologische Mikrobiologie **1**:264–269.

61. **Tindal BJ**. 2008. The type strain of *Lactobacillus casei* is ATCC 393, ATCC 334 cannot serve as the type because it represents a different taxon, the name *Lactobacillus paracasei* and its subspecies names are not rejected and the revival of the name "*Lactobacillus zeae*" contravenes Rules 51b (1) and (2) of the International Code of Nomenclature of Bacteria. Opinion 82. Int J Syst Evol Microbiol **58**:1764–1765.

**Table 3.1** Strains used in this study

| Organism (strain) | Study designation | Source | Origin | S-layer | Reference |
|---|---|---|---|---|---|
| *L. acidophilus* (NCFM) | NCK56 | - | Human intestinal isolate | + | (47) |
| *L. helveticus* (1846) | NCK230 | NCDO | Dairy isolate | + | (48) |
| ***L. helveticus*** (481-C) | NCK246 | NCDO | Dairy isolate | + | (49) |
| *L. helveticus* | NCK338 | NCDO | Dairy isolate | + | (50) |
| ***L. helveticus*** (CNRZ32) | NCK936 | CNRZ | Industrial cheese starter culture | + | (51) |
| ***L. helveticus*** (ATCC 15009) | NCK1088 | ATCC | Dairy isolate | + | (52) |
| ***L. crispatus*** (ATCC 33820) | NCK777 | ATCC | Human isolate | + | (53, 54) |
| ***L. crispatus*** | NCK953 | - | Chicken isolate | + | - |
| ***L. crispatus*** (CZ6) | NCK1351 | - | Human endoscopy isolate | + | (55) |
| ***L. amylovorus*** (ATCC 33620) | NCK776 | ATCC | Cattle feces | + | (56, 57) |
| *L. gallinarum* (ATCC 33199) | NCK778 | ATCC | Chicken isolate | + | (58) |
| *L. gallinarum* | NCK1560 | - | Chicken isolate | + | - |
| ***L. delbrueckii* subsp. *bulgaricus*** | NCK1561 | - | Dairy isolate | - | - |
| *L. gasseri* (ATCC 33323) | NCK334 | ATCC | Human isolate | - | (59) |
| *L. johnsonii* (ATCC 33200) | NCK779 | ATCC | Human isolate | - | (58) |
| *L. reuteri* (ATCC 23272) | NCK702 | ATCC | Human feces | - | (60) |
| ***L. casei*** (ATCC 393) | NCK125 | ATCC | Dairy isolate | - | (61) |

Proteins from bolded/underlined strains were proteomically identified using LC-MS/MS.
The line in the table separates the 15 *L. acidophilus* homology group members from the two species outside of the *L. acidophilus* homology group. Abbreviations: NCDO, National Collection of Dairy Organisms. ATCC, American Type Culture Collection. CNRZ, Centre National de Recherches Zootechniques

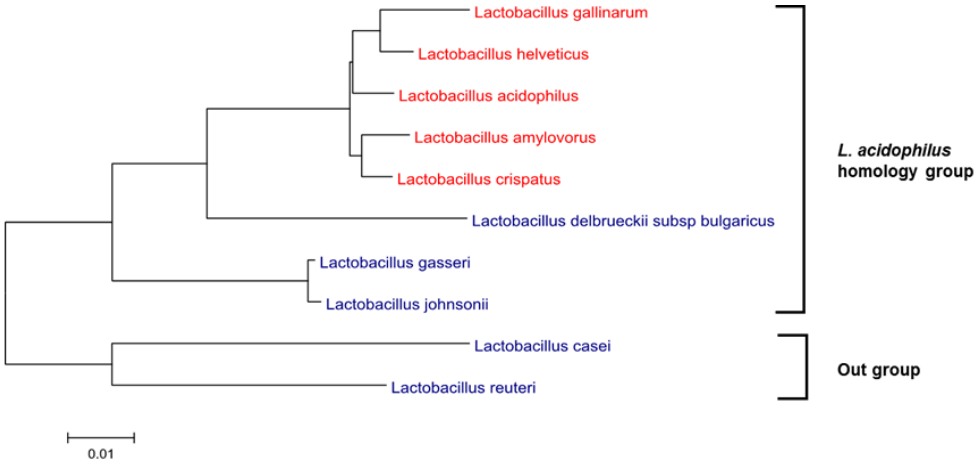


**Figure 3.1.** 16S rRNA dendogram of the S-layer (red) and non-S-layer (blue) forming species of the *L. acidophilus* homology group. The tree is rooted by the non-S-layer forming species, *L. casei* and *L. reuteri* which are not members of the *L. acidophilus* homology group.

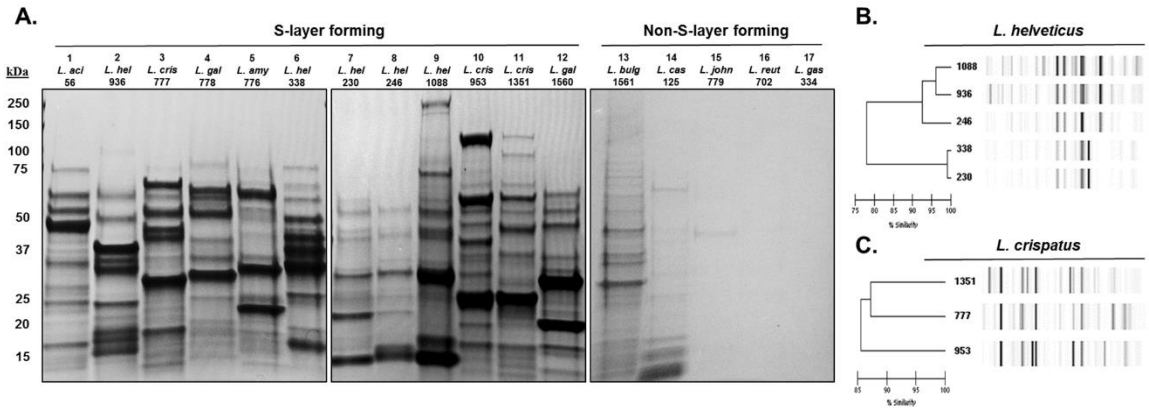


**Figure 3.2.** Non-covalently bound exoproteomes were extracted using LiCl and electrophoresed on SDS-PAGE. These gels are representative of protein extractions from two biological replicates of each strain. (A) The S-layer forming strains of the *L. acidophilus* homology group presented a diverse array of proteins in the LiCl extracts, including many anticipated S-layer associated proteins (SLAPs). By contrast, the non-S-layer forming species harbored very few proteins in the cell surface extracts. (B) Five strains of *L. helveticus* and (C) three strains of *L. crispatus* were typed using the rep-PCR based Diversilab typing system.

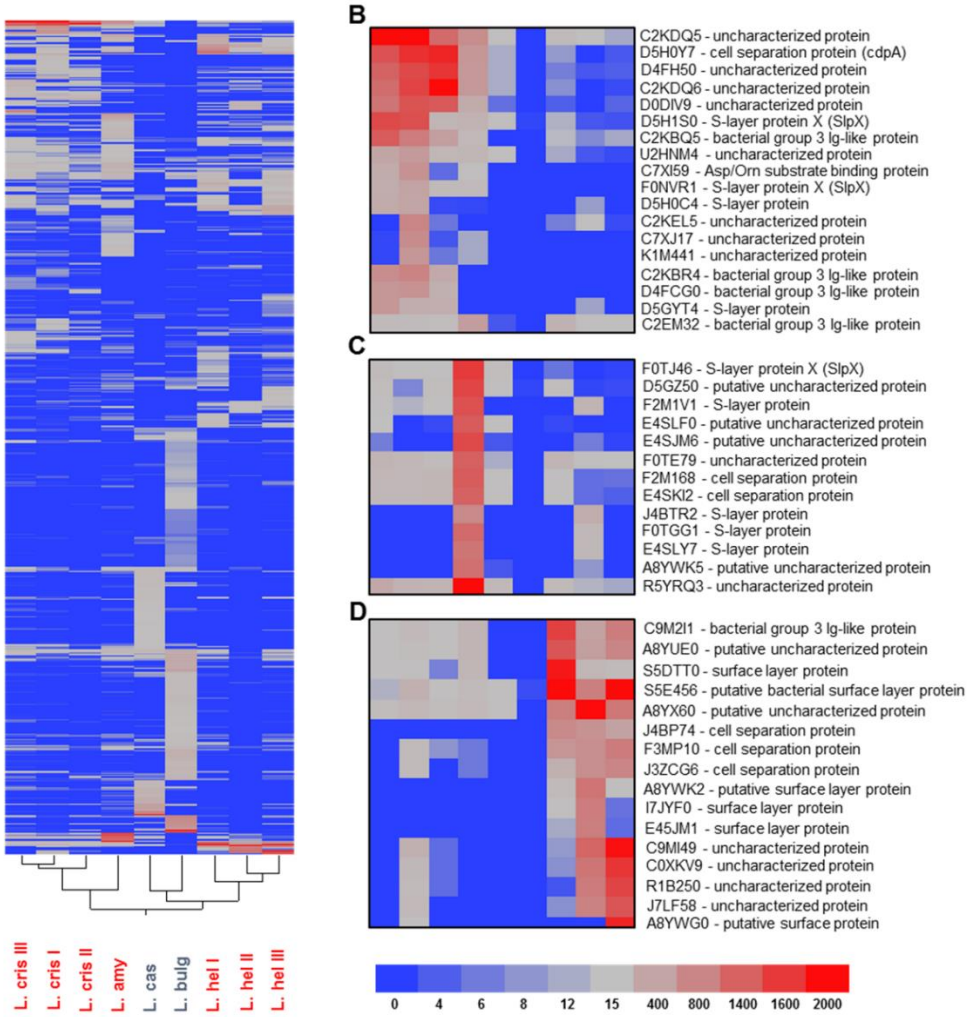


**Figure 3.3.** (A) 2,929 proteins were identified from the S-layer forming strains (in red), *L. crispatus*, *L. amylovorus*, and *L. helveticus*, as well as the non-S-layer forming strains (in blue) *L. delbrueckii* subsp. *bulgaricus* and *L. casei*. Two way clustering was performed on the identified proteins based on their similarity between strains, and visualized using a red-blue heat map. The colors in the heat map represent the spectral counts of the identified proteins (semi-quantitative measure of protein abundance), with red being the most present (400-1000 total spectral counts), grey being somewhat present (12- 400 total spectral counts), and blue being low or no presence (0-12 total spectral counts). Regarding the S-layer forming strains, there were three main clusters of proteins: SLAPs specific to (B) *L. crispatus*, SLAPs specific to (C) *L. amylovorus*, and SLAPs specific to (D) *L. helveticus*. These three clusters have been noted with the corresponding Uniprot and protein annotations of the identified proteins.

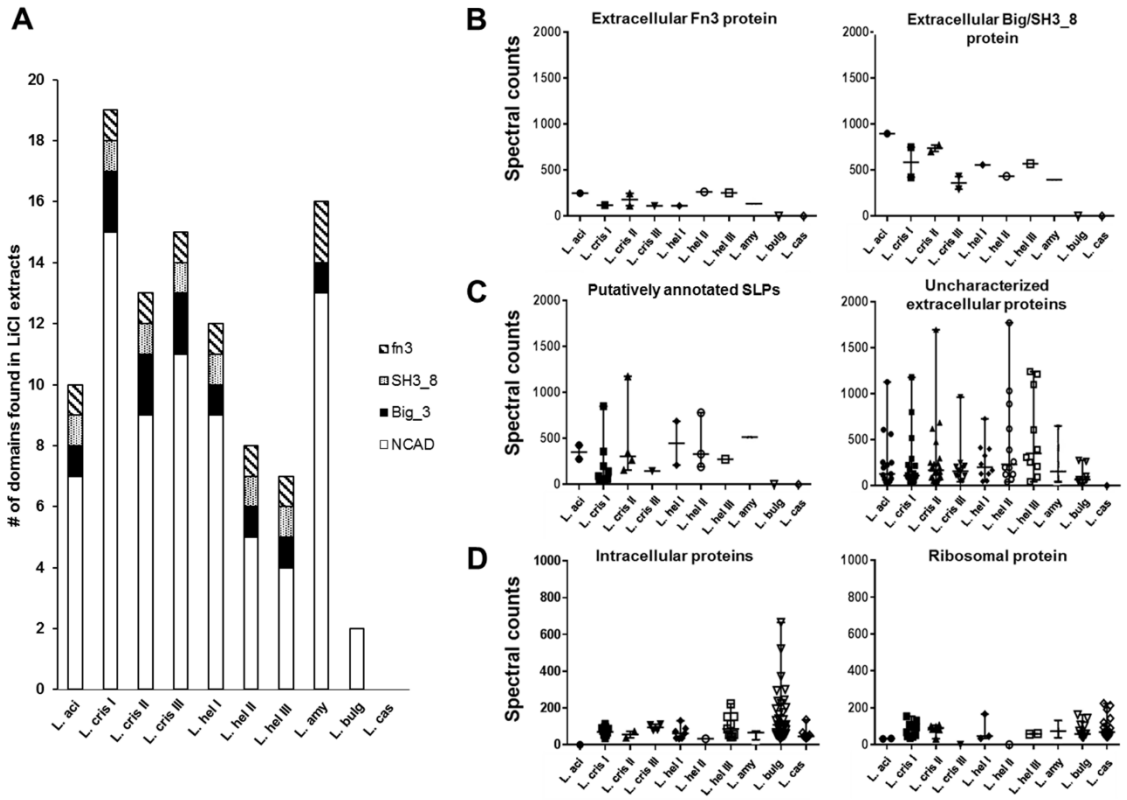


**Figure 3.4.** (A) Four protein domains found consistently in the proteins identified within the LiCl extracts: NCAD (white), BIg_3 (black), SH3_8 (dots), and fn3 (diagonal lines). Dot plots were created using the semi-quantitative total spectral counts from the identified proteins of each strain. Plotted are the (B) extracellular Fn3 proteins and Extracellular BIg_3/SH3_8 proteins; (C) Putative annotated S-layer proteins and Uncharacterized extracellular proteins; and (D) intracellular and ribosomal proteins. The proteins in (C) contain the NCAD domain, while the proteins in (D) do not.

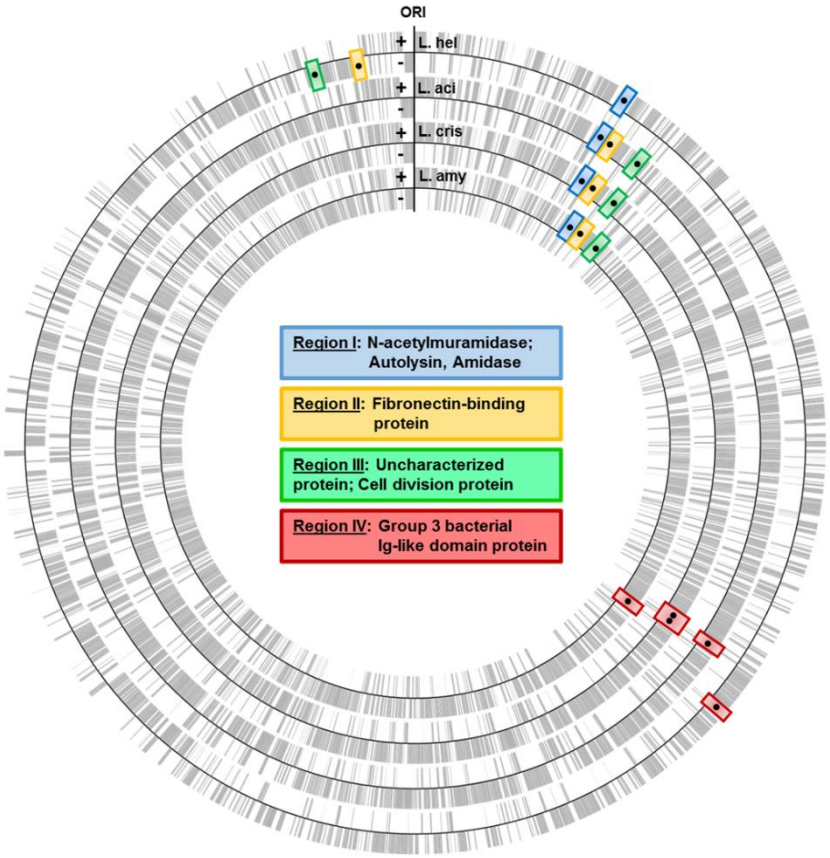


**Figure 3.5.** All ORFs from the positive (+) and negative (-) strand of *L. helveticus* CNRZ32, *L. acidophilus* NCFM, *L. crispatus* ST1, and *L. amylovorus* GRL1112 were mapped onto circular chromosomes with an annotated origin of replication (Ori). Four conserved SLAP gene regions were identified based on position between strains. Region I (blue), region II (yellow), region III (green), and region IV (red).

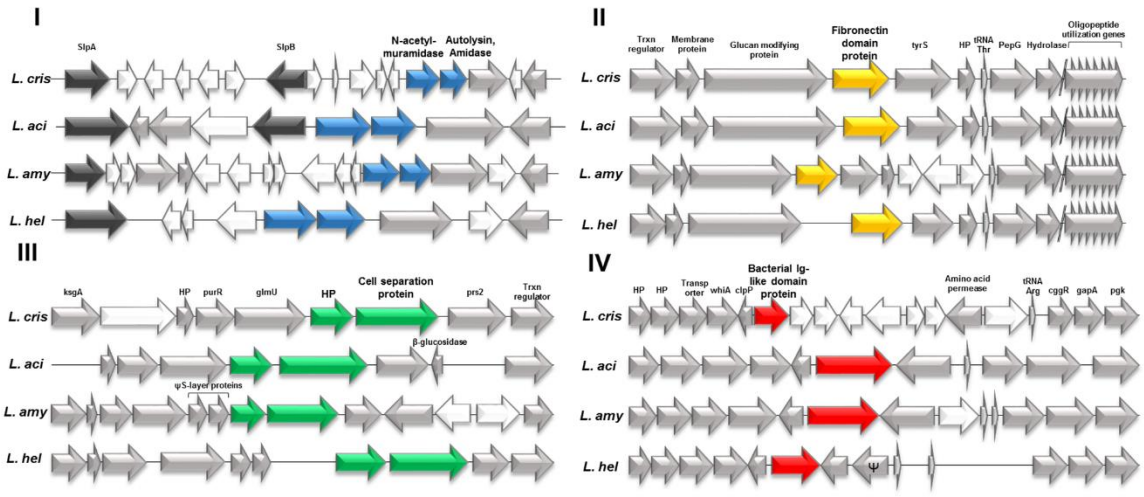


**Figure 3.6.** The genomic context of the five SLAP gene regions among the four strains of *Lactobacillus*: *L. crispatus* ST1, *L. acidophilus* NCFM, *L. amylovorus* GRL 1112, and *L. helveticus* CNRZ32. Grey genes represent conserved synteny between the four strains, while white genes represent divergence. Colored genes represent the SLAP gene regions as follows: blue (region 1), yellow (region II), green (region III), and red (region IV). Abbreviations: HP, hypothetical protein; Trxn, transcriptional.

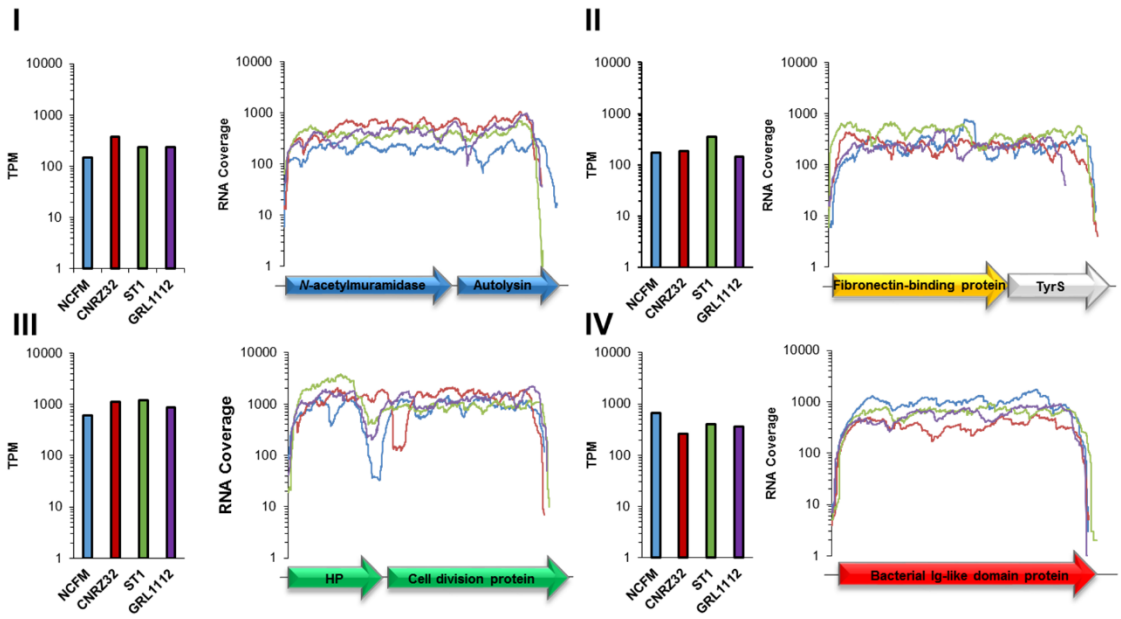


**Figure 3.7.** Transcription levels of the four conserved SLAP genomic regions was measured through RNA sequencing. Panels I-IV: illustrated expression in each regions; region I (blue), region II (yellow), region III (green), and region IV (red). Bar graphs for each panel present the normalized TPM while the line graphs present RNA coverage across each gene from the SLAP regions in *L. acidophilus* NCFM (light blue), *L. helveticus* CNRZ32 (dark red), *L. crispatus* ST1 (light green), and *L. amylovorus* GRL1112 (purple).

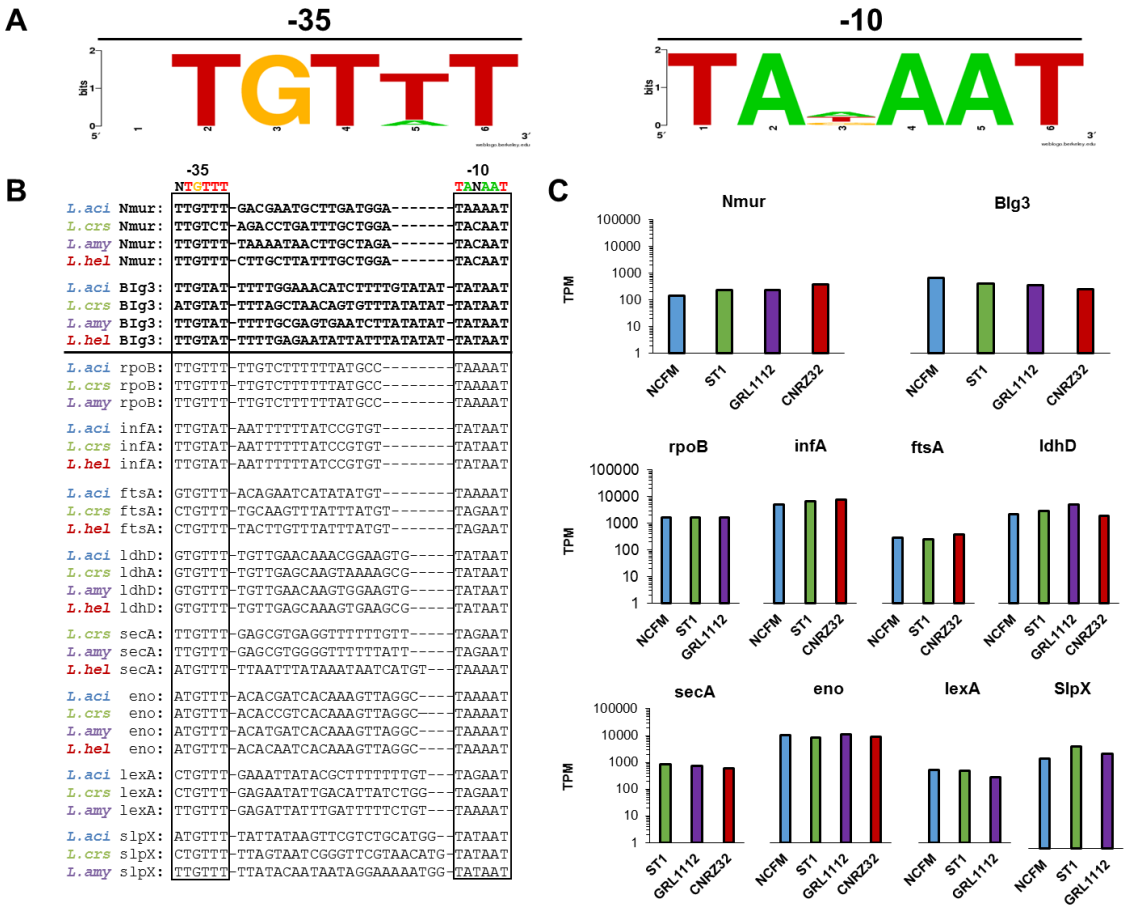


**Figure 3.8.** The putative promoter elements in the sequence directly upstream of the SLAP gene regions. Two similar promoter elements were discovered upstream of the *N*-acetylmuramidase (*Nmur*) of region I and the group 3 bacterial Ig-like domain gene (*BIg3*) of region IV. For both promoters the -10 region consisted of a TANAAT consensus motif, while the -35 region followed a NTGTNT consensus motif, in which N represents any nucleotide (A). There was a spacing of 17 nucleotides between the -35 and -10 sites of the *Nmur* promoter, but the spacing was 23 nucleotides for the *BIg3* promoter (B, bolded). The level of expression was comparable for each gene. In fact, the *BIg3* gene was more highly expressed than *Nmur*, notwithstanding the increased spacing between the -10 and -35 sites (C). The promoters containing the TANAAT and NTGTNT consensus motifs with a spacing of 16 to 23 base pairs in the upstream untranslated regions within the four genomes were curated (8B, non bolded). Numerous housekeeping genes were found to be downstream of the putative promoters, including the β subunit of RNA polymerase (*rpoB*), initiation factor A (*infA*), cell division protein, *ftsA*, *D*-lactate dehydrogenase (*ldhD*), the protein translocase subunit, *secA*, enolase (*eno*), *lex*A repressor, and *slpX*. The 8 housekeeping genes examined had varying levels of expression (C).

# CHAPTER 4

## Functional analysis of AcmB, an S-layer associated β-*N*-acetylglucosaminidase in *Lactobacillus acidophilus* NCFM.

Planned submission to Applied and Environmental Microbiology

## 4.1 Abstract

Autolysins, also known as peptidoglycan hydrolases (PGH), are enzymes that hydrolyze specific bonds within bacterial cell wall peptidoglycan during cell division and daughter cell separation. Within the genome of *Lactobacillus acidophilus* NCFM there are eleven genes encoding proteins with PGH catalytic domains, nine of which are predicted to be functional. Notably, five of the nine putative autolysins in *L. acidophilus* NCFM are S-layer associated proteins (SLAPs) non-covalently co-localized along with the S-layer at the cell surface. One of these SLAPs, AcmB, a β-*N*-acetylglucosaminidase encoded by the gene *lba0176* (*acmB*), was selected for functional analysis. Chromosomal deletion of *acmB* resulted in aberrant cell division, autolysis, and autoaggregation. Complementation of *acmB* in the Δ*acmB* strain restored the wild-type phenotype, confirming the role of this SLAP in cell division. The absence of AcmB within the exoproteome had a pleiotropic effect on the S-layer, which led to a decrease in the ability of the Δ*acmB* strain to bind to extracellular matrices, *in vitro*. As a scaffold for multiple autolysins, these data indicate that the S-layer is critical for cell division in *L. acidophilus* NCFM.

## 4.2 Introduction

Beneficial microorganisms such as probiotics are defined by the FAO/WHO as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (1). *Lactobacillus acidophilus* NCFM is a generally recognized as safe, industrially significant lactic acid bacteria which has been sold commercially and consumed in various probiotic food formulations for over 35 years (2). Predicated by the availability of a fully sequenced and annotated genome (3), *L. acidophilus* NCFM is one of the most studied and well characterized probiotic bacteria (2, 4-8). Most notably, the probiotic activity of *L. acidophilus* is mediated by cell surface-associated components which interact with the host gastrointestinal mucosa and immune system (9, 10).

As in other Gram-positive bacteria, the cell wall of *L. acidophilus* is characterized by a lipid membrane surrounded by a thick peptidoglycan sacculus with a complex assemblage of macromolecules including teichoic acids, polysaccharides, and proteins (11, 12). The peptidoglycan is composed of glycan chains consisting of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid, linked via β-1, 4 bonds and covalently crosslinked with peptide chains. Among the numerous functions of peptidoglycan are the maintenance of cell shape (13), integrity from osmotic pressure (14), and the presentation of proteins (11). Some of these proteins are covalently linked to the peptidoglycan via sortase and LPXTG motif recognition (15), while many others, including proteins which comprise the Surface (S-) layer, are non-covalently attached through cell wall binding domains (16).

S-layers are semi-porous, proteinaceous crystalline arrays comprised of self-assembling (glyco) protein subunits called S-layer proteins (SLPs). While S-layers can be found in all

prokaryotes, including Gram-positive, Gram-negative, and many species of Archaea, S-layers are not ubiquitous to all microorganisms (16). In *L. acidophilus,* the S-layer array is comprised of a dominant protein constituent, SlpA (46 kDa) with minor constituents SlpB (47 kDa) and SlpX (51 kDa; 17). Because the S-layer is presented as the outermost layer of proteins on the cell wall, it has been the target for functional analysis of probiotic-host interactions. In fact, *in vitro* studies using intestinal epithelial cell lines suggest the S-layer as a major factor in intestinal adhesion for *L. acidophilus* (18, 19). Furthermore, SlpA of *L. acidophilus* NCFM has been shown to bind dendritic cell C-type lectin receptors (20) and exert regulatory signals which mitigate inflammatory disease states and promote maintenance of healthy intestinal barrier function (21).

Despite the apparent importance of the S-layer for probiotic-host interactions, there is still a great deal unknown about the composition and evolutionary function of S-layers. Complete functional analysis of the S-layer has been limited due to the apparent essentiality of the S-layer for cell survival, and the ensuing difficulty of creating stable knockouts of the SLPs in *L. acidophilus* (Goh & Klaenhammer, unpublished). However, recent evidence has demonstrated that the S-layer is not a monomorphic layer of a single protein constituent, as previously believed. Exoproteomic analysis of *L. acidophilus* NCFM and other S-layer-forming lactobacilli has revealed the presence of numerous S-layer associated proteins (SLAPs) which are co-localized with the S-layer through non-covalent association with the cell wall (10, 22). In addition to uncharacterized proteins with putative probiotic activity, numerous autolysins were found in these SLAP fractions (10, 22).

Autolysins, also known as peptidoglycan hydrolases (PGH), are a class of enzymes responsible for peptidoglycan turnover during cell division and daughter cell separation (12). PGHs have numerous catalytic domains and are normally bound to the cell wall through LysM or SH3 anchoring domains (23, 24). Notably, the PGHs identified in the SLAP fractions of *L. acidophilus* NCFM and other S-layer forming *Lactobacillus* species are anchored to the cell wall with Non-Covalent Attachment Domains (NCAD, pfam03217), the same domains found in SLPs (10, 22, 25). In this study, an S-layer associated β-*N*-acetylglucosaminidase, AcmB, was selected for functional analysis in *L. acidophilus* NCFM. Chromosomal deletion of *acmB* resulted in aberrant cell division, autolysis, and autoaggregation, confirming the role of this SLAP in PGH activity. Further, the absence of AcmB within the exoproteome had a pleiotropic effect on the S-layer, which led to a decrease in the S-layer thickness of the Δ*acmB* strain compared to wild-type. Surprisingly, the ability of the Δ*acmB* strain to bind to mucin and extracellular matrices, *in vitro*, was significantly reduced. Here we present the S-layer as a scaffold for numerous proteins, including autolysins. Analysis of these S-layer associated autolysins will undoubtedly lead to a more comprehensive understanding of the evolutionary function of the S-layer in S-layer forming species of *Lactobacillus.*

## 4.3 Materials and Methods

### 4.3.1 Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *L. acidophilus* strains were propagated in de Man Ragosa Sharpe (MRS) broth (Difco) under aerobic conditions, statically or on MRS solid medium containing 1.5% (w/v) agar (Difco) under anaerobic conditions at 37 °C, and at 42 °C where indicated. Recombinant strains were selected in the presence of 2 μg/ml of erythromycin (Sigma-Aldrich, St. Louis, MO) and/or 2 to 5 μg/ml of chloramphenicol (Sigma). *Escherichia coli* strains were grown in brain heart infusion (Difco) medium at 37 °C, with aeration. *E. coli* EC101 was grown in the presence of 40 μg/ml kanamycin (Sigma-Aldrich) while NCK1911 and transformants were grown with 40 mg kanamycin and 150 μg/ml erythromycin. Counterselection of plasmid-free excision recombinants was performed using 5-fluorouracil-supplemented glucose semi-defined medium, as previously described (17).

### 4.3.2 DNA manipulation and transformation.

Genomic DNA from *L. acidophilus* strains was isolated using a Zymo Research Fungal/Bacterial DNA MiniPrep kit (Zymo Research). Plasmid DNA from *E. coli* was isolated using a QIAprep Spin Miniprep kit (Qiagen). Restriction enzyme digestions and ligations were performed using Roche restriction enzymes (Roche Diagnostics) and T4 DNA ligase (New England Biolabs), respectively. PCR primers were designed based on the genomic sequence data and synthesized by Integrated DNA Technologies. PCRs were carried out in Bio-Rad MyCycler thermocyclers (Bio-Rad Laboratories) using Choice-Taq Blue

DNA polymerase (Denville Scientific) for screening of recombinants and *PfuUltra* II fusion HS DNA polymerase (Agilent Technologies) for cloning purposes. PCR amplicons were analyzed on 0.8 % agarose gels and purified using QIAquick Gel Extraction kits (Qiagen).

*E. coli* EC101 cells were made competent using a rubidium chloride competent cell protocol (26). *L. acidophilus* cells were prepared for electrotransformation using a modified penicillin treatment protocol (17, 27-28).

### 4.3.3 *In silico* and RNA sequencing analyses.

Predicted peptidoglycan hydrolases were identified from genome of *L. acidophilus* NCFM (3; NCBI accession number NC_006814). Homologous sequences were identified and compared using the BLASTn and BLASTp features of NCBI, as well as SANSParallel (29). Signal peptidase cleavage sites for protein sequences were predicted using SignalP 4.1 (30). Protein domains were identified using UniProt and the Pfam protein family database (31, 32). RNA sequencing analysis from a previous study (10), was utilized to examine mRNA expression of the predicted peptidoglycan hydrolases. Gene expression was measured by the normalized transcripts per million (TPM) calculator within Geneious 8.0.5 (33).

### 4.3.4 Deletion of *AcmB* in *L. acidophilus* NCFM.

The *upp*-based counterselection gene replacement method (17) was used to create an internal deletion of 1103 bp in *acmB* (*lba0176*) of NCK1909, a *upp*-deficient background strain of *L. acidophilus* NCFM. Using splicing by overlap extension PCR (34), the 1 kb regions flanking the deletion target were spliced with BamHI restricted site added at the

upstream end and SacI at the downstream end. This construct was digested with BamHI and SacI, then ligated into the polylinker of the similarly digested integration plasmid pTRK935 and transformed into competent *E. coli* EC101. The resulting recombinant plasmid, pTRK1097, was transformed into *L. acidophilus* NCK1909 harboring the helper plasmid pTRK669 (NCK1910). Single crossover integrants were screened as described previously (17). Colonies with the Δ*acmB* genotype were screened among the double recombinants recovered on glucose semi-defined medium agar plates containing 5-fluorouracil. Deletion of *acmB* was confirmed by PCR and sequencing, and the resulting Δ*acmB* mutant was designated NCK2395.

### 4.3.5 Complementation of *acmB* in Δ*acmB* strain of *L. acidophilus* NCFM.

The Δ*acmB* strain of *L. acidophilus* NCFM was complemented using pTRK882, an expression plasmid with the promoter for *pgm* (*lba0185*), encoding a phosphoglyceromutase (35). The *acmB* gene, along with its native RBS, was amplified with EcoRI and BamHI restriction sites added to the 5' and 3' ends of the amplicon and subsequently cloned into the polylinker of pTRK882. The integrity of the insert was confirmed by DNA sequencing. The resulting recombinant plasmid, pTRK1098 was electroporated into the Δ*acmB* strain of *L. acidophilus* NCFM. Transformants were screened for erythromycin resistance, generating NCK2397 for phenotypic comparison.

### 4.3.6 LiCl extraction of SLAPs.

Non-covalently bound cell surface proteins, including S-layer proteins and S-layer associated proteins (SLAPs) were extracted from NCK1909 and Δ*acmB L. acidophilus* NCFM strains using LiCl denaturing salt, as described previously (22). Proteins were quantified via bicinchoninic acid assay kit (Thermo Scientific) and visualized via SDS-PAGE using precast 4–20% Precise Tris-HEPES protein gels (Thermo Scientific). The gels were stained using AcquaStain (Bulldog Bio) according to the instructions of the manufacturer.

### 4.3.7 Microscopic and morphological assessments.

Morphological assessment of *L. acidophilus* NCFM strain was performed using a phase-contrast light microscope at 40× magnification (Nikon Eclipse E600). Cells were observed over a growth period of 24 hours in MRS broth at 37 °C, or MRS broth with 5 μg/ml erythromycin for the complemented strain. Pictures were taken using a QImaging MicroPublisher 5.0 RTV camera attachment at 1, 4, 7, 14, and 24 hour time points. Cell chain length was measured using the Image-Pro Insight software (MediaCybernetics).

For transmission electron microscopy (TEM), *L. acidophilus* NCFM strains were grown in 35 ml of MRS to logarithmic and stationary phases. Cells were pelleted by centrifugation at 3,166 × *g* for 15 min at room temperature. Cell pellets were resuspended in a fresh 1:1 (vol/vol) fixative mixture of 6% glutaraldehyde and 0.2 M sodium cacodylate (pH 5.5) and stored at 4°C. Sample processing for TEM was performed by the Center for Electron Microscopy at North Carolina State University. TEM samples were viewed with a JEOL

100S transmission electron microscope. Surface layer thickness was measured by using a millimeter ruler and subsequently converted to nm scale based on the provided scale bar during TEM image processing.

### 4.3.8 Autoaggregation and autolysis assays.

Autoaggregation assays were performed as described previously (36). Bacteria were grown in MRS broth with 5 μg/ml erythromycin where necessary for 16 hours, harvested by centrifugation at 1,771 × *g* for 10 minutes, and washed twice with PBS (pH 7.4). Washed cells were resuspended in PBS to an adjusted $OD_{600}$ of 1. Cell suspensions were mixed by vortexing for 10s and autoaggregation was determined over 5 hours at room temperature. Every hour, 100 μl of the upper suspension was transferred to a cuvette with 900 μl PBS and the absorbance ($OD_{600}$) was measured. Autoaggregation percentages were calculated as follows: $1- (A_t/A_0) \times 100$, where $A_t$ is the $OD_{600}$ at time (t) = 1, 2, 3, 4, and 5 hours, and $A_0$ is the $OD_{600}$ at time 0.

Autolysis was performed as described previously (24) with some modifications. *L. acidophilus* NCFM strains were grown to late-exponential phase ($OD_{600}$ ~1.0) and were harvested via centrifugation at 1,771 × *g* for 10 minutes. Cells were washed once with PBS (pH 7.4) and resuspended in PBS (pH 7.4) supplemented with 0.05% Triton X-100. Suspensions were transferred to a sterile 96-well plate with transparent bottoms. $OD_{600}$ was assessed every 20 minutes for 24 hours using a FLUOStar Optima microtiter plate reader (BMG Technologies) at 37 °C. Autolysis was calculated as the decrease in $OD_{600}$ relative to the initial $OD_{600}$ at time 0.

### 4.3.9 Mucin and extracellular matrix (ECM) adherence assays.

ECM binding assays were performed as described previously (37). Mucin (type III from porcine stomach, Sigma) was dissolved in PBS to a final concentration of 10 mg/ml. Fibronectin (from human plasma, Sigma), collagen (type IV from human cell culture, Sigma), and laminin (from Engelbreth-Holm-Swarm murine sarcoma/basement membrane; Sigma) were diluted in 50 mM carbonate-bicarbonate buffer (pH 9.6, Sigma) to a final concentration of 10 μg/ml. For each assay, a Nunc Maxisorp 96-well microplate (Sigma) was coated with 100 μl/well of substrate and incubated at 4°C overnight. The wells were washed twice with PBS to remove excess substrate before blocking with 150 μl per well of 2% bovine serum albumin (BSA) solution (Sigma) for 2 h at 37°C. Excess BSA was removed by two washes with PBS (pH 7.4).

Bacterial cells were grown in MRS to stationary phase (16 hours) in preparation for the assay. Cultures were centrifuged (1,771 × *g*, 15 min, room temperature), washed once, and resuspended in PBS (pH 4.75). Cell density was adjusted to ~1 x $10^8$ CFU/mL based on previously calculated $OD_{600}$/CFU ratios. Cell suspensions (100 μl) were added to each mucin or ECM-coated well. Initial cell counts were enumerated on MRS plates. After incubation for 1 h at 37°C, the wells were gently washed five times with 200 μl/well PBS. Adhered cells were recovered by adding 100 μl of 0.05% Triton X-100 solution (FisherBiotech, prepared in PBS) to each well and agitating on an orbital shaker (200 rpm) for 15 min. Cell suspensions were transferred into 900 μl of 0.1X MRS before being further diluted and plated in duplicate on MRS plates. Colonies were enumerated and expressed as a percent of relative adherence (mutant CFU/parent CFU), where parent (NCK1909) CFU were defined as 100%.

## 4.4 Results

### 4.4.1 *In silico* analysis of autolysins within *L. acidophilus* NCFM.

Within the genome of *L. acidophilus* NCFM, there are eleven genes which encode putative autolysins with predicted PGH activity (Table 2, Figure 4.1). These PGH activities can be subdivided into four classes: *D* and *L* endopeptidases (pfam00877, Figure 4.1A, blue), β-*N*-acetylglucosaminidases (pfam01832, Figure 4.1A, red), β-*N*-acetylmuramidases (pfam01183, Figure 4.1A, green), and *N*-acetylmuramoyl-*L*-alanine amidases (pfam01510, Figure 4.1A, purple). The four classes of autolysins predicted in *L. acidophilus* have the required specificities to hydrolyze all components of the peptidoglycan (Figure 4.1B). Further, there is apparent redundancy in three of the PGH classes, with four endopeptidases, two β-*N*-acetylglucosaminidases, and four β-*N*-acetylmuramidases encoded within the genome (Figure 4.1A). Using RNA-seq data from a previous study (10), mid-logarithmic phase transcriptional profiles of the predicted autolysins could be compared to analyze which may be the primary autolysin of each class (Figure 4.1C). Based on these data, LBA1744, AcmB, and LBA1351 were found to be the most highly expressed endopeptidase, β-*N*-acetylglucosaminidase, and β-*N*-acetylmuramidase, respectively (Figure 4.1C). LBA0177 appears to be the sole *N*-acetylmuramoyl-*L*-alanine amidase of *L. acidophilus* NCFM. Two genes with very low expression, AcmA and LBA0616, encoding a β-*N*-acetylglucosaminidase and a β-*N*-acetylmuramidase, do not have predicted signal peptide sequences, and thus are likely not functional autolysins (Figure 4.1A and C).

Notably, five of the nine functional autolysins have been identified as SLAP constituents (Table 2) of the S-layer exoproteome in *L. acidophilus* NCFM and other S-layer

forming species of *Lactobacillus acidophilus* homology group (10, 22). In fact, these proteins have the Non-Covalent Attachment Domain (NCAD, pfam03217) found in other SLP, suggesting their co-localization to the cell surface along with the S-layer (Figure 4.1A, yellow). These SLAPs encompass all of the predicted functional β-*N*-acetylmuramidases, as well as the sole *N*-acetylmuramoyl-*L*-alanine amidase, LBA0177, and the β-*N*-acetylglucosaminidase, AcmB. The four endopeptidases were not found in the SLAP fractions of the previous studies and do not have the NCAD domains (Figure 4.1A). AcmB was selected for functional analysis based on its prevalence in the non-covalently bound exoproteome and conservation as a SLAP in *L. acidophilus* NCFM and other S-layer forming species of the homology group.

### 4.4.2 *In silico* genomic analysis of AcmB.

Although the gene encoding AcmB (*lba0176*) was previously annotated as a β-*N*-acetylmuramidase, the protein has a Mannosyl-glycoprotein endo-β-*N*-acetylglucosaminidase catalytic domain (pfam01832), suggesting that AcmB is a β-*N*-acetylglucosaminidase. Within the chromosome of *L. acidophilus* NCFM, *acmA* (*lba0527*) is the only other gene which also contains the β-*N*-acetylglucosaminidase domain (Figure 4.1A). However, *acmA* may be truncated and does not have a signal peptide sequence (Figure 4.1A), and demonstrates reduced transcriptional expression compared to *acmB*, which does contain a signal peptide sequence (Figure 4.1C). For these reasons, AcmB appears to be the primary β-*N*-acetylglucosaminidase for *L. acidophilus* NCFM.

AcmB orthologs are found in numerous *Lactobacillus* species, including *L. amylovorus* GRL1112 (81.7% amino acid identity), *L. helveticus* H10 (78.5%), *L. kefiranofaciens* ZW3 (75.8%), *L. crispatus* ST1 (73.3%), *L. melliventris* (61%), *L. amylolyticus* DSM 11664 (61.3%), and *L. gigeriorum* DSM 23908 (59.3%). Most notably, the AcmB orthologs are found exclusively in S-layer forming species of *Lactobacillus*, providing further evidence that AcmB is an S-layer associated protein. There are additional orthologs found in the genomic region surrounding AcmB in these S-layer forming species, compared to *L. acidophilus* NCFM (Figure 4.2). In fact, the genomic region directly downstream of AcmB appears to be syntenic and conserved among the seven S-layer forming species listed above (Figure 4.2). Among the orthologs with synteny in this region are genes encoding an S-layer associated *N*-acetylmuramoyl-*L*-alanine amidase, an uncharacterized $Na^+/H^+$ ion transporter, an oxidoreductase, a GMP synthetase, a phosphoglyceromutase, a pyrrolidine carboxypeptidase, and two hypothetical proteins (Figure 4.2). These genes were all found to be in the genomic region surrounding AcmB in the S-layer forming strains, with the exception of the pyrrolidine carboxypeptidase, which is absent in *L. amylolyticus* DSM 11664 and *L. gigeriorum* DSM 23908 (Figure 4.2). In *L. kefiranofaciens* ZW3, the position of the genes within the region is identical to *L. acidophilus* NCFM, and is likewise conserved in *L. crispatus* ST1, with the exception of a translocated pyrrolidine carboxypeptidase from the positive strand to the negative strand of the genome (Figure 4.2).

### 4.4.3 Deletion of *acmB* from the chromosome of *L. acidophilus* NCFM and corresponding complementation of AcmB in the Δ*acmB* strain.

Using a *upp*-based counterselective gene replacement method (17), a markerless chromosomal deletion of *acmB* was made in a *upp*-deficient background host of *L. acidophilus* NCFM. The Δ*acmB* genotype was confirmed using PCR and DNA sequencing with primers flanking the 1220 bp *acmB* gene (Figure 4.3A and B). Absence of AcmB from the SLAP fraction of the Δ*acmB* strain could not be observed visually by SDS-PAGE, due to multiple 45 kDa proteins in the fraction (Figure 4.3C). Furthermore, Δ*acmB* did not appear to have an altered SLAP profile compared to wild-type (Figure 4.3C). For phenotypic analysis, *acmB* was complemented in the Δ*acmB* strain using pTRK1098, an expression plasmid with the constitutive *pgm* promoter.

### 4.4.4 Phenotypic analyses of Δ*acmB*.

Following deletion confirmation, the Δ*acmB* strain was phenotypically assessed for: (i) cell morphology, (ii) autoaggregation and autolysis capacity, (iii) S-layer morphology, and (iv) the ability to bind to mucin and extracellular matrices (ECM) collagen, fibronectin, and laminin.

### 4.4.5 Cellular morphology of Δ*acmB*.

Cellular morphology of the Δ*acmB* strain was assessed by light microscopy over a 24 hour growth time course in MRS culture (Figure 4.4). Compared to the wild-type (WT) strain, the Δ*acmB* mutant had a distinctive morphological phenotype consisting of longer

cells, increased chain lengths, and increased autoaggregation (Figure 4.4A). Complementation of *acmB* in the Δ*acmB* strain (CΔ*acmB*) resulted in a return to WT morphology (Figure 4.4A). This cell division-related morphology was quantified by measuring the chain lengths of dividing cells throughout the 24 hour time course (Figure 4.4B). Both the WT and CΔ*acmB* strains, demonstrated prototypical chain lengths which followed a standard bacterial growth curve (Figure 4.4B, white and striped bars). Specifically, chain lengths increased from lag phase to logarithmic phase (hours 1-6), and decreased as a result of de-chaining during the transition to stationary phase (hours 7-24; Figure 4.4B). On the contrary, Δ*acmB* had a statistically significant increase in chain length across all measurements ($p < 0.001$; Figure 4.4B, gray bars). At the start of lag phase (hour 1), Δ*acmB* had a pronounced increase in chain length ($M = 30.32$ μm, $CI = 7.78$) compared to WT ($M = 8.83$ μm, $CI = 2.77$) and CΔ*acmB* ($M = 8.34$ μm, $CI = 1.79$), likely due to residual cells from inoculation transfer of previous stationary phase culture (Figure 4.4B). By early logarithmic phase, the difference in chain lengths were less evident between Δ*acmB* and the WT and CΔ*acmB* strains. However, by mid-logarithmic phase (hour 7) the chain lengths in the Δ*acmB* culture was considerably longer ($M = 47.0423$ μm, $CI = 6.17$) compared to WT ($M = 13.94$ μm, $CI = 0.58$) and CΔ*acmB* (17.85 μm, $CI = 1.91$; Figure 4.4B), suggesting aberrant de-chaining and daughter cell separation. For the remainder of the time course, the Δ*acmB* strain maintained increased chain lengths, while WT and CΔ*acmB* properly divided daughter cells for a concomitant decrease in chain lengths (Figure 4.4B).

### 4.4.6 Autoaggregation and autolysis of Δ*acmB*.

Based on the abnormal cellular morphology of Δ*acmB*, the autoaggregation and autolysis capacity of this strain was evaluated (Figure 4.5). The sedimentation rate of Δ*acmB*, WT, and the complemented CΔ*acmB* strains were measured over 5 h in PBS (Figure 4.5A). For the first two hours, the three strains had comparable autoaggregation rates. By 3 h, the autoaggregation of Δ*acmB* ($M$ = 49.24%, $CI$ = 7.16%) was significantly higher ($p < 0.001$) than WT ($M$ = 28.69%, $CI$ = 2.68%) and CΔ*acmB* ($M$ = 33.94%, $CI$ = 3.19%; Figure 4.5A). The differences were most pronounced at 5 h, in which Δ*acmB* had an autoaggregation percentage of 68.05% ($CI$ = 7.73%), compared to 45.42% ($CI$ = 10.35%) and 51.61% ($CI$ = 3.31%) for WT and CΔ*acmB*, respectively ($p < 0.01$; Figure 4.5A). To assess the autolytic behavior of the Δ*acmB*, WT, and CΔ*acmB* strains, Triton X-100-induced autolysis assays were performed (Figure 4.5B). The rate of autolysis in Δ*acmB* was significantly lower than in the WT and complemented strains ($p < 0.05$). Specifically, Triton X-100 induced cells resulted in 40% autolysis of the Δ*acmB* population, compared to 50% in WT and 52% in CΔ*acmB* (Figure 4.5B). These data demonstrate that the absence of AcmB in the Δ*acmB* strain cause an increase in autoaggregation, along with a decrease in stress-induced autolysis.

### 4.4.7 S-layer morphology and thickness in Δ*acmB*.

Transmission electron micrographs were taken to assess the S-layer morphology of the *acmB*-deficient strain (Figure 4.6). Compared to WT, the S-layer in Δ*acmB* was less pronounced (Figure 4.6A). As a metric to quantify the S-layer morphology, the S-layer thickness of each strain was measured at logarithmic and stationary phases of growth (Figure

4.6B). At logarithmic phase, the S-layer was thinner in Δ*acmB* (*M* = 10.89 nm, *CI* = 1.73) than in WT (*M* = 15.46 nm, *CI* = 1.10). Likewise, at stationary phase, the S-layer was thinner in Δ*acmB* (*M* = 17.82, *CI* = 1.46) compared to WT (*M* = 21.54, *CI* = 0.39). The changes in the S-layer thickness at both phases were statistically significant ($p < 0.001$; Figure 4.6B).

### 4.4.8 Adherence capacity of Δ*acmB*.

Extracellular proteins localized to the cell surface are important mediators of probiotic activity, including adhesion to host intestinal epithelial mucus layer and extracellular matrices (ECM). Because of the irregular cell and S-layer morphology of Δ*acmB,* the ability of this mutant to bind mucin and ECM including collagen, fibronectin, and laminin, was examined (Figure 4.7). The binding capacity of Δ*acmB* was significantly reduced relative to WT for mucin and all ECM tested (Figure 4.7). Specifically, there was a 50% reduction of cells bound to type III porcine mucin ($p < 0.002$), a 55% reduction of cells bound to type IV human collagen ($p < 0.001$), a 63% reduction to human plasma fibronectin ($p < 0.001$), and a 65% reduction in adherence to murine laminin ($p < 0.001$), relative to WT. These data suggest that the absence of AcmB on the cell surface has a pleiotropic effect on the cell surface, which results in decreased binding to various ECM.

## 4.5 Discussion

The Gram-positive cell wall is comprised of a thick peptidoglycan sacculus responsible for sustaining cell shape, resistance against environmental and osmotic stresses, and the covalent and non-covalent presentation of proteins (38). Extracellular proteins responsible for the turnover of peptidoglycan during cell division and daughter cell separation are known as peptidoglycan hydrolases (PGH), or autolysins (39). These PGH are divided into four classes: (i) β-*N*-acetylmuramidases, (ii) β-*N*-acetyl glucosamidases, (iii) *N*-acetylmuramoyl-*L*-amidases, and (iv) peptidases (23).

In this study, the PGH complement of *L. acidophilus* NCFM was identified and AcmB, an S-layer associated β-*N*-acetylglucosaminidase, was functionally characterized. Within the genome of *L. acidophilus* NCFM, eleven genes encoding putative PGH were identified, including four β-*N*-acetylmuramidases, two β-*N*-acetylglucosaminidases, one *N*-acetylmuramoyl-*L*-amidase, and four peptidases. Nine of these PGH are predicted to be functional based on the presence of signal peptide sequences and RNA transcription analyses. The redundancy of encoded autolysins is consistent with previous studies identifying the PGH complement in other lactobacilli. *L. casei* BL23 encodes 13 PGH (40), while *L. plantarum* WCFS1 encodes 12 (24), and the silage fermenting *L. buchneri* CD034 encodes 24 (41). Notably, the closely-related and S-layer forming cheese-ripening bacteria *L. helveticus* DPC 4571 encodes 9 autolysins, including 5 orthologs from the PGH identified in this study (42). Redundancy of PGH within bacterial genomes is widespread (39) due to the essentiality of autolysin activity for cell survival (43). Further, this redundancy may be due to the fact that many of these hydrolases have more than one function. PGH of *Bacillus subtilis*

have numerous described functions beyond autolytic hydrolase activity, including roles in protein turnover and secretion, motility, and competence (44). However, the characterization of autolysin activity in *Lactobacillus* species, to date, has been primarily focused on the hydrolysis of peptidoglycan sacculi (12).

Autolysins are associated to the cell wall via numerous cell wall binding domains (CWBD) including CHAP domains, GW domains, SH3 domains, and LysM domains (12, 39). Notably, in *L. acidophilus* the primary CWBD is the S-layer non-covalent attachment domain (NCAD; pfam03217), which is responsible for the non-covalent attachment of S-layer and S-layer associated proteins in *Lactobacillus* (10, 25). Five of the nine functional autolysins in *L. acidophilus* NCFM have the S-layer NCAD domain, suggesting extracellular co-localization with the S-layer. In fact, these five proteins were previously identified in the LiCl-purified SLAP fraction of the *L. acidophilus* NCFM exoproteome (22). Based on the *in silico* prediction of the PGH complement identified in this study and the previously identified SLAPs (22), all encoded β-*N*-acetylmuramidases, β-*N*-acetylglucosaminidases, and *N*-acetylmuramoyl-*L*-alanine amidases with a signal peptide sequence in *L.* acidophilus NCFM appear to be SLAPs. These findings are supported by previously published studies on autolysin activity in the S-layer forming strains *L. helveticus* ATCC 12046 and *L. helveticus* ISLC5, in which two autolysins were co-purified with the S-layer using LiCl (45, 46). It is also notable that there are two autolysins described in the PGH complement of *L. helveticus* DPC 4571 which contain the S-layer NCAD domain (42).

One of the five S-layer associated autolysins is AcmB, a 45 kDa protein with a β-*N*-acetylglucosaminidase catalytic domain. Further evidence of the association between this

autolysin and the S-layer can be seen through examination of the AcmB orthologs in *Lactobacillus* species. All known AcmB orthologs are found exclusively in S-layer forming species of the *L. acidophilus* homology group. Notably, AcmB orthologs are not found in the closely related, but non-S-layer forming members of the homology group, including *L. gasseri*, *L. johnsonii*, and the progenitor, *L. delbrueckii* subsp. *bulgaricus*. These data are supported by our recent exoproteomic survey of S-layer and non-S-layer forming species of the *L. acidophilus* homology group (10). Autolysins, including AcmB, were found in the non-covalently bound SLAP fractions of the S-layer forming *L. crispatus* ST1, *L. amylovorus* GRL1112, and *L. helveticus* CNRZ32, but were not found in the non-S-layer forming species tested (10).

There are two predicted β-*N*-acetylglucosaminidases in *L. acidophilus* NCFM, AcmA and AcmB. Because AcmA does not have a signal peptide sequence and has lower transcriptional expression compared to the other autolysins, AcmB appears to be the principal β-*N*-acetylglucosaminidase for *L. acidophilus* NCFM. To elucidate the role of AcmB in cell wall physiology and autolytic function, a Δ*acmB* isogenic mutant was created and complemented with an AcmB-expression vector. Results indicate that the Δ*acmB* strain presents an altered cellular morphology consisting of increased chain lengths and autoaggregation, as well as decreased stress induced autolysis. The phenotypes of the *L. acidophilus* NCFM Δ*acmB* strain are consistent with the characterization of a β-*N*-acetylglucosaminidase, Acm2, in *L. plantarum* WCFS1 which presented a similar de-chaining and autolysis phenotype (24). Collectively, these data suggest that AcmB is a functioning autolysin involved in peptidoglycan turnover and daughter cell separation during

cell division. Further work is necessary to characterize the specific hydrolytic activity of AcmB in *L. acidophilus* NCFM.

The Δ*acmB* strain appeared to be have pleiotropic effect on the S-layer morphology and subsequent presentation of S-layer and S-layer associated proteins. In the AcmB-deficient mutant, the S-layer thickness was significantly reduced compared to the S-layer of the parent strain during logarithmic and stationary phases. A recent analysis of an S-layer associated fibronectin binding protein FbpB revealed a similar S-layer phenotype in the FbpB-deficient strain (47) The FbpB mutant did not have an elongated cell length, suggesting that the absence of FbpB in the S-layer was responsible for the thinning of the S-layer compared to the parent. AcmB may be contributing to the S-layer morphology in a similar way. However, it remains to be determined whether this morphological phenotype is due to the increased cell and chain length or the physical absence of AcmB from the S-layer in the Δ*acmB* strain. Surprisingly, the altered S-layer morphology in the AcmB mutant lead to a diminished capacity for binding to mucin and extracellular matrices (ECM) collagen, fibronectin, and laminin, *in vitro*. The mechanism for the adhesion phenotype in Δ*acmB* remains unclear. It is possible that AcmB may directly interact with ECM in addition to its autocatalytic activity, not unlike the moonlighting proteins enolase, GAPDH, and GroEL, which have demonstrated secondary functions in adhesion to ECM in various *Lactobacillus* species (48-50). It seems more likely, however, that the reduced adhesion phenotype is due to the increased autoaggregation phenotype of Δ*acmB*, in which relevant adhesins at the cell surface may not be as exposed for contact with ECM.

In conclusion, we have shown that the SLAP, AcmB, is an autolysin involved in cell division and daughter cell separation in *L. acidophilus* NCFM. AcmB has an effect, directly or indirectly, on the adhesion of *L. acidophilus* NCFM to ECM, an important attribute for probiotic bacteria. Orthologs of AcmB are found exclusively in S-layer forming species of the *L. acidophilus* homology group. Further, many of these autolysins, including AcmB, have been identified as SLAPs in the non-covalent exoproteomes of the S-layer forming species of said homology group. There is a dearth of information regarding the evolutionary function of S-layers in bacteria, especially those in *Lactobacillus.* Here, we propose that the S-layer may function as scaffold for multiple proteins, including autolysins. Understanding the biological roles of these autolysins offers important evolutionary insights regarding the essentiality of the S-layer, as well as physiological insights regarding cell division and peptidoglycan hydrolysis in *Lactobacillus* species that produce S-layers.

## 4.6 Acknowledgments

This work was supported through funding from the North Carolina Agriculture foundation (Raleigh, NC) ad DuPont Nutrition & Health (Madison, WI). The authors wish to thank Dr. Yong Jun Goh and Dr. Sarah O'Flaherty for insightful discussion and critical reading of the manuscript.

## 4.7 References


1. **Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME**. 2014. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol **11**:506–514.

2. **Sanders ME, Klaenhammer TR**. 2001. Invited review: The scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic. J Dairy Sci **84**:319-331.

3. **Altermann E, Russell WM, Azcarate-Peril MA, Barrangou R, Buck BL, McAuliffe O, Souther N, Dobson A, Duong T, Callanan M, Lick S, Hamrick A, Cano R, Klaenhammer TR**. 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. Proc Natl Acad Sci USA **102**:3906–3912.

4. **Klaenhammer TR, Barrangou R, Buck BL, Azcarate-Peril MA, Altermann E**. 2005. Genomic features of lactic acid bacteria effecting bioprocessing and health. FEMS Microbiol Rev **29**:393-409.

5. **Pfeiler EA, Klaenhammer TR**. 2007. The genomics of lactic acid bacteria. Trends Microbiol **15**:546-553.

6. **Klaenhammer TR, Altermann E, Pfeiler EA, Buck BL, Goh YJ, O'Flaherty S, Barrangou R, Duong T**. 2008. Functional genomics of probiotic Lactobacilli. J Clin Gastroenterol **42**:160-162.

7. **O'Flaherty S, Klaenhammer TR**. 2010. The role and potential of probiotic bacteria in the gut, and the communication between gut microflora and gut/host. Int Dairy J **20**:262-268.

8. **Johnson BR, Klaenhammer TR**. 2014. Impact of genomics on the field of probiotic research: historical perspectives to modern paradigms. Antonie Van Leeuwenhoek **106**:141–156.

9. **Bron PA, Tomita S, Mercenier A, Kleerebezem M**. 2013. Cell surface-associated compounds of probiotic lactobacilli sustain the strain-specificity dogma. Curr Opin Microbiol **16**:262–269.

10. **Johnson BR, Hymes J, Sanozky-Dawes R, DeCrescenzo Henriksen E, Barrangou R, Klaenhammer TR**. 2016. Conserved S-layer-associated proteins revealed by exoproteomic survey of S-layer forming lactobacilli. Appl Environ Microbiol **82**:134-

145.

11. **Kleerebezem M, Hols P, Bernard E, Rolain T, Zhou M, Siezen RJ, Bron PA**. 2010. The extracellular biology of the lactobacilli. FEMS Microbiol Rev **34**:199-230.

12. **Chapot-Chartier MP, Kulakauskas S**. 2014. Cell wall structure and function in lactic acid bacteria. Microb Cell Fact **13**(Suppl 1):S9.

13. **Cabeen MT, Jacobs-Wagner C**. 2005. Bacterial cell shape. Nat Rev Microbiol **3**:601-610.

14. **Vollmer W, Seligman SJ**. 2010. Architecture of peptidoglycan: more data and more models. Trends Microbiol **18**:59-66.

15. **Call EK, Klaenhammer TR**. 2013. Relevance and application of sortase and sortase-dependent proteins in lactic acid bacteria. Front Microbiol **4**:73.

16. **Hynönen U, Palva A**. 2013. *Lactobacillus* surface layer proteins: structure, function and applications. Appl Microbiol Biotechnol **97**:5225–5243.

17. **Goh, YJ, Azcarate-Peril MA, O'Flaherty S, Durmaz E, Valence F, Jardin J, Lortal S, Klaenhammer TR.** 2009. Development and application of a *upp*-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. Appl. Environ. Microbiol. **75:**3093-3105.

18. **Buck BL, Altermann E, Svingerud T, Klaenhammer TR**. 2005. Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol **71**:8344–8351.

19. **Frece J, Kos B, Svetec IK, Zgaga Z, Mrsa V, Susković J**. 2005. Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. J Appl Microbiol **98**:285–292.

20. **Konstantinov SR, Smidt H, de Vos WM, Bruijns SCM, Singh SK, Valence F, Molle D, Lortal S, Altermann E, Klaenhammer TR, van Kooyk Y**. 2008. S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. Proc Natl Acad Sci USA **105**:19474–19479.

21. **Lightfoot YL, Selle K, Yang T, Goh YJ, Sahay B, Zadeh M, Owen JL, Colliou N, Li E, Johannssen T, Lepenies B, Klaenhammer TR, Mohamadzadeh M**. 2015. SIGNR3-dependent immune regulation by *Lactobacillus acidophilus* surface layer protein A in colitis. EMBO J **34**:881–895.

22. **Johnson B, Selle K, O'Flaherty S, Goh YJ, Klaenhammer T**. 2013. Identification of extracellular surface-layer associated proteins in *Lactobacillus acidophilus* NCFM. Microbiology **159**:2269–2282.

23. **Layec S, Decaris B, Leblond-Bourget N**. 2008. Diversity of Firmicutes peptidoglycan hydrolases and specificities of those involved in daughter cell separation. Res Microbiol **159**:507-515.

24. **Rolain T, Bernard E, Courtin P, Bron PA, Kleerebezem M, Chapot-Chartier MP, Hols P**. 2012. Identification of key peptidoglycan hydrolases for morphogenesis, autolysis, and peptidoglycan composition of *Lactobacillus plantarum* WCFS1. Microb Cell Fact **11**:137.

25. **Boot HJ, Kolen CP, Pouwels PH**. 1995. Identification, cloning, and nucleotide sequence of a silent S-layer protein gene of *Lactobacillus acidophilus* ATCC 4356 which has extensive similarity with the S-layer protein gene of this species. J Bacteriol **177**:7222–7230.

26. **Hanahan D**. 1983. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol **166**:557-580.

27. **Walker DC, Aoyama K, Klaenhammer TR**. 1996. Electrotransformation of *Lactobacillus acidophilus* group A1. FEMS Microbiol Lett **138**: 233-237.

28. **Wei MQ, Rush CM, Norman JM, Hafner LM, Epping RJ, Timms P**. 1995. An improved method for the transformation of *Lactobacillus* strains using electroporation. J Microbiol Methods **21**: 97-109.

29. **Somervuo P, Holm L**. 2015. SANSparallel: interactive homology search against Uniprot. Nucleic Acids Res **43**:W24-W29.

30. **Petersen TN, Brunak S, von Heijne G, Nielsen H**. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods **8**:785–786.

31. **The UniProt Consortium**. 2011. Reorganizing the protein space at the Universal Protein Resource (UniProt). Nucleic Acids Res **40**:D71-D75.

32. **Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, Heger A, Hetherington K, Holm L, Mistry J, Sonnhammer ELL, Tate J, Punta M**. 2014. Pfam: the protein families database. Nucleic Acids Res **42**:D222–230.

33. **Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond**

**A**. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics **28**:1647–1649.

34. **Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR**. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77**:61-68.

35. **Duong T, Miller MJ, Barrangou R, Azcarate-Peril MA, Klaenhammer TR**. 2011. Construction of vectors for inducible and constitutive gene expression in *Lactobacillus*. Microbiol Biotechnol **4**:357-367.

36. **Kos B, Suskovic J, Vukovic S, Simpraga M, Frece J, Matosic S**. 2003. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. J Appl Microbiol **94**:981-987.

37. **Goh YJ, Klaenhammer TR**. 2010. Functional roles of aggregation-promoting-like factor in stress tolerance and adherence of *Lactobacillus acidophilus* NCFM. Appl and Environ Microbiol **76**:5005-5012.

38. **Vollmer W, Blanot D, De Pedro MA**. 2008. Peptidoglycan structure and architecture. FEMS Microbiol Rev **32**:149-167.

39. **Vollmer W, Joris B, Charlier P, Foster S**. 2008. Bacterial peptidoglycan (murein) hydrolases. FEMS Microbiol Rev **32**:259-286.

40. **Regulski K, Courtin P, Meyran M, Claes IJJ, Lebeer S, Vanderleyden J, Hols P, Guillot A, Chapot-Chartier MA**. 2012. Analysis of the peptidoglycan hydrolase complement of *Lactobacillus casei* and characterization of the major γ-*D*-glutamyl-*L*-lysyl-endopeptidase. Plos One. **7**:e32301

41. **Anzenbruger J, Courtin P, Claes IJJ, Debreczeny M, Hofbauer S, Obinger C, Chapot-Chartier MA, Vanderleyden J, Messner P, Schäffer C**. 2014. Biochemical characterization of the major *N*-acetylmuramidase from *Lactobacillus buchneri*. Microbiology 160:1807-1819.

42. **Jebava I, Plockova M, Lortal S, Valence F**. 2011. The nine peptidoglycan hydrolases genes in *Lactobacillus helveticus* are ubiquitous and early transcribed. Int J Food Microbiol **148**:1-7.

43. **Typas A, Banzhaf M, Gross CA, Vollmer W**. 2012. From the regulation of peptidoglycan synthesis to bacterial growth and morphology. Nat Rev Microbiol **10**:123-136.

44. **Smith TJ, Blackman SA, Foster SJ**. 2000. Autolysins of *Bacillus subtilis*: multiple enzymes with multiple functions. Microbiology **146**:249-262.

45. **Lortal S, Rousseau M, Boyaval P, Heijenoort JV**. 1991. Cell wall and autolytic system of *Lactobacillus helveticus* ATCC 12046. Microbiology **137**:549-559.

46. **Valence F, Lortal S**. 1995. Zymogram and preliminary characterization of *Lactobacillus helveticus* autolysins. Appl Environ Microbiol **61**:3391-3399.

47. **Hymes J, Johnson BR, Barrangou R, Klaenhammer TR**. 2016. Functional analysis of an S-layer associated fibronectin-binding protein in *Lactobacillus acidophilus* NCFM. In review.

48. **Castaldo C, Vastano V, Siciliano RA, Candela M, Vici M, Muscariello L, Marasco R, Sacco M**. 2009. Surface displaced alpha-enolase of *Lactobacillus plantarum* is a fibronectin binding protein. Microb Cell Fact **8**:14

49. **Kainulainen V, Loimaranta V, Pekkala A, Edelman S, Antikainen J, Kylvaja R, Laaksonen M, Laakkonen L, Finne J, Korhonen TK**. 2012. Glutamine synthetase and glucose-6-phosphate isomerase are adhesive moonlighting proteins of *Lactobacillus crispatus* released by epithelial cathelicidin LL-37. J Bacteriol **194**:2509-2519.

50. **Kainulainen V, Korhonen TK**. 2014. Dancing to another tune – adhesive moonlighting proteins in bacteria. Biology **3**:178-204.

51. **Russell WM, Klaenhammer TR**. 2001. Efficient system for directed integration into the Lactobacillus acidophilus and Lactobacillus gasseri chromosomes via homologous recombination. Appl Environ Microbiol **67**:4361-4364.

**Table 4.1** Strains and Plasmids used in this study

| Strain or plasmid | Genotype or characteristics | Reference |
|---|---|---|
| ***L. acidophilus* strains** | | |
| NCFM | Human intestinal isolate | 2 |
| NCK1909 | NCFM carrying a 315-bp in-frame deletion within the *upp* gene | 17 |
| NCK1910 | NCK1909 harboring pTRK669; host for pORI-based counterselective integration vector | 17 |
| NCK2395 | NCK1909 carrying a 1,103-bp in-frame deletion within the *lba0176* gene | This study |
| NCK2397 | NCK2395 harboring pTRK1098 for complementation of *lba0176* | This study |
| ***E. coli* (EC101) strains** | | |
| NCK1911 | Host harboring pTRK935, $Kn^r$, $Em^r$ | 17 |
| NCK2394 | Host harboring pTRK1097, $Kn^r$, $Em^r$ | This Study |
| NCK2396 | Host harboring pTRK1098, $Erm^r$ | This Study |
| **Plasmids** | | |
| pTRK669 | Ori (pWV01), Cmr , $RepA^+$ thermosensitive | 51 |
| pTRK935 | pORI *upp*-based counterselective integration vector | 17 |
| pTRK882 | Δ*cat* derivative of pGK12 with MCS from pORI28 and cloned $P_{pgm}$, $Erm^r$ | 35 |
| pTRK1097 | pTRK935 with flanking regions of *lba0176* cloned into BamHI/SacI site | This study |
| pTRK1098 | pTRK882 with *lba0176* cloned into EcoRI/BamHI site | This study |

**Table 4.2** The autolysins of *L. acidophilus* NCFM

| ORF | ID | UniProt ID | PGH domain[a] | AA length | SignalP[b] | SLAP[c] |
|---|---|---|---|---|---|---|
| *Lba1744* | Putative glycosidase | Q5FIB9 | pfam00877 | 184 | + | - |
| *lba1741* | Cell wall-associated hydrolase | Q5FIC1 | pfam00877 | 299 | + | - |
| *lba1743* | Cell wall-associated hydrolase | Q5FIC0 | pfam00877 | 262 | + | - |
| *lba1883* | NLP-P60 secreted protein | Q5FHZ1 | pfam00877 | 160 | + | - |
| *acmA* (*lba0527*) | N-acetylglucosaminidase | Q5FLL6 | pfam01832 | 215 | - | - |
| *acmB* (*lba0176*) | N-acetylglucosaminidase | Q5FMJ9 | pfam01832 | 409 | + | + |
| *lba0616* | Putative uncharacterized protein | Q5FLC9 | pfam01183 | 234 | - | - |
| *LysA* (*lba1918*) | Lysin | Q5FHV9 | pfam01183 | 323 | + | + |
| *lba1140* | Lysin | Q5FJZ4 | pfam01183 | 382 | + | + |
| *lba1351* | Lysin | Q5FJE6 | pfam01183 | 404 | + | + |
| *lba0177* | Autolysin/amidase | Q5FMJ8 | pfam01510 | 364 | + | + |

[a]Catalytic domain determined by pfam (32).
[b]Signal peptidase cleavage site (30).
[c]Previously identified as an S-layer Associated Protein (22).

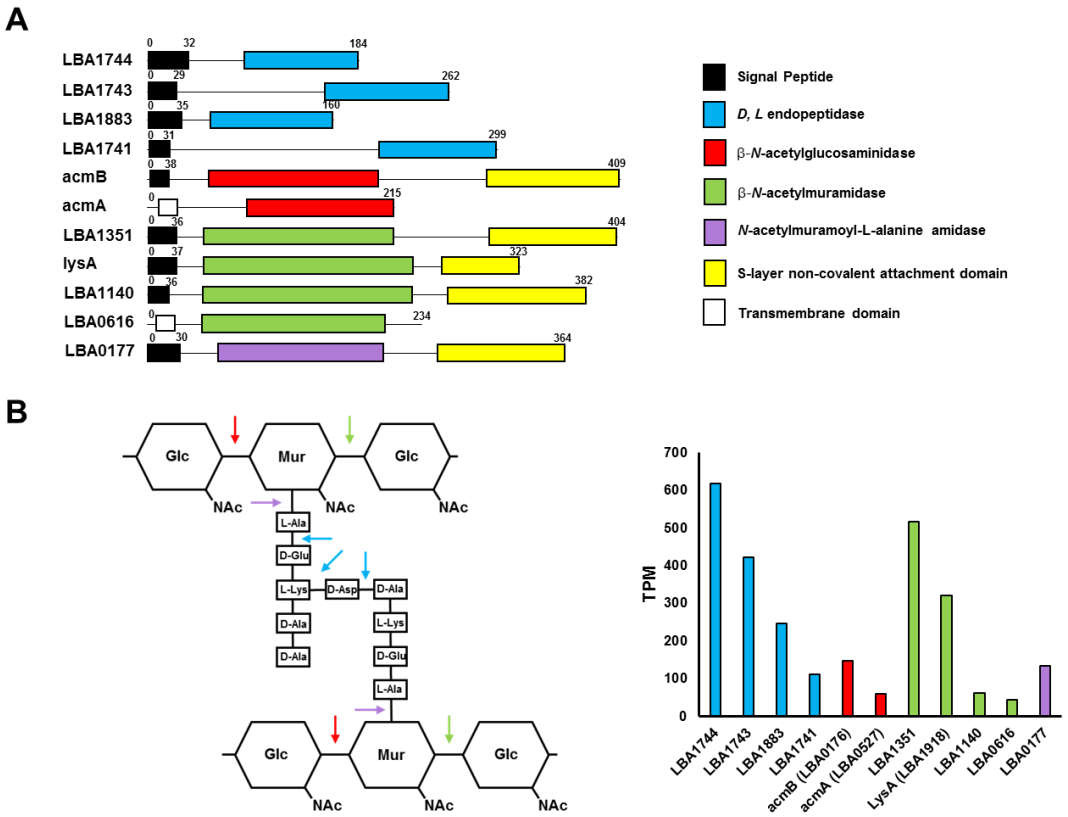


**Figure 4.1.** (A) The modular domain organization of the eleven predicted autolysins of *L. acidophilus* NCFM. The numbers indicate the corresponding amino acid length. The colored blocks represent the following domains: black, Signal peptide sequence; white, transmembrane region; blue, *D*/*L*-endopeptidase (pfam00877); red, β-*N*-acetylglucosaminidase (pfam01832); green, β-*N*-acetylmuramidase (pfam01183; purple, *N*-acetylmuramoyl-*L*-alanine amidase (pfam01510); and yellow, S-layer non-covalent attachment domain (pfam03217). (B) The corresponding specific activity of each catalytic domain on the peptidoglycan structure. (C) RNA-seq transcriptional analysis of the gene encoding each autolysin, with colors corresponding to mRNA transcripts from genes with the following catalytic activities: blue, endopeptidase; red, β-*N*-acetylglucosaminidase; green, β-*N*-acetylmuramidase; and purple, *N*-acetylmuramoyl-*L*-alanine amidase.

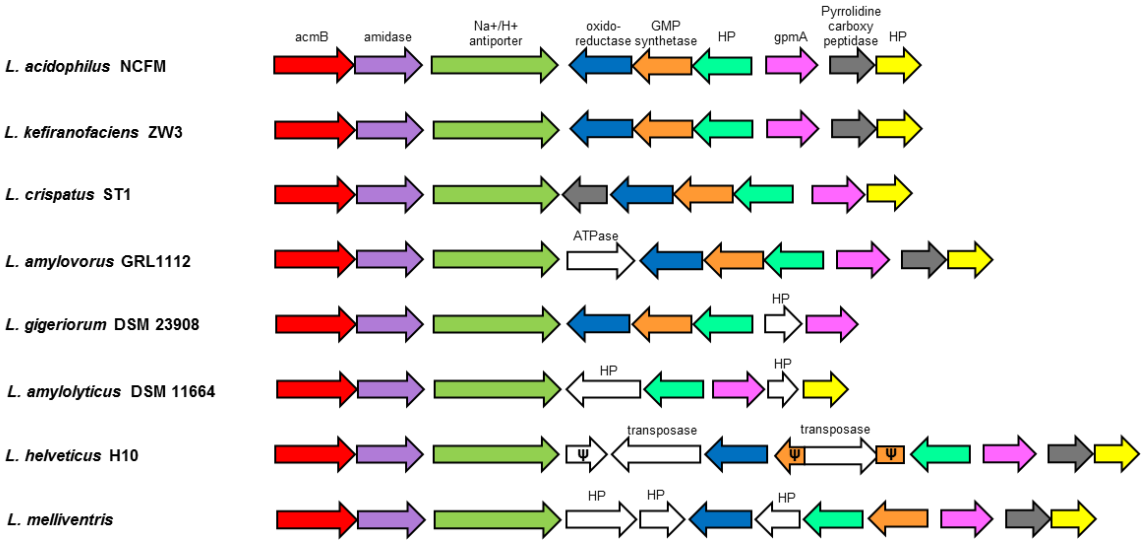


**Figure 4.2.** *acmB* orthologs were found in various S-layer forming strains of *Lactobacillus*, including *L. amylovorus* GRL1112, *L. helveticus* H10, *L. kefiranofaciens* ZW3, *L. crispatus* ST1, *L. melliventris*, *L. amylolyticus* DSM 11664, and *L. gigeriorum* DSM 23908. The genetic region surrounding *acmB* was highly syntenic in all species examined. Arrows represent genes, while the colors represented specific genes, as follows: red, *acmB*; purple, amidase; green, $Na^+/H^+$ ion transporter; blue, oxidoreductase; orange, GMP synthetase; teal, hypothetical protein (HP); pink, *gpmA*; dark grey, pyrrolidine carboxypeptidase; and yellow, HP. Genes in white are divergent genes unique to each species where indicated. Ψ indicates a truncated, pseudogene.

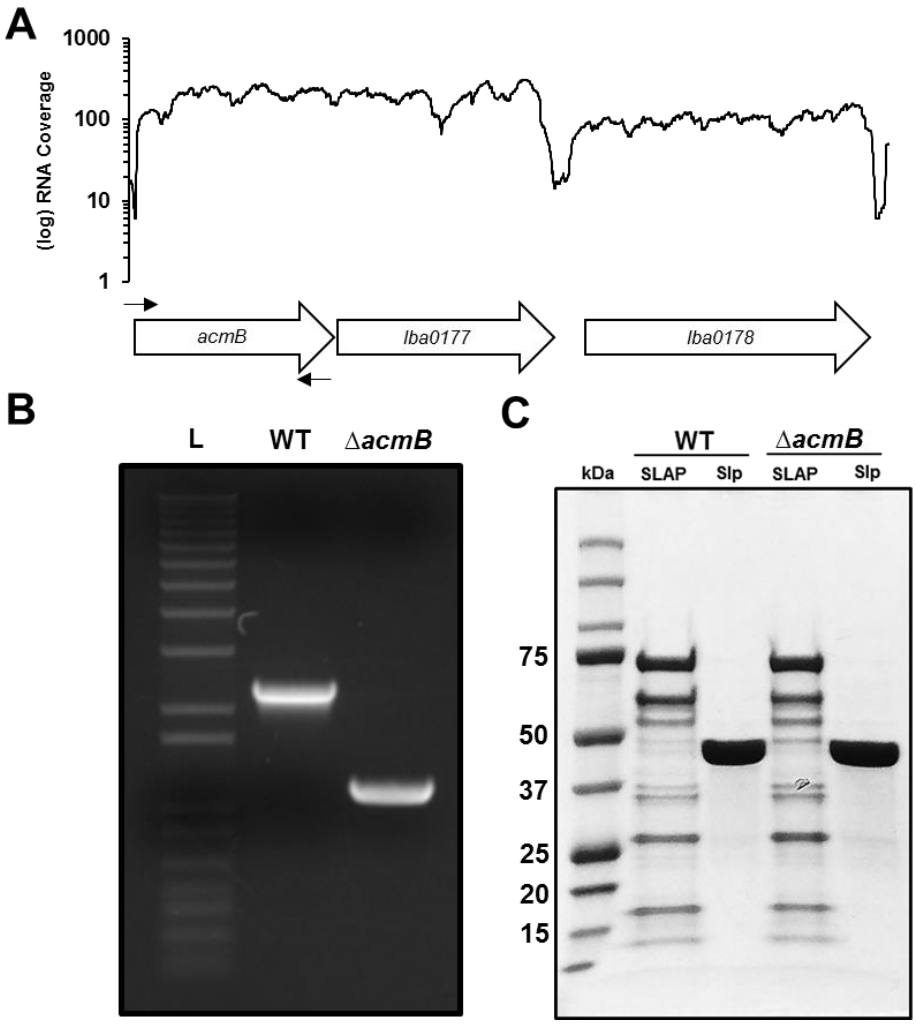


**Figure 4.3.** The gene encoding AcmB was deleted from the chromosome of *L. acidophilus* NCFM. (A) RNA-seq analysis demonstrates that *acmB* is polycistronically expressed with *lba0177*, which encodes an S-layer associated *N*-acetylmuramoyl-*L*-alanine amidase. Black arrows indicate the forward and reverse primer pair used to confirm the deletion of *acmB*. (B) Gel electrophoresis of PCR products using the primers indicated in (A) on the parent strain (WT) compared to the AcmB-deficient strain (Δ*acmB*). The deletion was confirmed by sequencing. (C) The non-covalently bound extracellular S-layer proteins (SLP) and S-layer associated proteins (SLAPs) were isolated from both WT and Δ*acmB*.

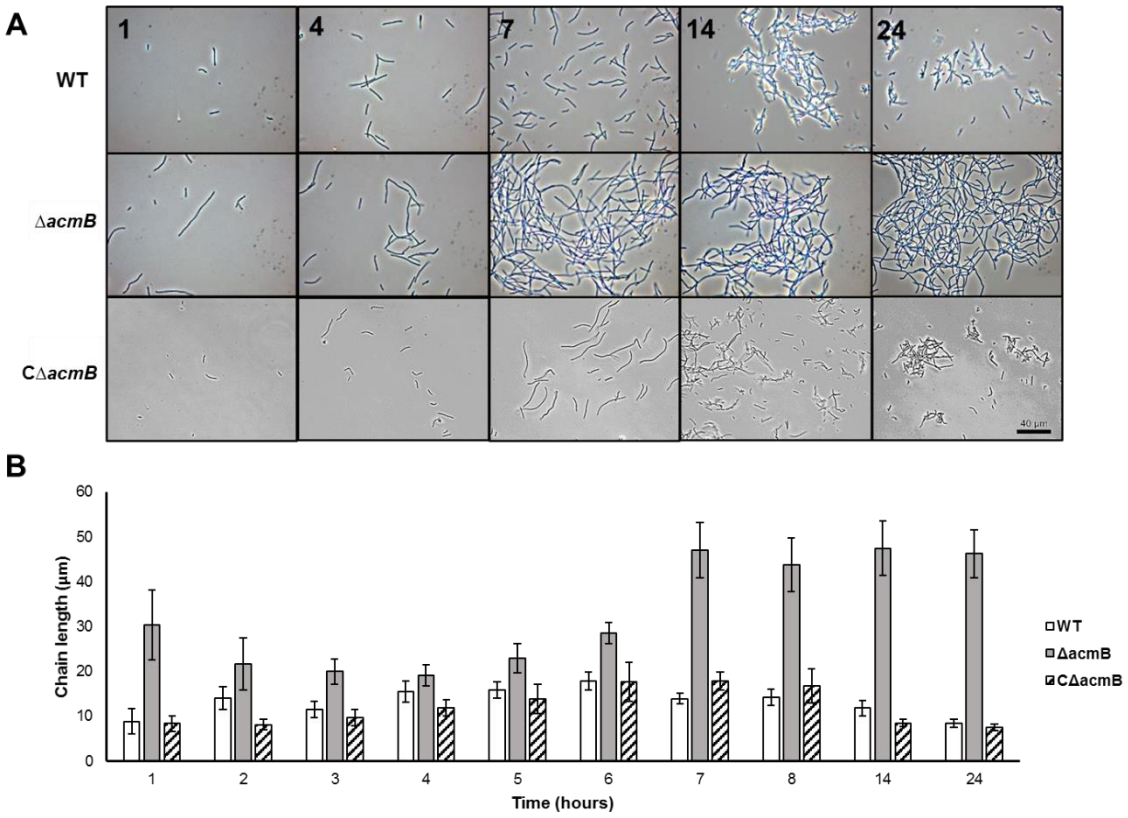


**Figure 4.4.** (A) The cellular morphology of the wild-type (WT), mutant (Δ*acmB*), and the *acmB* complemented strain (CΔ*acmB*) were assessed using phase contrast light microscopy over a 24 hour growth period. (B) Chain length measurements were taken for WT (white bar), Δ*acmB* (grey bar), and CΔ*acmB* (black and white striped bar). The chain length for Δ*acmB* was significantly higher than WT and CΔ*acmB* across all time points measured ($p < 0.001$). Error bars represent confidence intervals.

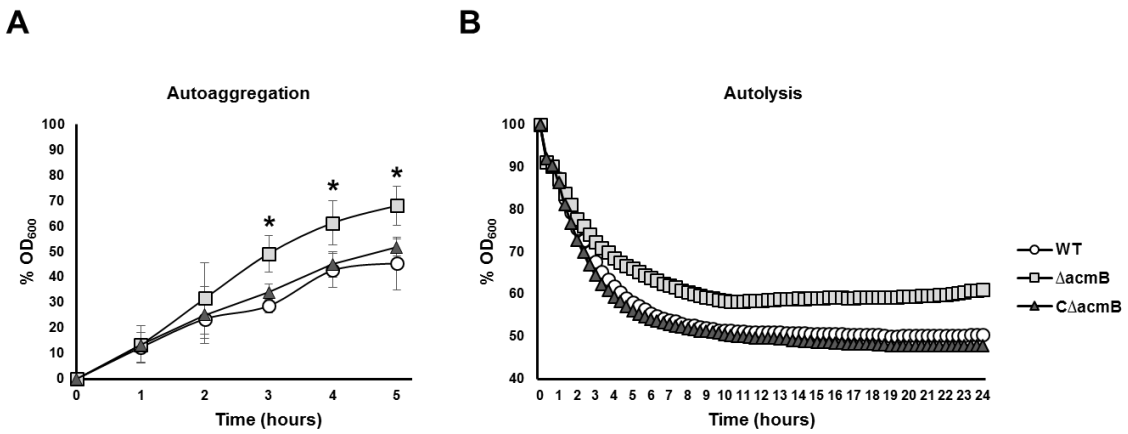


**Figure 4.5.** (A) Autoaggregation of WT (circle), mutant (Δ*acmB*; square), and complemented (CΔ*acmB*; circle) cells. Asterisks indicate statistical significance ($p < 0.001$). (B) Triton X-100 induced autolysis assays for WT (circle), Δ*acmB* (square), and complemented (circle) strains. The difference between Δ*acmB* and the WT and complemented strains are statistically significant ($p < 0.001$). Each assay was performed in triplicate; all error bars represent confidence intervals.

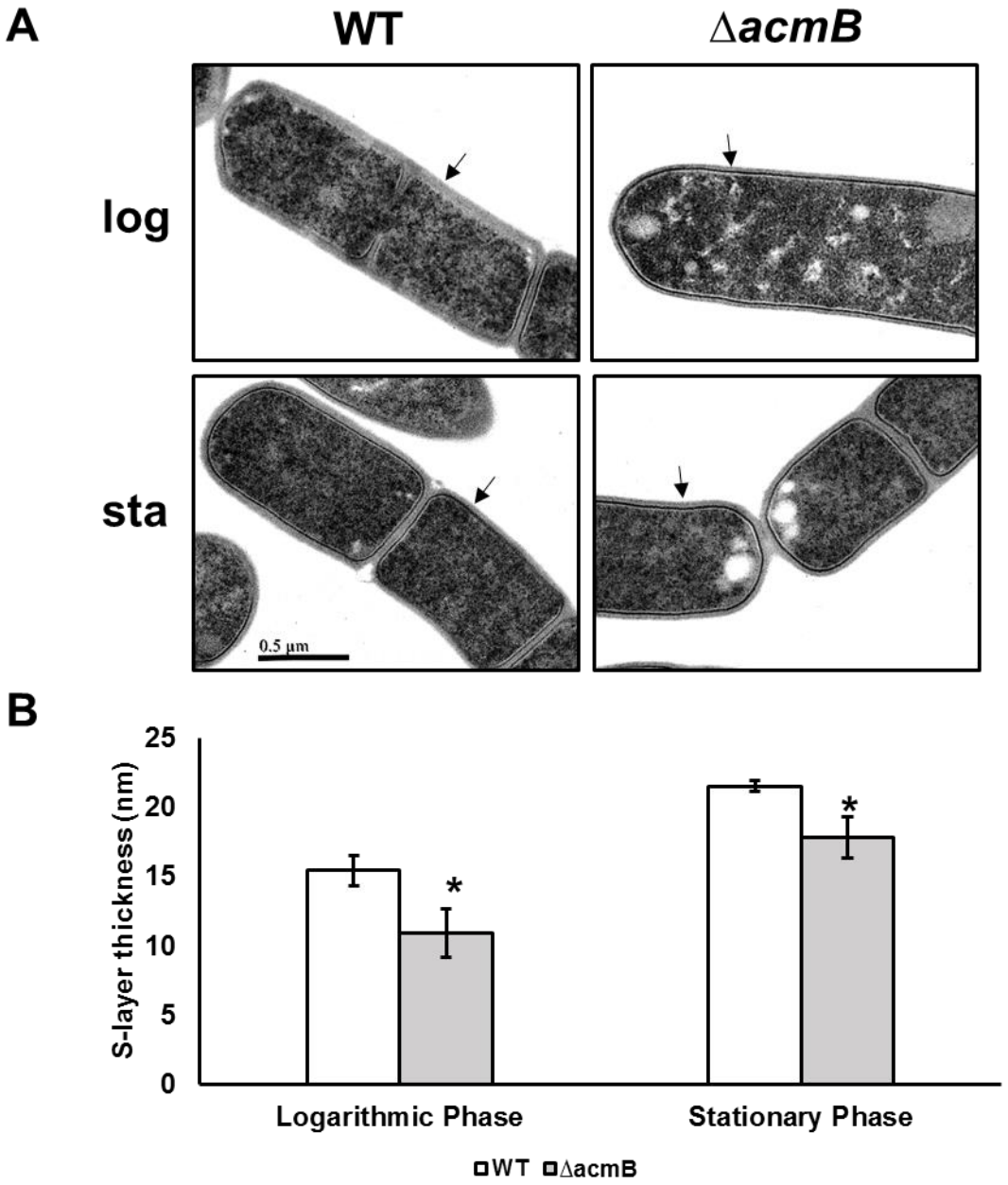


**Figure 4.6.** (A) Transmission electron micrographs of the wild-type reference (WT), compared to the AcmB-deficient strain (Δ*acmB*) at logarithmic (log) and stationary (sta) phase. The S-layer morphology, indicated with black arrows, appeared to be altered in the Δ*acmB* strain. (B) S-layer thicknesses were measured for WT and Δ*acmB* during logarithmic and stationary growth. Asterisks indicate statistical significance ($p < 0.001$). Error bars represent confidence intervals.

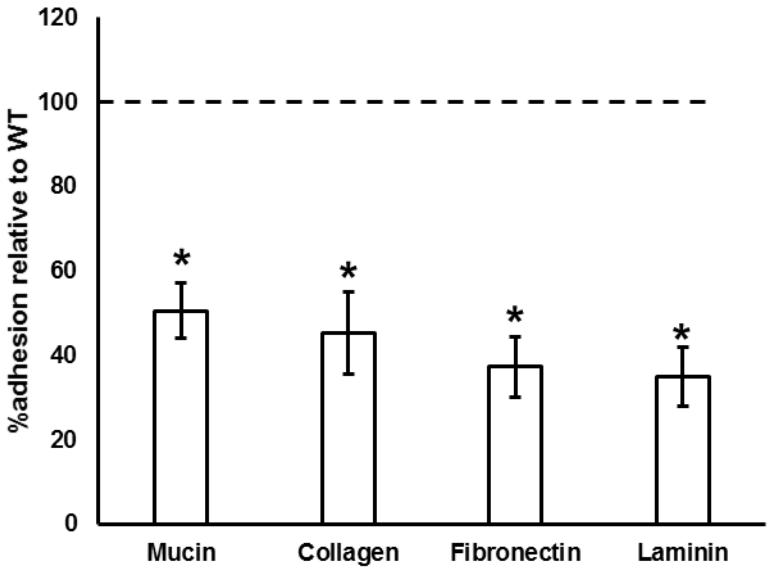


**Figure 4.7.** The ability of the Δ*acmB* mutant to bind to mucin and extracellular matrices (ECM) was assessed. Compared the wild-type reference (WT, dotted line), Δ*acmB* showed a significant reduction in binding to mucin, collagen, fibronectin, and laminin. Asterisks indicate statistical significance ($p < 0.001$). Adherence assays were performed in triplicate; all error bars represent confidence intervals.

# CHAPTER 5

## Characterization of an S-layer associated serine protease in *Lactobacillus acidophilus* NCFM

Planned submission to Frontiers in Microbiology

## 5.1 Abstract

Health-promoting aspects attributed to probiotic microorganisms, including adhesion to intestinal epithelia and modulation of the host mucosal immune system, are mediated by proteins found on the bacterial cell surface. Notably, certain probiotic and commensal bacteria contain a surface (S-) layer as the outermost stratum of the cell wall. S-layers are non-covalently bound semi-porous, crystalline arrays of self-assembling, proteinaceous subunits called S-layer proteins (SLPs). Recent evidence has shown that multiple proteins are non-covalently co-localized within the S-layer, designated S-layer associated proteins (SLAPs). In *Lactobacillus acidophilus* NCFM, SLP and SLAPs have been implicated in both mucosal immunomodulation and adhesion to the host intestinal epithelium. In this study, an S-layer associated serine protease, PrtX was characterized. The gene encoding PrtX (*prtX, lba1578*) was deleted from the chromosome of *L. acidophilus*. Compared to the parent strain, the PrtX-deficient strain (Δ*prtX*) demonstrated increased autoaggregation and an altered cellular morphology, which correlated to a pleiotropic increase in adhesion to mucin and fibronectin, *in vitro*. Furthermore, Δ*prtX* demonstrated increased immunomodulation of TNF-α, IL-6, and IL-10 compared to wild-type, when exposed to mouse dendritic cells. Finally, *in vivo* colonization of germ-free mice with Δ*prtX* resulted in an increase in epithelial barrier integrity. These data indicate that the S-layer associated PrtX is involved in protein turnover at the cell surface of *L. acidophilus* NCFM. The absence of PrtX in a Δ*prtX* strain led to an altered presentation of cell surface proteins, resulting in an amplification of some inherent probiotic properties of *L. acidophilus* NCFM.

## 5.2 Introduction

Lactic acid bacteria (LAB) are a diverse clade of Gram-positive, microaerophilic, and non-sporulating microbes which ferment hexoses primarily to lactic acid. Many of these bacteria, which include species from the genera *Lactococcus*, *Enterococcus*, *Pediococcus*, *Oenococcus*, *Streptococcus*, *Leuconostoc*, and *Lactobacillus*, have evolved through thousands of years of genome decay, to survive in and ferment numerous food and drink substrates (1-3). Predicated on this long history of consumption in foods, many LAB are generally recognized as safe and serve vital industrial roles as starter and adjunct cultures for fermentation in dairy, vegetable, meat, and wine foodstuffs (1, 4).

In addition to their evolution to food, some LAB are niche-associated with the mucosal surfaces of animals, including the gastrointestinal and urogenital tracts (3, 5). Some of these, most notably in the *Lactobacillus* genera, have been used as probiotics, which are defined by the FAO/WHO as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (6). One such organism is *Lactobacillus acidophilus* NCFM, which is an industrially significant probiotic, delivered commercially in various dairy and dietary supplement formulations for the past 35 years (7). In fact, *L. acidophilus* NCFM is one of the most studied and well characterized probiotic bacteria (7-10).

Proteins and macromolecules at the cell surface of probiotic bacteria play a critical role in mediating the strain-specific beneficial effects of probiotics towards the host (11, 12). For *L. acidophilus* NCFM, the specificity of probiotic activity on the host has been characterized for numerous cell surface components including lipoteichoic acid (13), sortase-

dependent mucus-binding proteins (14); an aggregation promoting factor (15), and a myosin-cross reactive protein (16). Most notably, proteins at the Surface (S-) layer, which are non-covalently bound to the outermost component of the cell wall, are of particular interest to the understanding of microbe --host interactions (3, 17).

S-layers are non-covalently bound, semi-porous, proteinaceous crystalline arrays comprised of self-assembling (glyco) protein subunits called S-layer proteins (SLPs; 18). In *L. acidophilus*, the S-layer array is comprised of a dominant protein constituent, SlpA (46 kDa) with minor constituents SlpB (47 kDa) and SlpX (51 kDa; 19). *In vitro* studies using intestinal epithelial cell lines suggest SLPs as a major factor in intestinal adhesion for *L. acidophilus* (20, 21). Furthermore, SlpA of *L. acidophilus* NCFM has been shown to bind dendritic cell C-type lectin receptors (22) and exert immunomodulatory signals which mitigate inflammatory disease states and promote maintenance of healthy intestinal barrier function (23). Recent evidence has also shown that there are additional proteins which non-covalently co-localize to the outermost stratum of the cell surface with the S-layer, called S-layer associated proteins (SLAPs; 24, 25). Due to the apparent importance of *Lactobacillus* cell surface proteins for probiotic roles, these SLAPs have been the target of recent investigations.

SLAPs were first identified in *L. acidophilus* NCFM (24), but have since been identified in *L. helveticus*, *L. crispatus*, *L. amylovorus*, and *L. gallinarum* (25). Notably, these SLAP-containing organisms are S-layer forming members of the *L. acidophilus*-*L.delbrueckii* homology group. However, no SLAPs were identified in the exoproteomes of the closely-related, non-S-layer forming members of the homology group, notably *L. gasseri*,

*L. johnsonii*, as well as the taxonomic progenitor *L. delbrueckii* subsp. *bulgaricus* (25). Preliminary functional analyses in *L. acidophilus* NCFM have revealed that SLAPs have a broad spectrum of both cellular and probiotic functionality, including cell division (26, 27), autolysin activity (27), immunomodulation of mouse dendritic cells (24), and adhesion to extracellular matrices (27, 28).

One of the most prevalent SLAPs identified in the exoproteome of *L. acidophilus* NCFM is a 72 kDa, uncharacterized serine protease encoded by the gene *lba1578* (24). In this study, the S-layer associated serine protease, which we designate PrtX, was selected for functional analysis and found to play an important role in cell division and protein turnover in the S-layer surface of *L. acidophilus* NCFM.

## 5.3 Materials and Methods

### 5.3.1 Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *L. acidophilus* strains were propagated in de Man Rogosa and Sharpe (MRS) broth (Difco) under aerobic conditions, statically or on MRS solid medium containing 1.5% (w/v) agar (Difco) under anaerobic conditions at 37 °C, and at 42 °C where indicated. Recombinant strains were selected in the presence of 2 μg/ml of erythromycin (Em; Sigma-Aldrich) and/or 2 to 5 μg/ml of chloramphenicol (Cm; Sigma). *Escherichia coli* strains were grown in brain heart infusion (Difco) medium at 37 °C with aeration. *E. coli* EC101 was grown in the presence of 40 μg/ml kanamycin (Kn; Sigma-Aldrich) while NCK1911 and transformants were grown with 40 μg/ml Kn and 150 μg/ml Em. Counterselection of plasmid-free excision recombinants was performed using 5-fluorouracil-supplemented glucose semi-defined medium, as previously described (19).

### 5.3.2 Molecular techniques.

Genomic DNA from *L. acidophilus* strains was isolated using a Zymo Research Fungal/Bacterial DNA MiniPrep kit (Zymo Research). Plasmid DNA from *E. coli* was isolated using a QIAprep Spin Miniprep kit (Qiagen). Restriction enzyme digestions and ligations were performed using Roche restriction enzymes (Roche Diagnostics) and T4 DNA ligase (New England Biolabs), respectively. PCR primers were designed based on the genomic sequence data and synthesized by Integrated DNA Technologies (Coralville, Iowa). PCRs were performed in Bio-Rad MyCycler thermocyclers (Bio-Rad Laboratories) using

Choice-Taq Blue DNA polymerase (Denville Scientific) for screening of recombinants and *PfuUltra* II fusion HS DNA polymerase (Agilent Technologies) for cloning. PCR amplicons were analyzed on 0.8 % agarose gels and purified using QIAquick Gel Extraction kits (Qiagen).

*E. coli* EC101 cells were made competent using a rubidium chloride competent cell protocol (29). *L. acidophilus* cells were prepared for electrotransformation using a modified penicillin treatment protocol (19, 30-31).

### 5.3.3 Construction of a Δ*prtX* strain of *L. acidophilus* NCFM.

The *upp*-based counterselection gene replacement method (19) was used to create an internal deletion of 1966 bp in *prtX* (*lba1578*) of NCK1909, a *upp*-deficient background strain of *L. acidophilus* NCFM. Using splicing by overlap extension PCR (32), the 1 kb regions flanking the deletion target were spliced with BamHI added on the upstream end and SacI on the downstream end. This construct was digested with BamHI and SacI, then ligated into the polylinker of the similarly digested integration plasmid pTRK935 and transformed into competent *E. coli* EC101. The resulting recombinant plasmid, pTRK1073, was transformed into *L. acidophilus* NCK1909 harboring the helper plasmid pTRK669 (NCK1910). Single crossover integrants were screened as described previously (19). Colonies with the Δ*prtX* genotype were screened among the double recombinants recovered on glucose semi-defined medium agar plates containing 5-fluorouracil. Deletion was confirmed by PCR and sequencing. The resulting Δ*prtX* strain was designated NCK2282.

### 5.3.4 LiCl extraction of extracellular S-layer associated proteins.

Non-covalently bound cell surface proteins, including SLPs and SLAPs were extracted from NCK1909 and Δ*prtX L. acidophilus* NCFM strains using LiCl denaturing salt, as described previously (24). Proteins were quantified via bicinchoninic acid assay kit (Thermo Scientific) and visualized via SDS-PAGE using precast 4–20% Precise Tris-HEPES protein gels (Thermo Scientific). Gels were stained using AcquaStain (Bulldog Bio) according to the instructions of the manufacturer.

### 5.3.5 Morphological assessment and electron microscopy.

Morphological assessment of *L. acidophilus* NCFM and Δ*prtX* was performed using a phase-contrast light microscope at 40× magnification (Nikon Eclipse E600). Cells were observed over a growth period of 24 hours in MRS broth at 37 °C. Pictures were taken using a QImaging MicroPublisher 5.0 RTV camera attachment at 1, 3, 5, 8, 10, and 13 hour time points.

For electron microscopy, *L. acidophilus* NCFM strains were grown in 35 ml of MRS to logarithmic and stationary phases. Cells were pelleted by centrifugation at 3,166 × *g* for 15 min at room temperature. Pellets were resuspended in a fresh 1:1 (vol/vol) fixative mixture of 6% glutaraldehyde and 0.2 M sodium cacodylate (pH 5.5) and stored at 4°C. Sample processing for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) was performed by the Center for Electron Microscopy at North Carolina State University. SEM samples were viewed with a JEOL JSM 5900LV scanning electron microscope at 15kV. TEM samples were viewed with a JEOL 100S transmission electron

microscope. Surface layer thickness was measured by using a millimeter ruler and subsequently converted to nm based on the provided scale bar during TEM image processing.

### 5.3.6 Adherence assays.

Mucin and extracellular matrices (ECM) binding assays were performed as described previously (15). Mucin (type III from porcine stomach, Sigma) was dissolved in PBS to a final concentration of 10 mg/ml. Fibronectin (from human plasma, Sigma), collagen (type IV from human cell culture, Sigma), and laminin (from Engelbreth-Holm-Swarm murine sarcoma/basement membrane; Sigma) were diluted in 50 mM carbonate-bicarbonate buffer (pH 9.6, Sigma) to a final concentration of 10 μg/ml. For each assay, a Nunc Maxisorp 96-well microplate (Sigma) was coated with 100 μl/well of substrate and incubated at 4°C overnight. The wells were then washed twice with PBS to remove excess substrate before blocking with 150 μl of 2% bovine serum albumin (BSA) solution (Sigma) for 2 h at 37°C. Excess BSA was removed by two washes with PBS.

Bacterial cells were grown in MRS to stationary phase (16 hr) in preparation for the assay. Cultures were centrifuged (1,771 × *g*, 15 min, room temperature), washed once with PBS, and resuspended in PBS (pH 4.75). Cell density was adjusted to ~1 x $10^8$ CFU/mL based on previously calculated $OD_{600}$/CFU ratios. Cell suspensions (100 μl) were added to each mucin or ECM-coated well. Initial cell counts of samples were adjusted to 1 x $10^8$ CFU/mL and enumerated on MRS plates. After incubation for 1 h at 37°C, the wells were gently washed five times with 200 μl/well PBS. Adhered cells were recovered by adding 100 μl of 0.05% Triton X-100 solution (FisherBiotech, prepared in PBS) to each well and

agitating on an orbital shaker (200 rpm) for 15 min. Cell suspensions were transferred into 900 µl of 0.1X MRS before being further diluted and plated in duplicate on MRS plates. Colonies were enumerated and calculated as a percent of relative adherence (mutant CFU/parent CFU), where parent (NCK1909) CFU were set at 100%.

### 5.3.7 Bacterial-dendritic cell co-incubation assay and cytokine measurement.

*In vitro* bacterial-dendritic cell (DC) co-incubation assays were performed as described previously (14, 24). Cytokine measurements for IL-6, IL-10 and IL-12 were quantified using Single-Analyte ELISArray kits (Qiagen), according to the manufacturer's instructions. Following cytokine quantification, the cytokine expression data were compared between the parent and mutant strains using student T-test.

### 5.3.8 Mono-colonization of WT and Δ*prtX L. acidophilus* strains in a germ-free mouse model.

Germ-free 129S6/SvEv mice utilized for *in vivo* experiments were taken from breeding colonies maintained at the North Carolina State University Gnotobiotic Core of the Center for Gastrointestinal Biology and Disease, as described previously (14). Mice were maintained in cages in germ-free flexible film isolators housed in a room with 12 h of light and darkness. They were provided access to a standard diet (Prolab RMH 3500, LabDiet) and water *ad libitum*. Germ-free status was evaluated at least once a month through culturing fecal samples in thioglycollate broth, blood agar, and Sabouraud agar. Prior to colonization with *L. acidophilus* strains, the mice were also verified germ-free by culturing fecal samples

aerobically and anaerobically on plate count agar and MRS agar. Animal use protocols were approved by the Institutional Animal Care and Use Committee of North Carolina State University.

In preparation for mono-colonization of the Germ-free mice, *L. acidophilus* NCK56 and Δ*prtX* strains were propagated in MRS broth at 37 °C overnight. Bacteria were harvested at stationary phase (16 h) via centrifugation (1735 × *g*, 10 min, room temperature). They were subsequently washed with and resuspended in PBS to an $OD_{600}$ corresponding to $5 \times 10^9$ CFU/ml for each strain. Germ free 129S6/SvEv mice (*n* = 12, 6 male and 6 female, 17-18 weeks old) were gavaged with ~$1 \times 10^9$ cells in 200 µl PBS per mouse. Fecal samples were collected from each mouse on the day of gavage (Day 0), day 2, day 5, and day 7. Fecal samples were weighed, resuspended in 1 ml of PBS, diluted and plated on MRS agar for enumeration.

### 5.3.9 *In vivo* FITC-dextran epithelial barrier integrity in a germ-free mouse model.

Germ-free 129S6/SvEv mice were mono-colonized with either WT or Δ*prtX* strains of *L. acidophilus* NCFM, as described above. On the day of the assay, mice were denied access to food but allowed access to water *ad libitum* for 3 h, after which 150 µl of 3-5 kDa Fluorescein isothiocyanate (FITC)-dextran at a concentration of 80 mg/ml was administered to each mouse using an oral gavage needle. Two hours after administration of the FITC-dextran, blood was collected using a 1 ml tiburculin syringe (BD) and stored in Microtainer serum separator tubes (BD). Blood was placed in the dark for 1 h and allowed to clot, after which the serum was separated via centrifugation (13,000 rpm, 4°C). Fluorescence in the

serum was measured using a FLOUStar Optima Microtiter plate reader (BMG Technologies) with the excitation filter set for 490 nm, the emission filter set for 520 nm, and a 1250 gain setting for fluorescence intensity. FITC-dextran fluorescence was measured in the serum collected from the WT mono-colonized mice ($n$ = 5), the Δ*prtX* mono-colonized mice ($n$ = 5), and Germ-free control mice ($n$ = 2). Using a standard of known concentrations of FITC-dextran in PBS, the concentration of FITC-dextran was calculated.

## 5.4 Results

### 5.4.1 PrtX is an S-layer associated serine protease unique to *L. acidophilus*.

One of the most prevalent SLAPs in the non-covalently bound exoproteome of *L. acidophilus* NCFM is a 72 kDa uncharacterized serine protease encoded by the gene *lba1578* (24). The protein is 684 amino acids in length and contains a predicted signal peptide sequence located between amino acid 34 and 35 (VKA-AD) *In silico* analysis of LBA1578 revealed that there are no homologs of this protein outside of the *L. acidophilus* species. The protein demonstrated discreet homology to a hypothetical protein in *L. acetotolerans* DSM 20749 (33% identity), a serine protease in *L. gigeriorum* DSM 23908 (32%), *L. pasteurii* DSM 23907 (31%), *L. amylovorus* DSM 20351 (31%), *L. kitasatonis* DSM 16761 (31%), and *L. amylolyticus* DSM 11664 (30%). Due to the uncharacterized nature of LBA1578, and the conserved annotation of serine protease in homologs and discreet homologs, this protein was designated PrtX.

Using RNA-seq transcriptional analysis from a previous study (25), it was determined that *lba1578* (*prtX*) mRNA is polycistronically expressed downstream of *murC*, which encodes a UDP-*N*-acetylmuramate-*L*-alanine ligase putatively involved in peptidoglycan biosynthesis (Figure 5.1A). Because PrtX is an uncharacterized serine protease which is prominently featured in the non-covalent exoproteome in *L. acidophilus*, it was selected for functional analysis. The *upp*-based counterselective gene replacement method (19) was used to create a markerless chromosomal deletion of 1103 base pairs in the coding region of *prtX* (Δ*prtX*) in *L. acidophilus* NCFM (Figure 5.1B). SDS-PAGE analysis (Figure 5.1C) of

extracted SLAPs confirmed the presence of PrtX in the non-covalently bound exoproteome of NCFM and its absence from the Δ*prtX* deletion mutant strain, compared to wild-type.

### 5.4.2 Growth and morphology of Δ*prtX*.

The cellular morphology of the Δ*prtX* was examined using light microscopy over a 24 hour period of growth in MRS culture (Figure 5.2A). Compared to the parent strain (WT), the Δ*prtX* strain demonstrated aberrant morphology consisting of longer cells and autoaggregation (Figure 5.2A). Specifically, during lag phase (1 hr – 3 hr; $OD_{600}$ 0.1 - 0.2) cells from Δ*prtX* were longer than WT cells. By logarithmic phase (5 hr – 10 hr; $OD_{600}$ 0.5 – 1.0) cells of the Δ*prtX* mutant were only slightly longer than WT. Furthermore, the Δ*prtX* cells presented an increased autoaggregation phenotype which was not observed in the WT strain (Figure 5.2A). The autoaggregated Δ*prtX* cells did not resolve by stationary phase (13 hr; $OD_{600}$ 1.4; Figure 5.2A).

For a more detailed observation of these morphological phenotypes, WT and Δ*prtX* strains were examined using scanning electron microscopy (SEM) at logarithmic phase ($OD_{600}$ 0.6) and stationary phase ($OD_{600}$ 1.9; Figure 5.2B). The SEM micrographs mirror the morphological assessment with the light microscope. In particular, the Δ*prtX* mutant cells were longer than the WT cells at both logarithmic and stationary phase (Figure 5.2B). Most notably, the SEM micrographs of Δ*prtX* cells at 8,500 × magnification, revealed that the cause of the increased autoaggregation appears to be a proteinaceous build-up at the cell surface (Figure 5.2B). The Δ*prtX* mutant and WT grew similarly, as measured by OD (Figure 5.1C), suggesting that the irregular morphology in Δ*prtX* does not affect the rate of growth

compared to WT. Finally, transmission electron microscopy (TEM) demonstrated that the S-layer thickness was significantly reduced in the Δ*prtX* mutant compared to WT (Figure 5.1D). However, this phenotypic difference was more pronounced at logarithmic phase than stationary phase (Figure 5.1D). Collectively, these morphological data indicate that PrtX may be involved in protein turnover at the surface during growth and cell division.

### 5.4.3 Adherence of Δ*prtX* to mucin and extracellular matrices.

Because of the apparent build-up of proteins at the cell surface in Δ*prtX*, the ability of this mutant to bind to mucin and the extracellular matrices (ECM) fibronectin, collagen, and laminin was assessed and compared to WT, *in vitro* (Figure 5.3). The Δ*prtX* strain demonstrated a statistically significant 40% increase in binding capacity to mucin, compared to the parent strain (Figure 5.3). Similarly, Δ*prtX* showed a statistically significant 20% increase in binding to fibronectin (Figure 5.3). While Δ*prtX* appeared to have an increased binding capacity for laminin by 25%, this increase was not statistically significant. Finally, relative to WT, Δ*prtX* did not show any statistically significant difference in binding to collagen (Figure 5.3).

### 5.4.4 *In vitro* immunomodulation of mouse DC cells exposed to Δ*prtX*.

The immunomodulatory potential of the Δ*prtX* mutant was assessed using an *in vitro* bacterial/murine DC co-incubation assay. Relative to the wild-type strain, Δ*prtX* demonstrated an overall increase in immunomodulation of the cytokines IL-6, IL-12, and IL-10 (Figure 5.4). Specifically, Δ*prtX* induced production of the pro-inflammatory cytokine IL-

6 in DC from 33,957 pg/ml in WT to 60,347 pg/ml (Figure 5.4, IL-6). The pro-inflammatory cytokine IL-12 was also induced in Δ*prtX* (47.2 pg/ml) compared to WT (35.8 pg/ml) in a mild, but statistically significant manner (Figure 5.4, IL-12). By far, the most significantly induced cytokine was the anti-inflammatory cytokine IL-10. In Δ*prtX* induced DC, IL-10 was produced to 3,115 pg/ml, compared to 1,529 pg/ml in WT-induced DC (Figure 5.4, IL-10). Although Δ*prtX* induced both the pro-inflammatory cytokine IL-12 and the anti-inflammatory cytokine IL-10, the IL-10/IL-12 ratio, which is a measure of the balance between pro-inflammatory and anti-inflammatory states (33, 34), was higher in Δ*prtX* than in WT (Figure 5.4, IL-10/IL-12).

### 5.4.5 *In vivo* mono-colonization and epithelial barrier integrity of germ-free mice.

Due to the increased adhesion and immunomodulation properties of Δ*prtX*, *in vitro*, the biological relevance of the Δ*prtX* strain was explored in an *in vivo* germ-free mouse model. Germ-free mice were colonized with ~$1 \times 10^9$ CFU of either WT or Δ*prtX* strains; this mono-colonization was measured over 7 days (Figure 5.5A). Both WT and Δ*prtX* strains colonized the germ-free mice at similar rates, and by day 7 the bacteria had colonized to $4.56 \times 10^8$ CFU for WT and $3.71 \times 10^8$ CFU for Δ*prtX* (Figure 5.5A). Notably, the mice colonized with Δ*prtX* were diminished in size and weight compared to the germ-free control mice and the mice colonized with the WT strain (Figure 5.5B). Specifically, mice colonized with Δ*prtX* weighed 23.6 g compared to the germ-free control mice at 27.9 g and the WT colonized mice at 27 g (Figure 5.5B). Next, the total gastrointestinal epithelial barrier integrity was examined for these mice (Figure 5.5C). WT and Δ*prtX* colonized mice were fed

FITC-dextran and the resulting FITC-dextran in the blood serum correlated to the gastrointestinal barrier integrity of the mouse. Compared to WT, serum from the Δ*prtX* colonized mice demonstrated a statistically significant reduction in FITC-dextran levels (Figure 5.5C). These data indicate that the intestinal epithelial barrier was enhanced in mice colonized with Δ*prtX*.

## 5.5 Discussion

Proteolytic systems of LAB are responsible for converting macromolecular proteins into peptides and amino acids for bacterial growth and survival, and are essential for organisms that occupy dairy niche environments. (35-38). These systems are organized into three components: (i) cell-envelope proteinases (CEPs; often serine proteases) which degrade extracellular oligopeptides and casein, (ii) peptide transporters which take up peptides into the cell, and (iii) intracellular peptidases that degrade peptides into amino acids (38). Because milk proteolysis is an industrially significant aspect of dairy food fermentations and flavor development, the starter cultures which encode these systems have been extensively explored genetically and biochemically (36-38). The most extensively studied CEP in LAB include PrtP from *Lactococcus lactis* (39), PrtS from *Streptococcus thermophilus* (40), PrtH and PrtH2 from *L. helveticus* (41-43), PrtB from *L. delbrueckii* subsp. *bulgaricus* (44), and PrtR from *L. rhamnosus* (45).

Within the genome of *L. acidophilus* NCFM are genes encoding a proteolytic system for growth in milk, including *prtP*, the *opp1* and *opp2* oligopeptide transport systems, and numerous peptidases (8, 46). As is the case for the Prt-like proteinases in other LAB, the PrtP in *L. acidophilus* is predicted to be cell membrane bound through a cell membrane anchor domain. However a recent analysis of 213 *Lactobacillus* genomes revealed a surprising diversity of CEP beyond those described in the prototypical milk oligopeptide proteolytic systems (47). Notably, of the 60 CEPs described, 17 were found to incorporate a non-covalent attachment domain, which is responsible for the non-covalent attachment of SLPs and SLAPs (25, 47-48). Further, 13 of the 60 CEPs had no discernable cell wall binding

domain (47). Our recent work identified one of these CEPs, a 72 kDa serine protease, as a prominent SLAP, non-covalently co-localized at the cell surface along with the S-layer (24). In this study, the serine protease, which we called PrtX, was functionally and phenotypically characterized. Following the creation of a clean chromosomal deletion of *prtX*, the corresponding strain (Δ*prtX*) was assessed for cellular morphology, *in vitro* mucin and ECM adhesion, *in vitro* immunomodulation, and *in vivo* mouse intestinal epithelial barrier integrity.

Morphological assessment of the Δ*prtX* isogenic mutant revealed a visible increase in autoaggregation, compared to the parent strain. This increased autoaggregation phenotype was further evaluated using scanning electron microscopy, in which Δ*prtX* cells were observed to present aberrant cellular topology through an apparent increase of protein debris at the cell surface. These data suggest that PrtX is responsible for protein turnover at the cell surface of *L. acidophilus* NCFM. Notably, gene deletion studies of CEPs in other *Lactobacillus* species, including *prtB* in *L. delbrueckii* subsp. *bulgaricus* and *prtH* in *L. helveticus*, have not reported a similar autoaggregation phenotype (41-44). A similar increased autoaggregation phenotype was reported during the functional analysis of an S-layer associated autolysin, AcmB, in *L. acidophilus* NCFM (27).

The absence of PrtX in the Δ*prtX* mutant also appeared to have a pleiotropic effect on the thickness of the S-layer, as measured by transmission electron microscopy. At logarithmic phase the S-layer was significantly thinner in Δ*prtX* compared to WT. However, by stationary phase the difference between the S-layer thicknesses of Δ*prtX* cells was not nearly as stark. The relationship between the S-layer and the autoaggregation capacity of *L.*

*acidophilus* has been previously established (27, 49). Furthermore, previous mutational analysis of genes encoding the SLAPs AcmB and the fibronectin binding protein, FbpB, demonstrated similar S-layer phenotypes compared to WT (27, 28). Still, it is not fully understood if the altered S-layer thickness is due to the physical absence of these SLAPs on the cell surface or other morphological factors. In the case of PrtX, both factors appear to be at play. Logarithmic Δ*prtX* cells are longer than WT cells which corresponds to a thinner S-layer phenotype. By stationary phase, however, WT and Δ*prtX* cells are the same length, suggesting that the more modest discrepancy between the S-layer thickness in WT and Δ*prtX* may be due to the physical absence of the 72 kDa PrtX from the cell surface.

Proteins localized at the cell surface of *L. acidophilus* NCFM are important mediators of adhesion to host intestinal epithelial mucus layer and extracellular matrices, *in vitro* (14-16, 27-28, 46). In this study, the Δ*prtX* strain demonstrated a significant increase in binding to mucin and fibronectin. These results are consistent with a recent analysis of PrtS in *S. thermophilus*, in which a PrtS-deficient strain demonstrated doubly-efficient binding to Caco-2 epithelial cells (50). Furthermore, previous analysis of the S-layer associated fibronectin binding protein, FpbB, in *L. acidophilus* NCFM revealed that FpbB has specificity for fibronectin and mucin, *in vitro* (28). Though the exact mechanism remains unclear, it is possible that the PrtX-deficient strain is not efficiently turning proteins over at the cell surface of the bacteria, including SLAPs such as FpbB, resulting in increased binding to mucin and fibronectin, specifically. However, it is also possible that the generalized increase in autoaggregation may result in the increased binding, non-specifically.

In addition to their role in adhesion, cell surface proteins of *L. acidophilus* NCFM, including SLP and SLAPs, have also been increasingly examined for roles in immunomodulation (13-14, 22-24). Previous research regarding immunomodulation and CEPs, specifically, have focused on the production of bioactive compounds released during casein proteolysis in milk (51-53). For example, milk fermented with a non-proteolytic variant of *L. helveticus* was found to have a suppressed mucosal immune response compared to milk fermented by the wild-type strain of *L. helveticus* (55). Recent evidence, however, has pointed to a more specific immunomodulatory role of CEPs in *Lactobacillus*. An extracellular lactocepin serine protease in *Lactobacillus paracasei* was found to exert anti-inflammatory effects by selectively degrading pro-inflammatory chemokines, such as IP-10 (55). In this study, we found that the absence of PrtX in the Δ*prtX* mutant resulted in a generalized increase in DC expression of cytokines IL-6, IL-12, and IL-10, compared to WT. The cytokine most profoundly induced in DC exposed to Δ*prtX* was the anti-inflammatory IL-10, which has been proposed as a biological therapy for chronic irritable bowel disease (IBD), including Chron's disease and colitis (56). It is possible that PrtX may directly degrade certain cytokines, resulting in the increased immunomodulation of the PrtX-deficient strain, though this direct mechanism remains to be discovered. It is more likely that the immunomodulation in Δ*prtX* is due to the aggregated proteins at the cell surface that are no longer being turned over.

Previous work has been performed in *L. acidophilus* NCFM using an *in vivo* germ-free mouse model. Mutational analysis of sortase, an enzyme which covalently couples extracellular proteins containing an LPXTG motif to the cell surface, was found to contribute

to gut retention of *L. acidophilus* NCFM (14). Similarly, the glycogen biosynthesis pathway in *L. acidophilus* NCFM was found to contribute to gut fitness and retention, *in vivo* (57). In the present study, the biological relevance of PrtX as a mediator of probiotic activity was examined in germ-free mice. Surprisingly, mice colonized with Δ*prtX* were found to demonstrate significant weight loss compared to the parent strain. Weight loss is normally a clinical characteristic associated with inflammation in IBD and colitis mouse models (58). It is possible that the increased immunomodulatory phenotype in DC exposed to Δ*prtX*, *in vitro*, including pro-inflammatory cytokines IL-6 and IL-12, translated to inflammation in the germ-free mice mono-colonized with Δ*prtX*. However, it is also possible that PrtX may participate in the digestion of the provided mouse diet, such that in mice colonized with the Δ*prtX* mutant, the nutrients in the food are not as bioavailable than in the WT colonized mice. More work is needed to characterize the specific mechanisms leading to the weight loss of mice colonized with the Δ*prtX* strain.

Finally, intestinal epithelial barrier integrity was assessed in germ-free mice colonized with either Δ*prtX* or the WT strains. Dysbiosis of the normal enteric gastrointestinal microbiome has been demonstrated as a key factor in the initiation and amplification of IBD (59). Recent evidence has implicated enteric and commensal proteases as one such mechanism for pathogenesis in IBD (60, 61). In *Enterococcus faecalis*, a Gram-positive commensal bacteria of the gastrointestinal tract, extracellular proteases have been shown to mediate intestinal epithelial barrier disruption and contribute to intestinal inflammation (62, 63). Here, we show that the Δ*prtX* mutant causes increased epithelial barrier integrity in the germ-free mice compared to WT colonized mice. PrtX may directly

interact with various ECM components of intestinal epithelia causing a direct effect on intestinal epithelial integrity, but this specific mechanism has not been explored. It is also possible that the indirect immunomodulatory effects of Δ*prtX* in the germ-free mouse model resulted in the increased barrier integrity, as cytokines can modulate tight junction structure and function in intestinal epithelial cells (64).

In conclusion, we have demonstrated that PrtX is an extracellular, S-layer associated serine protease unique to *L. acidophilus*. Deletion of *prtX* from the chromosome of *L. acidophilus* NCFM revealed a distinct autoaggregation phenotype which resulted in increased binding to mucin and fibronectin, as well as increased immunomodulation of IL-6, IL-12, and IL-10, *in vitro*. Furthermore, colonization of the Δ*prtX strain* in a germ-free mouse model lead to weight loss, along with an overall increased gastrointestinal epithelial barrier integrity. Collectively, these data suggest that PrtX is involved in protein turnover at the cell surface, and that the absence of PrtX in results in increased presentation of cell surface proteins which mediate some host-microbe interactions of *L. acidophilus* NCFM.

## 5.6 Acknowledgements

This research was funded, in part, by the North Carolina Agricultural Foundation and DuPont Nutrition & Health. The authors would like to thank Dr. Sue Tonkonogy for technical assistance and support. We also thank Karen Flores of the Gnotobiotic Core, College of Veterinary Medicine, North Carolina State University for assistance with experiments using germ-free and gnotobiotic mice. The Gnotobiotic Core is a facility of the Center for Gastrointestinal Biology and Disease, funded by NIH grant P30 DK034987.

## 5.7 References


1. **Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S, Huang K, Goodstein DM, Hawkins T, Plegvidhya V, Welker D, Hughes J, Goh Y, Benson A, Baldwin K, Lee JH, Diaz-Muniz I, Dosti B, Smeianov V, Wechter W, Barabote R, Lorca G, Altermann E, Barrangou R, Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O'Sullivan D, Steele J, Unlu G, Saier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B, Mills D**. 2006. Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci USA **103**:15611-15616.

2. **Douglas GL, Klaenhammer TR**. 2010. Genomic evolution of domesticated microorganisms. Annu Rev Food Sci Technol **1**:397-414.

3. **Johnson BR, Klaenhammer TR**. 2014. Impact of genomics on the field of probiotic research: historical perspectives to modern paradigms. Antonie Van Leeuwenhoek **106**:141–156.

4. **Wood B**. 1998. Microbiology of fermented foods. Blackie Academic & Professional, Glasgow, Scotland.

5. **O'Flaherty S, Klaenhammer TR**. 2010. The role and potential of probiotic bacteria in the gut, and the communication between gut microflora and gut/host. Int Dairy J **20**:262-268.

6. **Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME**. 2014. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol **11**:506–514.

7. **Sanders ME, Klaenhammer TR**. 2001. Invited review: The scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic. J Dairy Sci **84**:319-331.

8. **Altermann E, Russell WM, Azcarate-Peril MA, Barrangou R, Buck BL, McAuliffe O, Souther N, Dobson A, Duong T, Callanan M, Lick S, Hamrick A, Cano R, Klaenhammer TR**. 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. Proc Natl Acad Sci USA **102**:3906–3912.

9. **Klaenhammer TR, Barrangou R, Buck BL, Azcarate-Peril MA, Altermann E**. 2005. Genomic features of lactic acid bacteria effecting bioprocessing and health.

FEMS Microbiol Rev **29**:393-409.

10. **Klaenhammer TR, Altermann E, Pfeiler EA, Buck BL, Goh YJ, O'Flaherty S, Barrangou R, Duong T**. 2008. Functional genomics of probiotic Lactobacilli. J Clin Gastroenterol **42**:160-162.

11. **Lebeer S, Vanderleyden J, De Keersmaeker SCJ**. 2010. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol **8**:171-184.

12. **Bron PA, Tomita S, Mercenier A, Kleerebezem M**. 2013. Cell surface-associated compounds of probiotic lactobacilli sustain the strain-specificity dogma. Curr Opin Microbiol **16**:262–269.

13. **Mohamadzadeh M, Pfeiler EA, Brown JB, Zadeh M, Gramarossa M, Managlia E, Bere P, Sarraj B, Khan MW, Chaitanya Pakanati K, Javeed Ansari M, O'Flaherty S, Barrett T, Klaenhammer TR**. 2011. Regulation of induced colonic inflammation by *Lactobacillus acidophilus* deficient in lipoteichoic acid. Proc Natl Acad Sci USA **108**:4623-4630.

14. **Call EK, Goh YJ, Selle K, Klaenhammer TR, O'Flaherty S**. 2015. Sortase-deficient lactobacilli: effect on immunomodulation and gut retention. Microbiology **161**:311-321.

15. **Goh YJ, Klaenhammer TR**. 2010. Functional roles of aggregation-promoting-like factor in stress tolerance and adherence of *Lactobacillus acidophilus* NCFM. Appl and Environ Microbiol **76**:5005-5012.

16. **O'Flaherty SJ, Klaenhammer TR**. 2010. Functional and phenotypic characterization of a protein from *Lactobacillus acidophilus* involved in cell morphology, stress tolerance and adherence to intestinal cells. Microbiology **156**:3360-3367.

17. **Hynönen U, Palva A**. 2013. *Lactobacillus* surface layer proteins: structure, function and applications. Appl Microbiol Biotechnol **97**:5225–5243.

18. **Sára M, Sleytr UB**. 2000. S-layer proteins. J Bacteriol **182**:859-868.

19. **Goh, YJ, Azcarate-Peril MA, O'Flaherty S, Durmaz E, Valence F, Jardin J, Lortal S, Klaenhammer TR.** 2009. Development and application of a *upp*-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. Appl. Environ. Microbiol. **75:**3093-3105.

20. **Frece J, Kos B, Svetec IK, Zgaga Z, Mrsa V, Susković J**. 2005. Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. J Appl Microbiol **98**:285–292.

21. **Buck BL, Altermann E, Svingerud T, Klaenhammer TR**. 2005. Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol **71**:8344–8351.

22. **Konstantinov SR, Smidt H, de Vos WM, Bruijns SCM, Singh SK, Valence F, Molle D, Lortal S, Altermann E, Klaenhammer TR, van Kooyk Y**. 2008. S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. Proc Natl Acad Sci USA **105**:19474–19479.

23. **Lightfoot YL, Selle K, Yang T, Goh YJ, Sahay B, Zadeh M, Owen JL, Colliou N, Li E, Johannssen T, Lepenies B, Klaenhammer TR, Mohamadzadeh M**. 2015. SIGNR3-dependent immune regulation by *Lactobacillus acidophilus* surface layer protein A in colitis. EMBO J **34**:881–895.

24. **Johnson B, Selle K, O'Flaherty S, Goh YJ, Klaenhammer T**. 2013. Identification of extracellular surface-layer associated proteins in *Lactobacillus acidophilus* NCFM. Microbiology **159**:2269–2282.

25. **Johnson BR, Hymes J, Sanozky-Dawes R, DeCrescenzo Henriksen E, Barrangou R, Klaenhammer TR**. 2016. Conserved S-layer-associated proteins revealed by exoproteomic survey of S-layer forming lactobacilli. Appl Environ Microbiol **82**:134-145.

26. **Altermann E, Buck LB, Cano R, Klaenhammer TR**. 2004. Identification and phenotypic characterization of the cell-division protein CdpA. Gene **342**:189-197.

27. **Johnson BR, Klaenhammer TR**. 2016. Functional analysis of AcmB, an S-layer associated β-*N*-acetylglucosaminidase in *Lactobacillus acidophilus* NCFM. In review.

28. **Hymes J, Johnson BR, Barrangou R, Klaenhammer TR**. 2016. Functional analysis of an S-layer associated fibronectin-binding protein in *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol. In press.

29. **Hanahan D**. 1983. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol **166**:557-580.

30. **Walker DC, Aoyama K, Klaenhammer TR**. 1996. Electrotransformation of *Lactobacillus acidophilus* group A1. FEMS Microbiol Lett **138**: 233-237.

31. **Wei MQ, Rush CM, Norman JM, Hafner LM, Epping RJ, Timms P**. 1995. An improved method for the transformation of *Lactobacillus* strains using electroporation. J Microbiol Methods **21**: 97-109.

32. **Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR**. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77**:61-68.

33. **Hamer HM, Jonkers DMAE, Vanhoutvin SAWL, Troost FJ, Rijkers G, de Bruine A, Bast A, Venema K, Brummer RJM**. 2010. Effect of butyrate enemas on inflammation and antioxidant status in the colonic mucosa of patients with ulcerative colitis in remission. Clin Nutr **29**:697-842.

34. **O'Garra A, Murphy KM**. 2009. From IL-10 to IL-12: how pathogens and their products stimulate APCs to induce $T_H1$ development. Nat Immunol **10**:929-932.

35. **Kunji ERS, Mierau I, Hagting A, Poolman B, Konings WN**. 1996. The proteolytic systems of lactic acid bacteria. Antonie Van Leeuwenhoek **70**:187-221.

36. **Law J, Haandrikman A**. 1997. Proteolytic enzymes of lactic acid bacteria. Int Dairy J **7**:1-11.

37. **Savijoki K, Ingmer H, Varmanen P**. 2006. Proteolytic systems of lactic acid bacteria. Appl Microbiol Biotechnol **71**:394-406.

38. **Liu M, Bayjanov JR, Renckens B, Nauta A, Siezen RJ**. 2010. The proteolytic system of lactic acid bacteria revisited: a genomic comparison. BMC Genomics **11**:36.

39. **Kok J, Leenhouts J, Haandrikman AJ, Ledeboer AM, Venema G**. 1988. Nucleotide sequence of the cell wall proteinase gene of *Streptococcus cremoris* Wg2. Appl Environ Microbiol **54**:231-238.

40. **Fernandez-Espla MA, Garault P, Monnet V, Rul F**. 2000. *Streptococcus thermophilus* cell wall-anchored proteinase: release, purification, and biochemical and genetic characterization. Appl Environ Microbiol **66**:4772-4778.

41. **Pederson JA, Mileski GJ, Weimer BC, Steele JL**. 1999. Genetic characterization of a cell envelope-associated proteinase from *Lactobacillus helveticus* CNRZ32. J Bacteriol **181**:4592-4597.

42. **Genay M, Sadat L, Gagnaire V, Lortal S**. 2009. *prtH2*, not *prtH*, is the ubiquitous cell wall proteinase gene in *Lactobacillus helveticus*. Appl Environ Microbiol **75**:3238-

3249.

43. **Sadat-Mekmene L, Jardin J, Corre C, Molle D, Richoux R, Delage MM, Lortal S, Gagnaire V**. 2011. Simultaneous presence of PrtH and PrtH2 proteinases in *Lactobacillus helveticus* strains improves breakdown of the pure $\alpha_{s1}$-casein. Appl Environ Microbiol **77**:179-186.

44. **Gilbert C, Atlan D, Blanc B, Portailer R, Germond JE, Lapierre L, Mollet B**. 1996. A new cell surface proteinase: sequencing and analysis of the *prtB* gene from *Lactobacillus delbrueckii* subsp. *bulgaricus*. J Bacteriol **178**:3059-3065.

45. **Pastar I, Tonic I, Golic N, Kojic M, van Kranenburg R, Kleerebezem M, Topisirovic L, Jovanovic G**. 2003. Identification and genetic characterization of a novel proteinase, PrtR, from the human isolate *Lactobacillus rhamnosus* BGT10. Appl Environ Microbiol **69**:5802-5811.

46. **Azcarate-Peril MA, Tallon R, Klaenhammer TR**. 2009. Temporal gene expression and probiotic attributes of *Lactobacillus acidophilus* during growth in milk. J Dairy Sci **92**:870-886.

47. **Sun Z, Harris HMB, McCann A, Guo C, Argimon S, Zhang W, Yang X, Jeffery IB, Cooney JC, Kagawa TF, Liu W, Song Y, Salvetti E, Wrobel A, Rasinkangas P, Parkhill J, Rea MC, O'Sullivan O, Ritari J, Douillard FP, Ross RP, Yang R, Briner AE, Felis GE, de Vos WM, Barrangou R, Klaenhammer TR, Caufield PW, Cui Y, Zhang H, O'Toole PW**. 2015. Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. Nat Comm **6**:8332.

48. **Boot HJ, Kolen CP, Pouwels PH**. 1995. Identification, cloning, and nucleotide sequence of a silent S-layer protein gene of *Lactobacillus acidophilus* ATCC 4356 which has extensive similarity with the S-layer protein gene of this species. J Bacteriol **177**:7222–7230.

49. **Kos B, Suskovic J, Vukovic S, Simpraga M, Frece J, Matosic S**. 2003. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. J Appl Microbiol **94**:981-987.

50. **Kebouchi M, Galia W, Genay M, Soligot C, Lecomte X, Awussi AA, Perrin C, Roux E, Dary-Mourot A, Le Roux Y**. 2016. Implication of sortase-dependent proteins of *Streptococcus thermophilus* in adhesion to human intestinal epithelial cell lines and bile salt tolerance. Appl Microbiol Biotechnol **100**:1-13.

51. **Korhonen H, Pihlanto A**. 2006. Bioactive peptides: production and functionality. Int Dairy J **16**:945-960.

52. **Shah NP**. 2007. Functional cultures and health benefits. Int Dairy J **17**:1262-1277.

53. **Silva SV, Malcata FX**. Caseins as source of bioactive peptides. Int Dairy J **15**:1-15.

54. **Matar C, Valdez JC, Medina M, Rachid M, Perdigon G**. 2001. Immunomodulating effects of milks fermented by *Lactobacillus helveticus* and its non-proteolytic variant. J Dairy Res **68**:601-609.

55. **von Schillde MA, Hormannsperger G, Weiher M, Alpert CA, Hahne H, Bauerl C, van Huynegem K, Steidler L, Hrncir T, Perez-Martinez G, Kuster B, Haller D**. 2012. Lactocepin secreted by *Lactobacillus* exerts anti-inflammatory effects by selectively degrading proinflammatory chemokines. Cell Host Microbe **11**:387-396.

56. **Li MC, He SH**. 2004. IL-10 and its related cytokines for treatment of inflammatory bowel disease. World J Gastroenterol **10**:620-625.

57. **Goh YJ, Klaenhammer TR**. 2014. Insights into glycogen metabolism in *Lactobacillus acidophilus*: impact on carbohydrate metabolism, stress tolerance and gut retention. Microb Cell Fact **13**:94.

58. **Perse M, Cerar A**. 2012. Dextran sodium sulphate colitis mouse model: traps and tricks. J Biomed Biotechnol. **2012**:1-13.

59. **Tamboli CP, Neut C, Desreumaux P, Colombel JF**. 2004. Dysbiosis in inflammatory bowel disease. Gut **53**:1-4.

60. **Pruteanu M, Hyland NP, Clarke DJ, Kiely B, Shanahan F**. 2011. Degradation of extracellular matrix components by bacterial-derived metalloproteases: implications for inflammatory bowel diseases. Inflamm Bowel Dis **17**:1189-1200.

61. **Carroll IM, Maharshak N**. 2013. Enteric bacterial proteases in inflammatory bowel disease-pathophysiology and clinical implications. Wolrd J Gastroenterol **19**:7531-7543.

62. **Steck N, Hoffmann M, Sava IG, Kim SC, Hahne Hannes, Tonkonogy SL, Mair K, Krueger D, Pruteanu M, Shanahan F, Vogelmann R, Schemann M, Kuster B, Sartor RB, Haller D**. 2011. *Enterococcus faecalis* metalloprotease compromises epithelial barrier and contributes to intestinal inflammation. Gastroenterol **141**:959-971.

63. **Maharshak N, Young Huh E, Paiboonrungruang C, Shanahan M, Thurlow L, Herzog J, Djukic Z, Orlando R, Pawlinski R, Ellermann M, Borst L, Patel S, Dotan I, Sartor RB, Carroll IM**. 2015. *Enterococcus faecalis* gelatinase mediates intestinal permeability via protease-activated receptor 2. Infect Immun **83**:2762-2770.

64. **Walsh SV, Hopkins AM, Nusrat A**. 2000. Modulation of tight junction structure and function by cytokines. Adv Drug Deliv Rev **41**:303-313.

65. **Russell WM, Klaenhammer TR**. 2001. Efficient system for directed integration into the *Lactobacillus acidophilus* and *Lactobacillus gasseri* chromosomes via homologous recombination. Appl Environ Microbiol **67**:4361-4364.

**Table 5.1** Bacterial strains and plasmids used in this study.

| Strain or plasmid | Genotype or characteristics | Reference |
|---|---|---|
| ***L. acidophilus* strains** | | |
| NCFM | Human intestinal isolate | 7 |
| NCK1909 | NCFM carrying a 315-bp in-frame deletion within the upp gene | 19 |
| NCK1910 | NCK1909 harboring pTRK669; host for pORI-based counterselective integration vector | 19 |
| NCK2282 | NCK1909 carrying a 1,103-bp in-frame deletion within the *lba1578* gene | This study |
| ***E. coli* (EC101) strains** | | |
| NCK1911 | Host harboring pTRK935, $Kn^r$, $Em^r$ | 19 |
| NCK2281 | Host harboring pTRK1073, $Kn^r$, $Em^r$ | This Study |
| **Plasmids** | | |
| pTRK669 | Ori (pWV01), Cmr , $RepA^+$ thermosensitive | 65 |
| pTRK935 | pORI upp-based counterselective integration vector | 19 |
| pTRK1073 | pTRK935 with a mutated copy of *lba1578* cloned into BamHI/SacI site | This study |

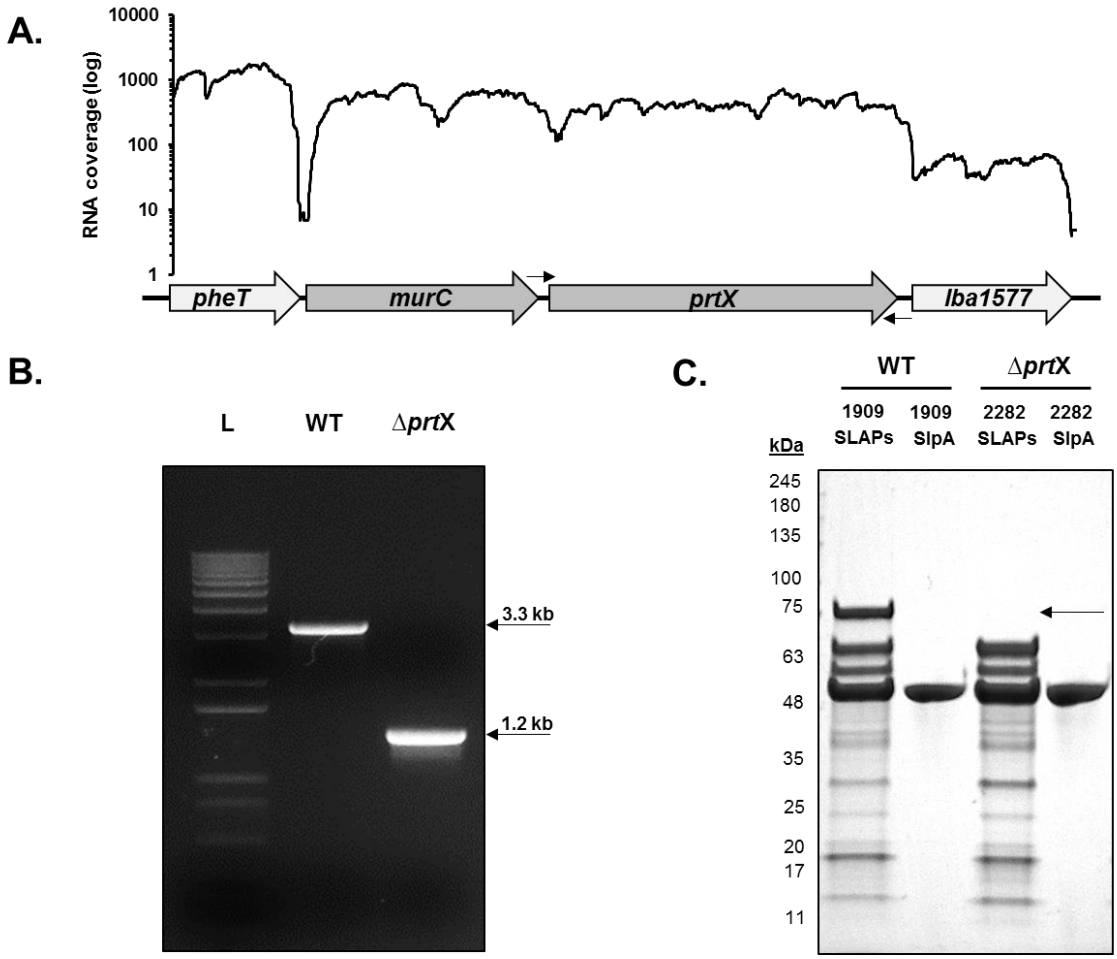


**Figure 5.1**: The gene encoding PrtX was deleted from the chromosome of *L. acidophilus* NCFM. (A) RNA-seq analysis demonstrates that *prtX* is polycistronically expressed with *murC*, which encodes for a UDP-*N*-acetylmuramate-*L*-alanine ligase putatively involved in peptidoglycan biosynthesis. Black arrows indicate the forward and reverse primer pair used to confirm the deletion of *prtX*. (B) Gel electrophoresis of PCR products using the primers indicated in (A) for the parent strain (WT; 3.3 kb) compared to the PrtX-deficient strain (Δ*prtX*; 1.2 kb). The deletion was confirmed by sequencing. (C) SDS-PAGE of the non-covalently bound extracellular S-layer proteins (SLP) and S-layer associated proteins (SLAPs) were isolated from both WT and Δ*prtX*. Absence of the 72 kDa band corresponding to PrtX within the SLAP extracts from Δ*prtX* confirmed the absence of PrtX from the exoproteome of Δ*prtX*.

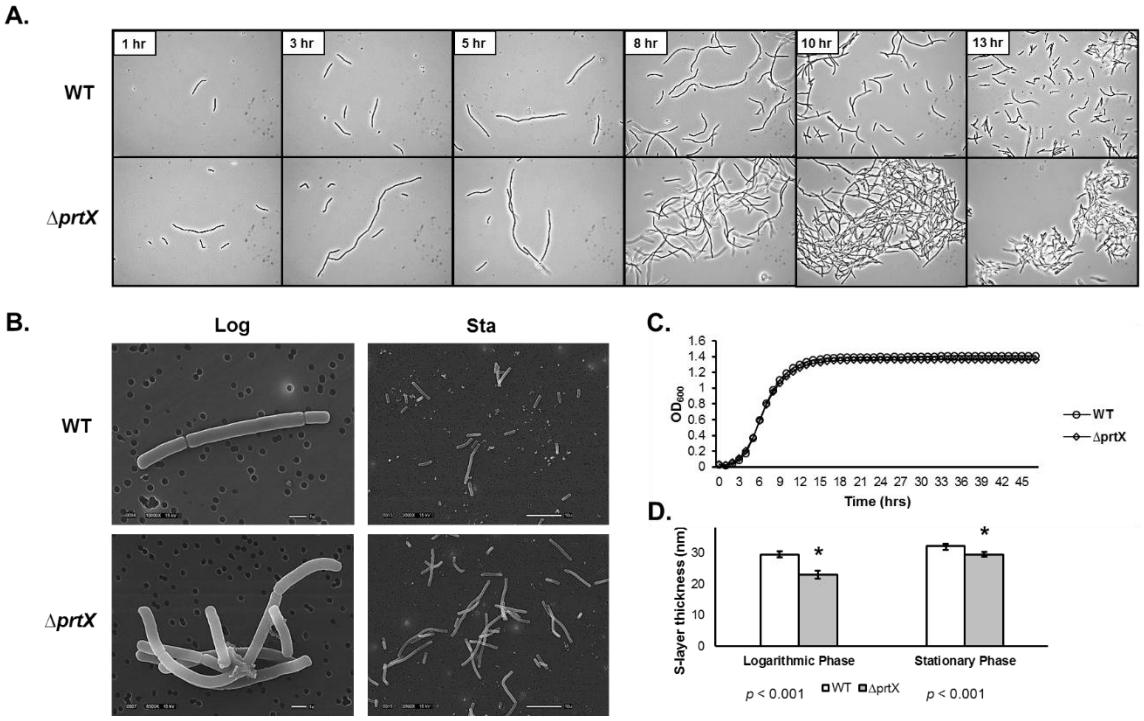


**Figure 5.2**: (A) The cellular morphology of the wild-type (WT) and mutant (Δ*prtX*) strains were assessed using phase contrast light microscopy over a 13 hour growth period. (B) Scanning electron micrographs were taken of WT and Δ*prtX* at logarithmic and stationary phases. (C) Growth rate of WT and Δ*prtX* was assessed over 48 hours using optical density ($OD_{600}$). (D) The S-layer thickness was measured for WT and Δ*prtX* using transmission electron micrographs at logarithmic and stationary phases. The differences between the S-layer thickness in WT and Δ*prtX* were statistically significant for measurements at logarithmic and stationary phase ($p < 0.001$). Error bars represent the confidence intervals from at least 25 measurements per condition.

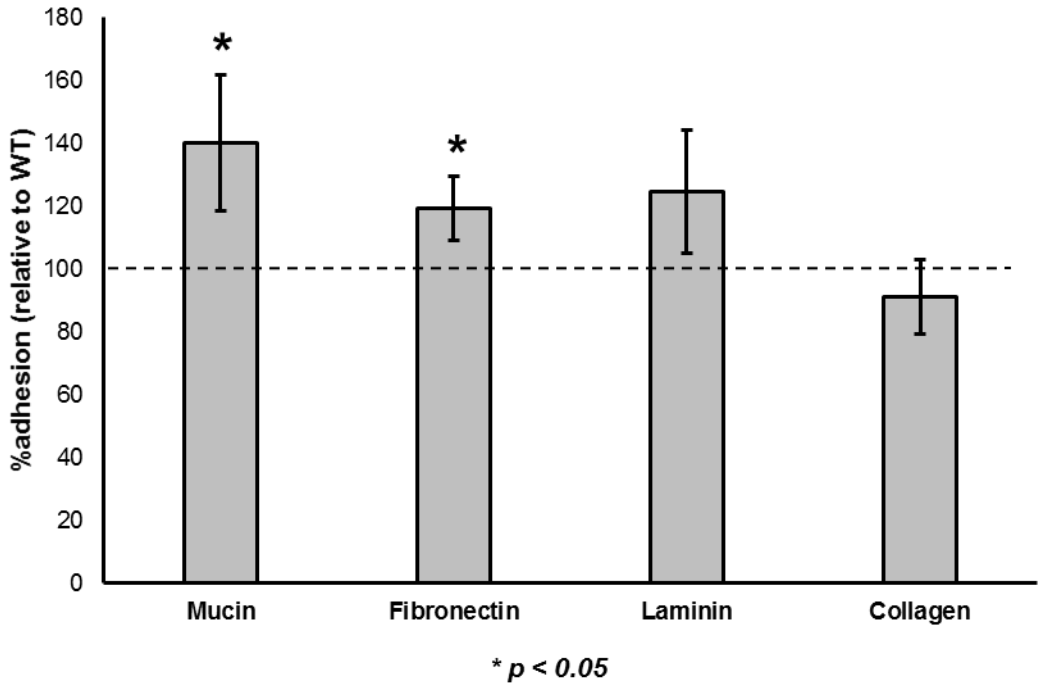


**Figure 5.3**: The ability of the Δ*prtX* mutant to bind to mucin and extracellular matrices (ECM) was assessed. Compared the wild-type reference (WT, dotted line), Δ*prtX* showed a significant increase in binding to mucin and fibronectin. Asterisks indicate statistical significance ($p < 0.05$). Adherence assays were performed in triplicate; all error bars represent confidence intervals.

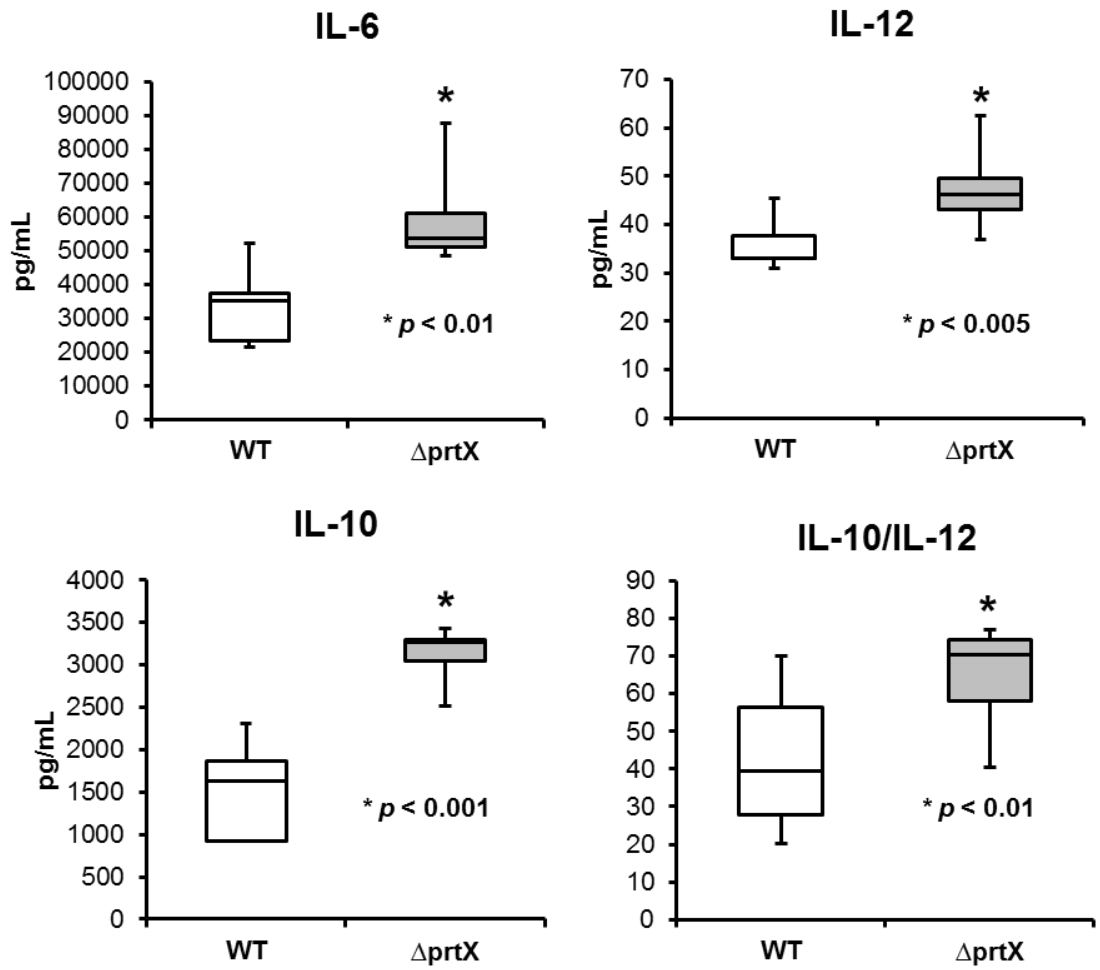


**Figure 5.4**: The immunomodulatory phenotype of Δ*prtX* (gray) compared to WT (white) was measured using a murine dendritic cell co-incubation assay. Cytokines IL-6, IL-12 and IL-10 were assessed using enzyme-linked immunosorbent assays. For all cytokines measured, Δ*prtX* demonstrated a significant increase in immunomodulation compared to WT (IL-6, $p < 0.01$; IL-12, $p < 0.006$; IL-10, $p < 0.001$). The IL-10/IL-12 ratio was also significantly increased in Δ*prtX* ($p < 0.01$). Co-incubation assays were performed in duplicate or triplicate; bars on the box-whisker plots represent the range.

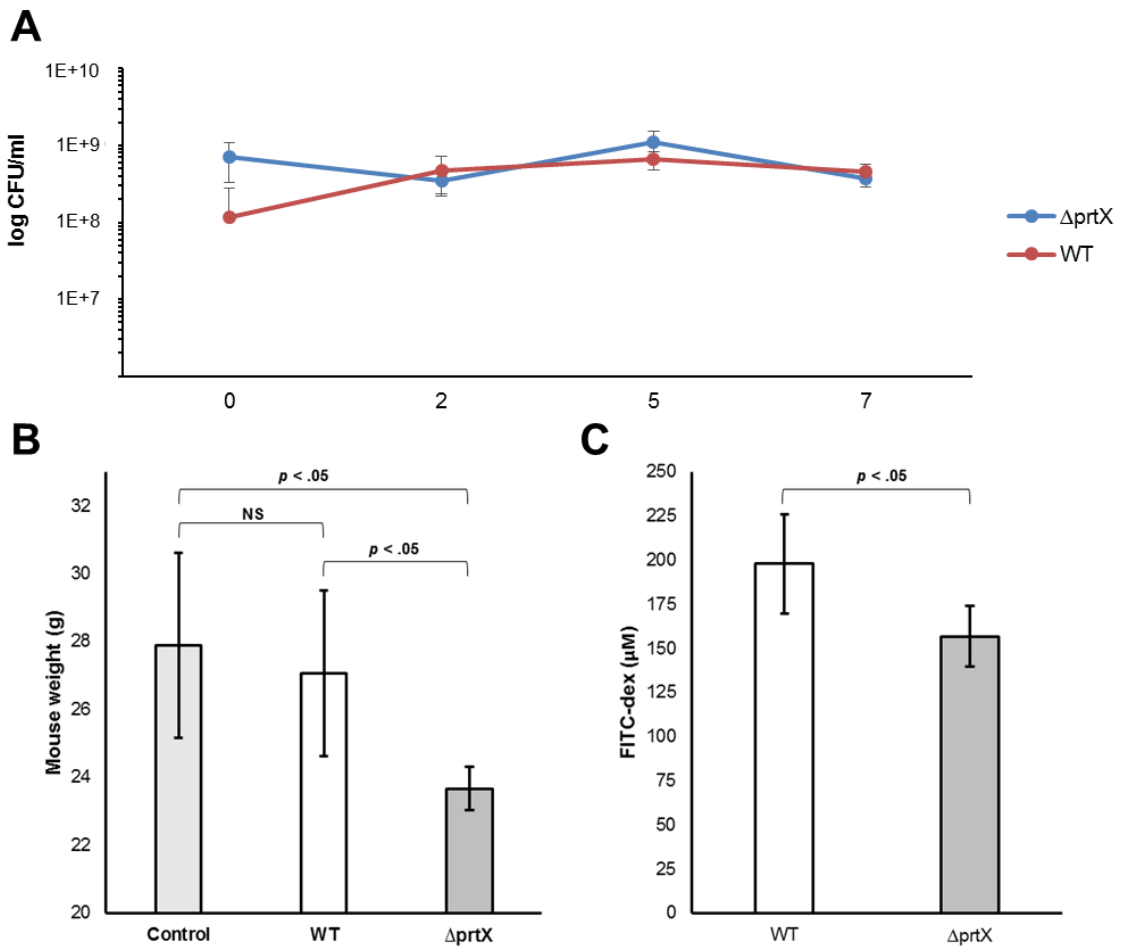


**Figure 5.5**: *In vivo* assessment of germ-free mice colonized with either WT or Δ*prtX* strains of *L. acidophilus* NCFM. (A) Colonization of mice monocolonized with WT (red, $n = 5$) or Δ*prtX* (blue, $n = 5$) over seven days was measured by plating fecal samples on MRS agar. (B) Body weight of the mice were recorded for mice colonized with WT (white; $n = 5$) and Δ*prtX* (dark gray; $n = 5$), along with the germ-free control mice ($n = 2$). There was no statistical difference between control mice and WT-colonized mice, however Δ*prtX*-colonized mice demonstrated a statistically signficiant decline in body weight after 7 days of colonization ($p < 0.05$). (B) Total gastrointestinal epithelial barrier integrity was assessed through the measurement of FITC-dextran in the serum of mice previously fed FITC-dex. Δ*prtX* colonized mice had a statistically significant reduction in FITC-dex in the serum, indicating an increase in epithelial barrier integrity ($p < 0.05$).

# APPENDICES

***Appendix A***

Reprint from:

**Johnson BR, Klaenhammer TR**. 2014. Impact of genomics on the field of probiotic research: historical perspectives to modern paradigms. *Antonie van Leeuwenhoek* **106**: 141-156

Antonie van Leeuwenhoek (2014) 106:141–156
DOI 10.1007/s10482-014-0171-y

INVITED REVIEW

Antonie van Leeuwenhoek 80th Anniversary Issue

# Impact of genomics on the field of probiotic research: historical perspectives to modern paradigms

**Brant R. Johnson · Todd R. Klaenhammer**

Received: 22 January 2014 / Accepted: 4 April 2014 / Published online: 20 April 2014


**Abstract** For thousands of years, humans have safely consumed microorganisms through fermented foods. Many of these bacteria are considered probiotics, which act through diverse mechanisms to confer a health benefit to the host. However, it was not until the availability of whole-genome sequencing and the era of genomics that mechanisms of probiotic efficacy could be discovered. In this review, we explore the history of the probiotic concept and the current standard of integrated genomic techniques to discern the complex, beneficial relationships between probiotic microbes and their hosts.

**Keywords** Probiotic · Lactic acid bacteria · Fermentation · Genomics · Lactobacilli · Bifidobacteria

B. R. Johnson · T. R. Klaenhammer (✉)
Department of Microbiology, North Carolina State University, Raleigh, NC, USA
e-mail: Klaenhammer@ncsu.edu

T. R. Klaenhammer
Department of Food, Bioprocessing, and Nutrition Science, North Carolina State University, Raleigh, NC, USA

## Introduction

### History of probiotic bacteria and the probiotic concept

A multitude of autochthonous (naturally occurring) commensal bacterial species inhabit the mucosal surfaces of the gastro-intestinal tract (GIT), as well as those of the nose, mouth and vagina. It has long been held that the consumption of allochthonous (transient) beneficial bacteria, either through food products or supplements, has a positive influence on general health and well-being of the host via commensal interactions with the GIT immune system and resident microbiota. These beneficial microorganisms, known as probiotics, are defined by the World Health Organization as "live microorganisms, which when administered in adequate amounts, confer a health benefit upon the host" (FAO/WHO 2002). Over the past four decades, there has been substantial research in the field of probiotics and, more specifically, into the mechanism of probiotic action within the host. However, the probiotic concept is not novel to the twentieth century and twenty-first centuries.

For millennia, humans have consumed microorganisms via fermented foods, which served to prevent putrefaction as well as increase sensory aspects in the food. Some of the first fermentations were likely the result of serendipitous contaminations in favourable environments resulting in soured milk products such as kefir, leben, koumiss, yogurt and sour cream—

products that are still consumed worldwide (Hosono 1992). Furthermore, through the continued practice of milk souring along with back slopping techniques, humans inadvertently aided in the domestication of certain microorganisms to diverse food environments over time (Douglas and Klaenhammer 2010). Not only were these products safe to consume, fermented dairy foods were culturally significant, as evidenced by their mention in the Bible and early sacred Hindu texts, as well as therapeutically consumed (Hosono 1992; Bibel 1988; Shortt 1999).

In the late nineteenth century, French biochemist Louis Pasteur premiered significant discoveries leading to a greater scientific understanding of fermentation (Fig. 1). Upon studying wine and beer fermentations, Pasteur demonstrated that fermentation reactions are carried out by microorganisms. Furthermore, he established that the growth of these microbes is not a product of spontaneous generation, as was the prevailing scientific and cultural consensus, but is instead due to biogenesis, which posits that all living things come only from other living things. On the foundation of Pasteur's research, Russian Nobel laureate, Élie Metchnikoff first popularized the concept of probiotics around the turn of the twentieth century. In his book, *The Prolongation of Life: Optimistic Studies*, Metchnikoff (1907) proposed that putrefaction in the intestines correlated with shortened life expectancy. Reconciling long-held observations involving lactic acid food fermentations with microbial feeding studies in animals and humans, Metchnikoff proposed that lactic acid-producing microorganisms may act as anti-putrefactive agents in the gastrointestinal tract when consumed. In fact, he hypothesized that by transforming the "wild population of the intestine into a cultured population… the pathological symptoms may be removed from old age, and… in all probability, the duration of the life of man may be considerably increased" (Metchnikoff 1907). His theory was bolstered upon observing a higher prevalence of centenarians in Bulgaria, a region known for the consumption of soured milk. Michel Cohendy, a colleague at the Pasteur Institute, provided experimental data to support Metchnikoff's hypothesis. In two feeding trials of human subjects, Cohendy found that the Bulgarian bacillus (now known as *Lactobacillus delbrueckii* subsp. *bulgaricus*) was recoverable from faeces; reduced the prevalence of putrefactive toxins; and aided in the treatment of colitis following transplantation to the large intestine (Cohendy 1906a, b). The aforementioned studies on *L. bulgaricus* enthralled the health-conscious society of Europe in the early 20$^{th}$ century and soon the Pasteur Institute of Paris began selling the *Lactobacillus* under the label of "Le Ferment" (Shortt 1999; Bibel 1988).

Despite the promising observations made by Metchnikoff and colleagues at the genesis of the probiotic concept, there was still meager scientific evidence suggesting any definitive probiotic strains or their purported effector mechanisms. In fact, Leo Rettger and coworkers at Yale University found that *L. bulgaricus* could not survive gastric passage to colonize the small intestine (Rettger 1915). This study called into question which strain(s) may have been

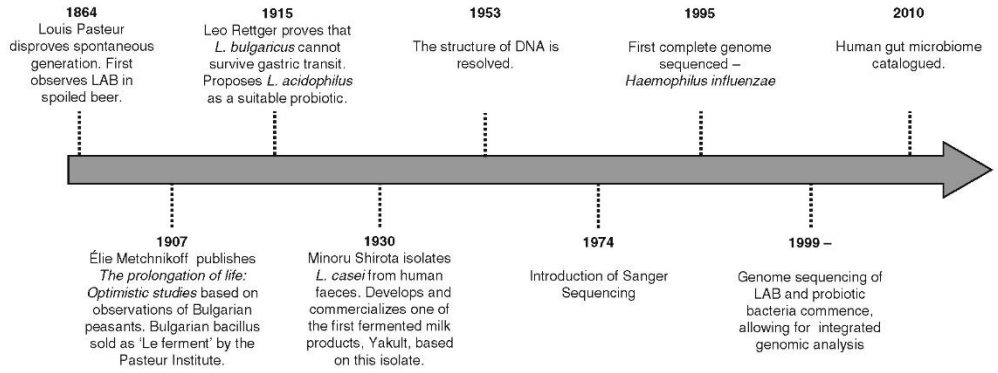


**Fig. 1** Seminal milestones contributing to the functional characterization of probiotic lactic acid bacteria

present in the original therapeutic administration studies performed by Cohendy, and subsequently sold as "Le Ferment." Instead, *Lactobacillus acidophilus* was touted to be a more suitable candidate for therapeutic applications because of its ability to survive gastric passage and transform the intestinal flora in conditions of lactose and dextrin supplementation (Rettger and Cheplin 1921). It is based on these seminal studies that the foundation of therapeutic treatment with *L. acidophilus* originated. However, even rigorous studies such as these were limited by the techniques and technologies of their time. *L. acidophilus* could not be distinguished from other aciduric commensal lactobacilli, such as *Lactobacillus gasseri*, until electrophoretic DNA–DNA hybridization studies on *Lactobacillus* lactate dehydrogenase enzymes were performed in the 1970s (Gasser 1970; Gasser et al. 1970). Therefore, it is unknown whether the cultures administered during these studies were indeed pure *L. acidophilus*, or mixed culture with *L. acidophilus*, *L. gasseri* and other aciduric lactobacilli.

After examining the burgeoning experimental evidence of probiotic bacteria, a Japanese physician named Minoru Shirota sought to isolate a human-derived strain of *Lactobacillus* for therapeutic application. And thus, in 1930, Shirota selected a species of *Lactobacillus* (now known as *Lactobacillus casei* Shirota) from human faeces that could survive passage through the GIT (Shortt 1999). From this culture, Shirota developed and commercialized one of the first fermented milk products, Yakult (Shortt 1999). Not only was this a major advancement for the commercial dairy industry, but one of the first products to deliver a pure, defined strain-cultured product. Yakult remains a staple product in Japanese, Korean, Australian and European markets. Since then, there has been a massive expansion of the functional food market, especially in fermented dairy products containing probiotic bacteria (Sanders and in't Huis-Veld 1999). In fact, a recent global market analysis on probiotics revealed a 7 % annual growth during the 2012 fiscal year, with a forecast of $48 billion in earnings within the next 5 years (Global Industry Analysis Report 2012). Furthermore, probiotics are expanding from functional food markets to pharmaceutical, therapeutic markets. This market increase correlates to the advancements of the scientific and regulatory aspects of probiotic mechanisms and delivery (Foligne et al. 2013). Considering that there are still a great number of scientific questions to explore concerning probiotic activities and interactions in the GIT, there remains a bright future for the field of probiotic research and the market thereof.

## Modern use of probiotic bacteria

Despite the long, storied history of probiotic discovery and therapeutic application, resounding clinical and experimental evidence for the use of probiotic bacteria has only recently come to a head (Table 1). One prominent example is the use of probiotics to treat functional gastrointestinal disorders (FGID). For many FGID, such as irritable bowel syndrome (IBS), there are few pharmacological treatment options due to low efficacy and serious side effects (Shen and Nahas 2009). Furthermore, IBS is quite common and is thought to be caused by changes in the gastrointestinal microbiome (Porter et al. 2011). Recently, a systematic review of successful clinical interventions using probiotics to treat various FGID has been compiled as a reference for clinicians to make evidence-based treatment decisions (Hungin et al. 2013). This systematic analysis reflects a notable caveat that must be made in probiotic research; namely, that probiotic activities are strain-specific (Hungin et al. 2013; Sanders et al. 2013). Because evidence clearly suggests not only the efficacy of probiotic therapy, but also the importance of understanding each strain, the paradigm of probiotic research is rightfully shifting towards understanding the mechanistic action of each specific strain.

Among the most studied species of probiotic bacteria are those from the genera *Lactobacillus* and *Bifidobacterium* (Table 2). The genus *Lactobacillus* is comprised of a diverse clade of Gram-positive, anaerobic/microaerophilic, non-sporulating, low G + C content lactic acid bacteria (LAB) belonging to the phylum *Firmicutes* (Pot et al. 1994). Biochemically, they are strictly fermentative; sugar fermentations result in either the sole production of lactic acid, or the production of lactic acid in conjunction with smaller amounts of carbon dioxide and ethanol/acetic acid (Hammes and Vogel 1995; Pot et al. 1994). Lactobacilli inhabit diverse ecological niches including the GIT of humans and animals, as well as vegetable, plant and dairy food environments. While *Lactobacillus* species are not dominant members of the colonic microbiotia, many are probiotic because of

**Table 1** Roles and benefits of probiotic bacteria in the GIT

| Probiotic role/benefit | Reference |
|---|---|
| Protection against infection | Corr and O'Neill (2009) |
| Symptom relief from irritable bowel syndrome | Hungin et al. (2013) |
| Lactose digestion for lactose-intolerant individuals | Mattila-Sandholm et al. (1999) |
| Lowered incidence of diarrhea | Leyer et al. (2009) |
| Lowered risk of antibiotic-associated diarrhea | Gao et al. (2010) |
| Lowered risk of *C. difficile*-associated diarrhea | Plummer et al. (2004), Gao et al. (2010) |
| Reduction in intestinal bloating | Ringel-Kulka et al. (2011) |
| Abdominal pain analgesic (via μ-opiod and cannabinoid receptors) | Rousseaux et al. (2007) |
| Lowered levels of cold and influenza-like symptoms in children | Leyer et al. (2009) |
| Antimicrobial activity | Ryan et al. (2009) |
| Competitive exclusion of pathogens | Lee et al. (2003) |
| Inhibition of *H. pylori* growth | Ushiyama et al. (2003); Fujimura et al. (2012) |
| Reduced incidence of necrotizing enterocolitis | Deshpande et al. (2010) |
| Prevention of upper respiratory infections | Hao et al. (2011) |
| Immune tolerance | van Baarlen et al. (2009) |
| Reduction in colorectal cancer biomarkers | Rafter et al. (2007) |
| Return to pre-antibiotic baseline flora | Engelbrektson et al. (2009) |
| Epithelial barrier function | Mennigen and Bruewer (2009) |
| Increased natural killer cell activity | Takeda and Okumura (2007) |
| Increased humoral immunity via secretion of IgA | Viljanen et al. (2005) |
| Lowered blood cholesterol levels | Ataie-Jafari et al. (2009) |
| Reduction in irritable bowel disease symptoms | MacFarlane et al. (2009) |
| Delivery of therapeutics | Wells and Mercenier (2008) |

Modified from O'Flaherty and Klaenhammer (2010a)

their ability to survive in the less-diverse small intestine. Members of the genus *Bifidobacterium*, of the phylum *Actinobacteria,* are Gram-positive, non-motile, anaerobic bacteria, with low levels of genomic and phylogenetic diversity (Ventura et al. 2006). They

**Table 2** Common probiotic *Lactobacillus* sp. and *Bifidobacterium* sp.

| Probiotic (strain designation) | Genome sequence reference (accession number) |
|---|---|
| *Lactobacillus* | |
| *L. acidophilus* (NCFM, La-1) | Altermann et al. (2005) (NC_006814.3) |
| *L. casei* (BL23) | Maze et al. (2010) (NC_010999.1) |
| *L. johnsonii* (NCC 533) | Pridmore et al. (2004) (NC_ 005632.1) |
| *L. plantarum* (JDM1) | Zhang et al. (2009) (NC_012984.1) |
| *L. reuteri* (SD2112) | (NC_015697.1) |
| *L. rhamnosus* (GG) | Kankainen et al. (2009) (NC_013198.1) |
| *L. salivarius* (UCC118) | Claesson et al. (2006) (NC_007929.1) |
| *L. bulgaricus* (ATCC 11842) | van de Guchte et al. (2006) (NC_008054.1) |
| *Bifidobacterium* | |
| *B. animalis* subsp. *lactis* (B1-04) | Barrangou et al. (2009) (NC_012814.1) |
| *B. breve* (UCC2003) | O'Connell Motherway et al. (2011) (NC_020517.1) |
| *B. longum* (NCC 2705) | Schell et al. (2002) (NC_004307.2) |

were originally isolated from the faeces of breast-fed infants (Tissier 1900) and nearly 50 species isolated from the GIT of humans animals and insects have since been classified (Velez et al. 2007). In fact, bifidobacteria are among the most prominent commensal bacteria found in the human colon and dominate the developing microbiome in breast-fed infants (Turroni et al. 2008; Favier et al. 2002).

Since the resolution of the first bacterial genome sequence (*Haemophilus influenzae*), an exponential advancement in sequencing processing, genome assembly and annotation technologies, at increasingly economical pricing, has yielded well over a thousand publicly available genomes (Fleischmann et al. 1995; Lagesen et al. 2010). Notably, many of these genomes are derived from lactic acid bacteria used as probiotics or starter cultures for food fermentations (Klaenhammer et al. 2002; Lukjancenko et al. 2012). Access to these data has revolutionized the molecular view of probiotic bacteria, as well as the way research questions related to probiotic mechanisms are formulated. Specifically, advancements in genomic tools

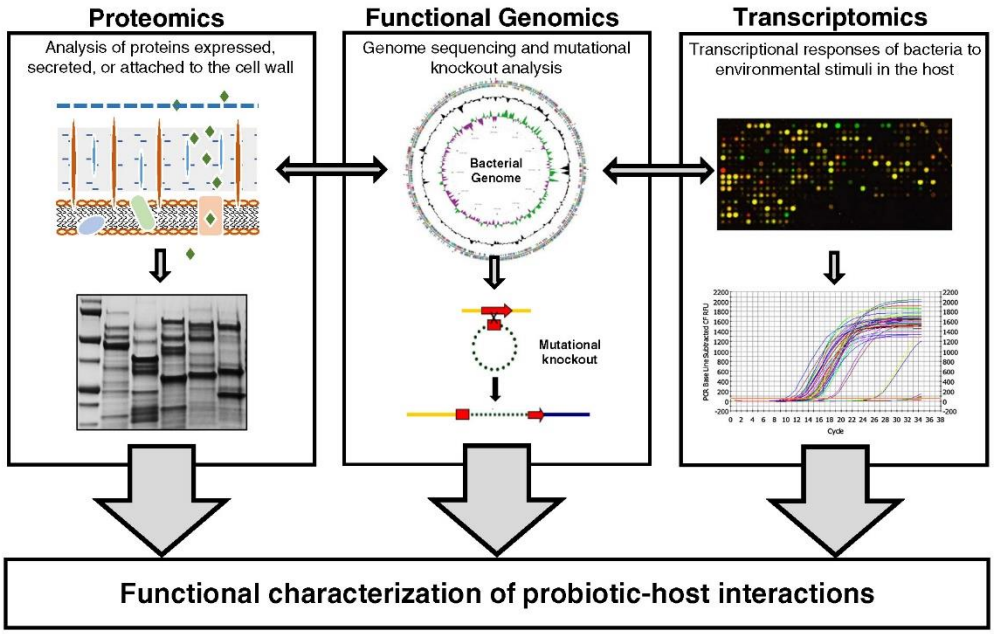


**Fig. 2** With the advent of genome sequencing, integrated genomic techniques including proteomics, transcriptomics and functional genomics have collectively characterized the mechanism of probiotic host-interactions. These analyses rely on access to annotated sequence data from whole genome sequencing. Genetic systems for deletions and mutational knockouts allow for phenotyping specific genetic loci. Proteomic approaches involve the characterization of proteins expressed, secreted, and/or attached to the cell wall. In this way, proteins are isolated, characterized by mass spectrometry, and mapped back to the proteome and corresponding genome for functional analysis. Finally, transcriptomic profiling using DNA microarrays, RNA sequencing, and RT-qPCR can measure the transcriptional responses of both bacteria and host cells in response to one another, via measurement of mRNA

including functional genomics, transcriptomics, proteomics and secretomics, have hastened research deciphering the interactions between probiotics and the GIT (Fig. 2). These techniques are being used to bridge the mechanistic gap between what has been seen clinically and anecdotally for hundreds of years.

## Characterizing probiotic mechanisms using genomic tools

Referencing the genome sequences of probiotic bacteria, the mechanism and interaction of probiotics with the host GIT are being discovered through the integration of functional genomic techniques. Within this context, there are three points of focus relating to probiotic action: (i) survival through gastrointestinal transit and adhesion to intestinal epithelia; (ii) competitive exclusion and antimicrobial activity; and (iii) modulation of the host GIT immune system (Fig. 3).

## Survival in and adhesion to the GIT

One of the most essential qualities of a probiotic microorganism is the ability to survive the varied environments of the GIT (Fig. 3a). The probiotic must be able to adapt to acidic gastric juices and bile in the small intestine. Like many aciduric bacteria, the lipid membranes of lactobacilli exposed to acid and bile are altered in order to increase survival. The lipid membrane of *Lactobacillus casei* demonstrated a marked increase of mono-unsaturated fatty acids in response to acidification (Fozo et al. 2004). Similarly, the lipid membrane of *Lactobacillus reuteri* exposed

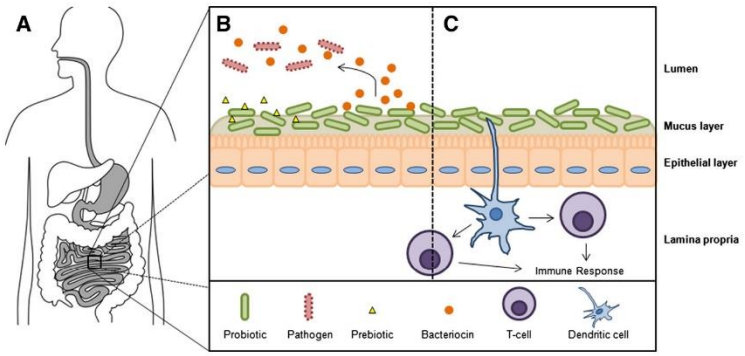


**Fig. 3** **a** Probiotic microbes delivered orally must survive varying environments encountered through gastrointestinal transit, including acidic gastric juices (pH ~2) in the stomach, and bile in the small intestines. **b** At the intestinal epithelia, probiotics have been reported to adhere in high numbers, leading to competitive exclusion of pathogens. The growth of certain probiotics can be stimulated by the presence of complex prebiotic oligosaccharides. Additionally, some probiotics produce bacteriocins and other antimicrobial agents which may antagonize pathogens in the lumen. **c** Probiotics bound in the mucus and epithelial layers are proximal to dendritic cells of the mucosal immune system, leading to immunomodulation

to bile salts and cholesterol increased the number of mono-unsaturated fatty acids compared to saturated fatty acids (Taranto et al. 2003). Considering these observations, a recent study using the probiotic *Lactobacillus rhamnosus* GG found that an exogenous oleic acid [C18:1 (cis-9)] source significantly increased acid survival by incorporating the oleic acid into the membrane, which is reduced to stearic acid (C18:0) in the acidified environment (Corcoran et al. 2007). Aside from the biochemical changes to the lipid membranes, the *Lactobacillus* species have global transcriptional responses to these stressors, usually through two-component regulatory systems (2CRS; Lebeer et al. 2008b). Numerous transcriptomic analyses have been used in lactobacilli to identify differentially expressed genes, such as those corresponding to 2CRS, surface proteins and proton efflux systems, in response to gastric acid stress (Azcarate-Peril et al. 2005; Pieterse et al. 2005) and bile stress (Bron et al. 2006; Pfeiler et al. 2007). Bacteria quickly sense and respond to changing environmental conditions via 2CRS through the sensing domains of a transmembrane histidine protein kinase (HPK). Upon receiving the environmental signal, the HPK is activated to autophosphorylate a specific histidine residue which is transferred to the regulatory domain of the response regulator (RR), a DNA-binding transcriptional regulator. Therefore, 2CRS can be predicted from bacterial genome sequence annotations based on the presence of putative HPK and RR in close proximity to one another (Altermann et al. 2005; Morita et al. 2009). In *L. acidophilus* NCFM, a gene (*lba1524*) encoding a functional HPK was knocked out, resulting in a mutant with increased sensitivity to acid stress compared to the parent strain. Furthermore, transcriptomic analysis via DNA microarray comparing the *lba1524* mutant to wild-type demonstrated an impact on 80 genes (Azcarate-Peril et al. 2005). Notably, one upregulated gene in the HPK mutant was the LuxS homolog of the autoinducer-2 quorum sensing compound, important for survival in gastric juices and adhesion to intestinal epithelial cell lines (Lebeer et al. 2008a; Buck et al. 2009).

The response of lactobacilli to bile salts has also been measured through microarray analysis. In *Lactobacillus plantarum* a DNA-microarray was performed after exposure to porcine bile, resulting in the identification of bile response genes encoding stress response proteins, cell envelope proteins and an $F_0F_1$ ATPase (Bron et al. 2006). A similar transcriptomic profiling of *L. acidophilus* revealed multiple genes involved in bile tolerance, including a 2CRS and multi-drug resistance (MDR) transporter efflux pumps (Pfeiler et al. 2007). Mutants with insertionally inactivated genes for the bile inducible 2CRS HPK and RR were more sensitive to bile compared to parent strains, confirming their role in bile tolerance (Pfeiler et al. 2007). A recent comparative proteomic analysis on bile sensitive and

bile tolerant strains of *L. plantarum* corroborated these transcriptomic data and elucidated potential biomarkers for the selection of bile tolerant probiotic strains (Hao et al. 2011). Additionally, the role of efflux pumps and MDR transporters in probiotic bile tolerance are beginning to be recognized. Functional genomic analyses of MDR transporters in probiotic strains of *L. reuterii* and *L. acidophilus* demonstrated roles in bile tolerance (Whitehead et al. 2008; Pfeiler and Klaenhammer 2009). Furthermore, a MDR transporter gene in *Bifidobacterium longum*, *betA* (bile efflux transporter), was recently identified through in silico genome analysis and functionally characterized (Gueimonde et al. 2009). Heterologous expression of *betA* in *Escherichia coli* conferred bile tolerance through active efflux of bile salts.

Survivability and enhancement of beneficial microbes in the GIT can be accomplished by providing selectively utilizable carbohydrates, called prebiotics (Roberfroid 2007; Andersen et al. 2013). These carbohydrates, including $\beta$-galactooligosaccharide (GOS), lactulose, fructo-oligosaccharide and inulin, are resistant to gastric acidity, hydrolysis and gastrointestinal absorption (Roberfroid et al. 2010). As growth substrates, prebiotic carbohydrates are preferentially metabolized by species of health-promoting bacteria. Recently, differential transcriptomic and functional genomic analyses have demonstrated the capabilities of the probiotic bacteria *L. acidophilus* NCFM (Andersen et al. 2012) and *Bifidobacterium lactis* B1-04 (Andersen et al. 2013) to utilize prebiotic oligosaccharides. With these data, novel symbiotic formulations of corresponding prebiotics for *L. acidophilus* and *B. lactis* can be created to aid in the survival and probiotic effectiveness in the host small intestines and colon, respectively. In a similar vein, there is compelling evidence to suggest glycogen metabolism is a colonization factor for probiotic LAB. Glycogen is a large molecular mass, soluble $\alpha$-1,4-linked glucose polymer with numerous $\alpha$-1,6-linked branches. It has multiple physiological functions in various bacteria and has been theorized to function as a carbon capacitor for the regulation of energy flux (Belanger and Hatfull 1999). Recent work by Goh and Klaenhammer (2013) demonstrated the functionality of a putative glycogen metabolism operon found in the genome sequence. Remarkably, through a series of chromosomal deletions and phenotypic assays, glycogen metabolism was found to regulate growth maintenance, bile tolerance and complex carbohydrate utilization in *L. acidophilus* (Goh and Klaenhammer 2013).

Beyond surviving gastrointestinal transit, a second key factor for probiotic activity is through adhesion to intestinal epithelia of the GIT. Preliminary in vitro studies using Caco-2 human intestinal epithelial cell lines revealed multiple probiotic lactobacilli with adhesive capabilities (Chauviere et al. 1992; Tuomola and Salminen 1998). Notably, there has also been work demonstrating the adhesiveness of *Bifidobacterium* spp. to human intestinal mucus (He et al. 2001). However, access to genome sequence data, paired with integrated genomic techniques, elucidated mediators of probiotic adhesion. The majority of these factors are secreted or attached to the cell wall in a sortase-dependent manner, in order to interface with the intestinal epithelia (reviewed by Velez et al. 2007 Lebeer et al. 2008b). In a study using *L. plantarum* WCFS1, two of these sortase-dependent proteins (SDP) were found to be induced in the murine GIT (Bron et al. 2004a, b). Mutational analysis of one of these genes (*lp_2940*) resulted in a 100- to 1,000-fold decrease in persistence capacity of the *L. plantarum* lp_2940 knockout mutant in a mouse model. In *L. acidophilus* NCFM, in silico genome screening lead to the selection of five putative adhesion cell surface proteins, including a fibronectin binding protein (FbpA), S-layer protein (SlpA), mucin-binding protein (Mub) and two R28 homologues involved in streptococcal adhesion (Buck et al. 2005). Through mutational analysis, FbpA, Mub, and SlpA were all found to contribute to adhesion to Caco-2 epithelial cell lines. Similarly, a stress response protein and an aggregation-promoting factor (both cell surface proteins) were found in later studies to contribute to adherence to Caco-2 cells (O'Flaherty and Klaenhammer 2010b; Goh and Klaenhammer 2010). In *Lactobacillus crispatus* JCM5810, the S-layer protein (CbsA) contains domains that bind to laminin and collagens (Antikainen et al. 2002). Genome screening and secretome analysis of *Lactobacillus salivarius* UCC118 led to the identification of three SDPs with mucus-binding domains. A sortase-deficient strain was created, resulting in significantly reduced adherence to Caco-2 and HT-29 cell lines in vitro (van Pijkeren et al. 2006). Notably, genomic analysis between two strains of *L. rhamnosus* revealed the presence of a

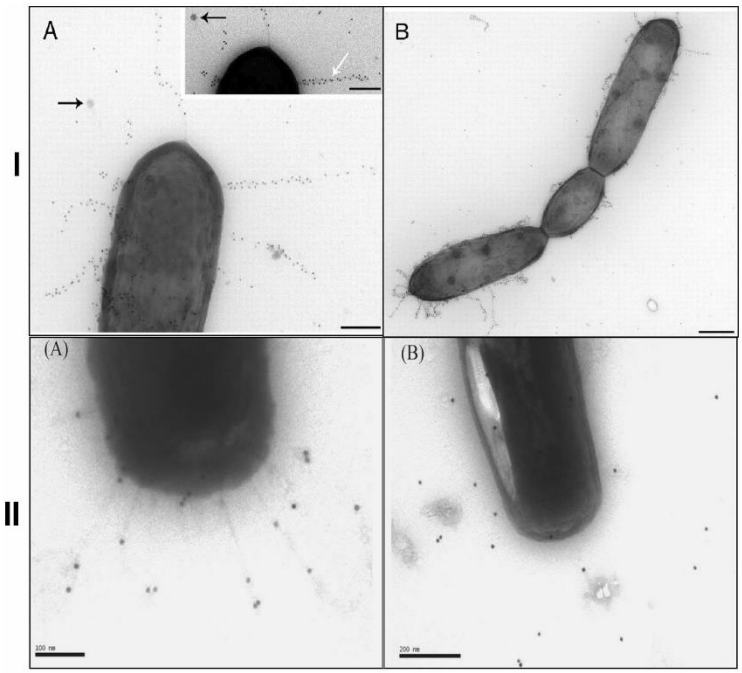


**Fig. 4** Identification of pili structures in *Lactobacillus rhamnosus* GG (**I**) and *Bifidobacterium breve* UCC2003 (**II**). Images were obtained using transmission electron microscopy on negatively stained, immunogold-labeled anti-*SpaC* pili in *L. rhamnosus* (**I**) and anti-$Flp_{2003}$ pili in *B. breve* (**II**). Reprinted with permission from Kankainen et al. (2009), copyright © 2009 National Academy of Sciences, USA; and O'Connell Motherway et al. (2011), copyright © 2011 National Academy of Sciences USA

genomic island in *L. rhamnosus* GG that contained three secreted, sortase-dependent pilins encoded by *spaCBA* (Kankainen et al. 2009). Immunoblotting and immunogold electron microscopy confirmed the formation of cell wall-bound pili (Fig. 4). Furthermore, mutational analysis of the *spaC* gene abolished the adherence capability of *L. rhamnosus* GG to human intestinal mucus, implicating the role of these unique pili structures in adherence and retention in the GIT. Since this initial report, a type IVb tight adherence (Tad) pilus-encoding gene cluster has been identified in *Bifidobacterium breve* UCC2003 (O'Connell Motherway et al. 2011; Fig. 4). Mutational analysis demonstrated that the Tad gene cluster was essential for colonization in a murine model. Collectively, these data suggest that there are multiple cell surface factors which contribute to probiotic adherence to human intestinal epithelia.

## Competitive exclusion and antimicrobial activity

Another health-promoting aspect of probiotic bacteria is the prevention of pathogenic infection (Fig. 3b). When probiotic lactobacilli are ingested, they temporarily coat the mucosal layer and epithelia of the small intestine (see above) leading to both physical and chemical barriers against harmful bacteria (Servin 2004). Initial studies demonstrated that lactobacilli inhibited adherence of Gram-negative uropathogens when uroepithelial cells were pre-incubated with whole, viable *Lactobacillus* (Chan et al. 1985). Furthermore, in vivo mice models demonstrated that *L. casei* GR1 was capable of preventing urinary tract infections from *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonus aeruginosa* (Reid et al. 1985). In both cases, the mechanism of pathogenic antagonism was due to the ability of lactobacilli to adhere to the

urogenital epithelia, thus preventing infection through competitive exclusion of the pathogen. These studies and others suggested that similar competitive exclusion could be possible in the human GIT using probiotic lactobacilli and bifidobacteria. In fact, numerous studies have demonstrated the in vitro inhibition of numerous gastrointestinal pathogens through competitive exclusion of probiotic lactobacilli and bifidobacteria using intestinal cell lines (reviewed by: Servin 2004).

In addition to competitive exclusion of pathogens, probiotic bacteria produce numerous chemical antimicrobials which may prevent pathogenic infection. These include: hydrogen peroxide (St Amant et al. 2002; Pridmore et al. 2008), lactic acid (Fayol-Messaoudi et al. 2005), biosurfactants (Velraeds et al. 1996), immunomodulatory products (Ryan et al. 2009) and bacteriocins (Dobson et al. 2012). Bacteriocins are bacterially derived antimicrobial peptides that are active against other bacteria, but to which the producing bacterium is immune (Cotter et al. 2005). Lactic acid bacteria produce numerous broad-spectrum bacteriocins which are divided into three main classes: class I bacteriocins (lantibiotics; Schnell et al. 1988), small peptides possessing lanthionine residues; class II bacteriocins, which are heat-stable and do not contain lanthionine residues; and bacteriolysins, which are large, heat-labile murein hydrolases (Cotter et al. 2005; Fig. 5). Historically, scientists have sought to characterize the genetics and biochemistry of bacteriocins produced by LAB, in part due to their safety implications in the dairy fermentation industries (Klaenhammer 1993; Nes et al. 1996). In fact, one of the most industrially relevant bacteriocins is nisin, a lantibiotic produced by *Lactococcus lactis* (Delves-Broughton et al. 1996). Nisin has two modes of bacteriocidal activity (Fig. 5). First, it can bind lipid II, the main transporter of peptidoglycan subunits, disrupting cell wall synthesis (Breukink et al. 1999). Nisin also targets lipid II as a docking mechanism for pore formation, leading to rapid cell death due to disruption of the proton motive force (Wiedemann et al. 2001). Notably, Gram-positive bacteriocins generally have a narrow range of toxicity, as they are primarily lethal to closely related bacterial species such as *Staphylococcus*, *Listeria* and other LAB (Servin 2004). Most research involving LAB-associated bacteriocins has been in vitro. However, a landmark study by Corr et al. (2007) demonstrated that a bacteriocin produced by *L. salivarius* UCC118 caused in vivo protection in mice challenged with the food-borne pathogen *Listeria monocytogenes*. Using a functional genomics-based mutational analysis, generating a stable *L. salivarius* UCC118 strain deficient in bacteriocin production, undoubtedly established the role of this bacteriocin in protection against *L. monocytogenes* infection.

## Probiotic modulation of the gastrointestinal mucosal immune system

Perhaps one of the most important aspects of probiotic bacteria is the ability to modulate the host GIT mucosal immune system locally and systemically (Fig. 3c). The interaction between the probiotic microbe with the resident microbiota, gastrointestinal epithelia and gut immune cells to produce an immunomodulatory response is quite complex, and has been reviewed exhaustively (Lebeer et al. 2010; O'Flaherty and Klaenhammer 2010a; Reid et al. 2011; Bron et al. 2012; Klaenhammer et al. 2012; Selle and Klaenhammer 2013). Probiotic microbes modulate mucosal immunity through the interaction of proteinacious microorganism-associated molecular patterns (MAMPs) with pattern recognition receptors (PRRs) on antigen-presenting cells (APCs), such as dendritic cells and macrophages. Upon exposure to MAMPs, the PRRs (including NOD-like receptors, Toll-like receptors, and C-type lectin receptors) activate nuclear factor (NF)-κB and mitogen-activated protein kinase signaling cascades, which modulate the expression of cytokine and chemokine genes. The most common MAMPs from probiotic microorganisms are lipoteichoic acids (LTA), peptidoglycan and S-layer proteins (Bron et al. 2012). Multiple studies have explored the immunomodulatory effect of these MAMPs using functional genomic techniques. In a seminal study, the probiotics *L. casei* and *L. reuteri* were found to induce IL-10 producing regulatory T-cells through the modulation of the DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN; Smits et al. 2005). Targeting of DC-SIGN by probiotic bacteria is potentially an important factor for treatment of inflammatory conditions via the production of anti-inflammatory IL-10. The S-layer protein (SlpA) of *L. acidophilus* NCFM was found to bind DC-SIGN, which regulate immature DC and T cell functionality (Konstantinov et al. 2008). Using *L. plantarum* NCIMB8826, cell wall composition was examined for immunomodulatory effects by creating a

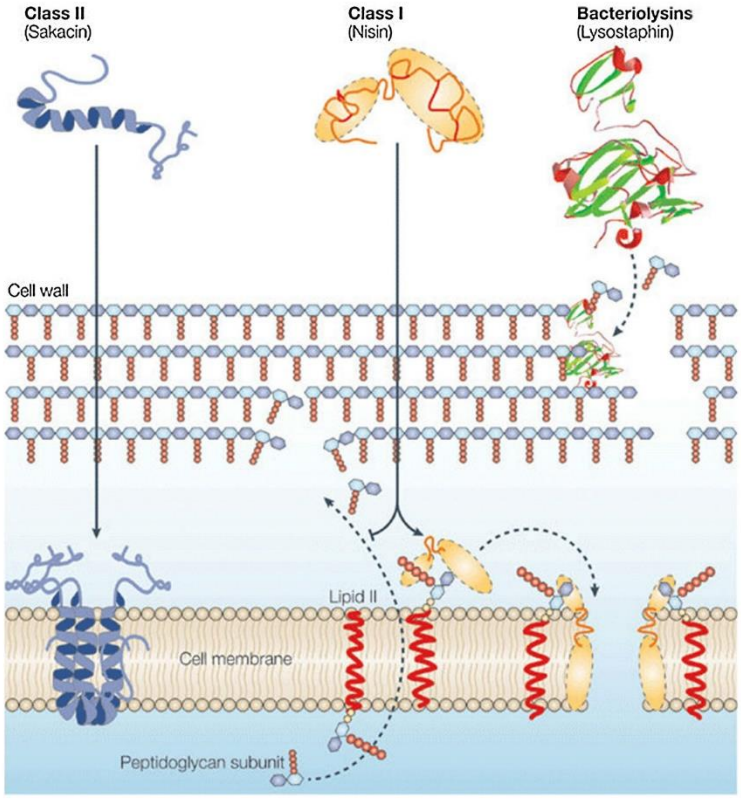


**Fig. 5** Bacteriocins produced by LAB are grouped into three classes based on structure and function: *class I* (lantibiotics), *class II*, and bacteriolysins. *Class I* lantibiotics, such as nisin, can have two modes of action. First, they bind lipid *II* to prevent peptidoglycan subunit transport, disrupting peptidoglycan synthesis and cell division. Second, they dock at *lipid II* to create pores in the cytoplasmic membrane of the bacteria. *Class II* bacteriocins, such as sakacin, often contain amphiphilic helical structures which can insert into the cell membrane, leading to cell lysis. Bacteriolysins, such as lyostaphin, are large hydrolases which directly degrade the peptidoglycan cell wall. Reprinted with permission of Macmillan Publishers, Ltd, from Cotter et al. (2005), copyright © 2005 Nature Publishing Group

mutant (*dlt* $^{-}$) which produced modified teichoic acids with less D-alanine than the parent strain (Grangette et al. 2005). The mutant demonstrated a significant reduction in production of proinflammatory cytokines compared to wild type, along with a simultaneous increase in anti-inflammatory IL-10. Furthermore, the *dlt* $^{-}$mutant was more protective in an in vivo murine colitis model than the parent strain (Grangette et al. 2005). An LTA-deficient strain of *L. acidophilus* NCFM, created by a clean deletion of the *lba0447* phosphoglycerol transferase, was able to abate induced colonic-inflammation in a colitis mouse model through the down regulation of pro-inflammatory IL-12 and TNF-α and the up regulation of anti-inflammatory IL-10 (Mohamadzadeh et al. 2011). Additionally, this same mutant reduced colonic polyposis in a colon cancer mouse model, through the normalization of pathogenic immune responses (Khazaie et al. 2012).

Like many probiotic effectors, most MAMPs are found on the cell surface of Gram-positive microbes. Recently, the genomes and proteomes of several lactobacilli were bioinformatically screened to create a secretome database cataloging the various extracellular proteins in LAB (Kleerebezem et al. 2010; Zhou et al. 2010). Consequently, using in silico genome analysis and by reference to the LAB secretome, a putative MAMP can be selected and validated through mutagenesis (Bron et al. 2012). Indeed, a recent study of *L. acidophilus* used a proteomic-based method to identify S-layer associated proteins (SLAPs) in situ (Johnson et al. 2013). After extraction, the SLAPs were identified through mass spectrometry and referenced to the LAB secretome. Mutational analysis of one SLAP (*lba1029*), revealed an immunomodulatory phenotype using in vitro bacterial-DC co-incubation assays, suggesting the potential of multiple unknown

MAMPs associated with the S-layer of *L. acidophilus* NCFM. Researchers are also trying to understand the complex dynamic of host-microbe crosstalk by using whole transcriptome profiling of human intestinal epithelia upon exposure to probiotics. In one study, transcriptomes were obtained from the mucosa of the proximal small intestines of healthy volunteers exposed to probiotic *L. acidophilus*, *L. casei*, and *L. rhamnosus* (van Baarlen et al. 2011). The transcriptional networks induced by each probiotic were unique to each strain and remarkably similar to response profiles obtained from bioactive components and drug treatments. In vitro transcriptome profiling of Caco-2 intestinal epithelial cell lines exposed to *L. acidophilus* NCFM corroborated these data (O'Flaherty and Klaenhammer 2012). Similarly, *Bifidobacterium bifidum* PRL2010 transcriptome analyses with both in vitro human cell lines and in vivo murine models demonstrated the capacity for strain PRL2010 to modulate host innate immunity (Turroni et al. 2014).

## Conclusions and future directions

While the paradigm of discovery based genomics in probiotic LAB has uncovered vital aspects of probiotic mechanisms, it has also revealed the complexity of these interactions with the resident microbiota and the mucosal immune system. But with this challenge has come great opportunity. For example, probiotic bacteria are now being explored as suitable models for vaccine/drug delivery, due to their close association with host immunity and immunomodulatory action (Kajikawa et al. 2011; Stoeker et al. 2011; Kajikawa et al. 2012). Furthermore, recent discoveries are also demonstrating that the roles of probiotic bacteria and the resident microbiota extend far beyond gastrointestinal health. Specifically, studies on the bi-directional crosstalk between the GIT and the brain (the gut-brain axis) are revealing the neurochemical importance of gut homeostasis (Cryan and Mahony 2011; Bercik et al. 2012). Along with these advancements, it is important that human clinical trials continue with experimental designs that are well-controlled and well-defined, reflecting the great progress that has been made in the field of probiotic and GIT microbiome research (reviewed by Sanders et al. 2013). With more than a century passing since Metchnikoff's observations, keen experimental design using integrated genomics has led to a clearer definition of probiotic bacteria, as well as a model for continued discovery.

**Acknowledgments** Research on the functional genomics of probiotic microbes in the Klaenhammer group is partially supported by the North Carolina Agricultural Foundation and Danisco/Dupont Nutrition and Health. The authors wish to thank Dr. Sarah O'Flaherty for helpful discussion on figure design.



## References

Altermann E, Russell WM, Azcarate-Peril MA, Barrangou R, Buck BL, McAuliffe O, Souther N, Dobson A, Duong T, Callanan M, Lick S, Hamrick A, Cano R, Klaenhammer TR (2005) Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. Proc Natl Acad Sci USA 102(11):3906–3912. doi:10.1073/0409188102

Andersen JM, Barrangou R, Hachem MA, Lahtinen SJ, Goh YJ, Svensson B, Klaenhammer TR (2012) Transcriptional analysis of prebiotic uptake and catabolism by *Lactobacillus acidophilus* NCFM. PLoS ONE 7(9):e44409. doi:10.1371/0044409

Andersen JM, Barrangou R, Abou-Hachem M, Lahtinen SJ, Goh YJ, Svensson B, Klaenhammer TR (2013) Transcriptional analysis of oligosaccharide utilization by *Bifidobacterium lactis* Bl-04. BMC Genom 14:312. doi:10.1186/1471-2164-14-312

Antikainen J, Anton L, Sillanpaa J, Korhonen TK (2002) Domains in the S-layer protein CbsA of *Lactobacillus crispatus* involved in adherence to collagens, laminin and lipoteichoic acids and in self-assembly. Mol Microbiol 46(2):381–394. doi:10.1046/1365-2958.2002.03180

Ataie-Jafari A, Larijani B, Alavi-Majd H, Tahbaz F (2009) Cholesterol-lowering effect of probiotic yogurt in comparison with ordinary yogurt in mildly to moderately hypercholesterolemic subjects. Ann Nutr Metab 54(1):22–27. doi:10.1159/000203284

Azcarate-Peril MA, McAuliffe O, Altermann E, Lick S, Russell WM, Klaenhammer TR (2005) Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in *Lactobacillus acidophilus*. Appl Environ Microbiol 71(10):5794–5804. doi:10.1128/71.10.5794-5804.2005

Barrangou R, Briczinski EP, Traeger LL, Loquasto JR, Richards M, Horvath P, Coute-Monvoisin AC, Leyer G, Rendulic S, Steele JL, Broadbent JR, Oberg T, Dudley EG, Schuster S, Romero DA, Roberts RF (2009) Comparison of the complete genome sequences of *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and Bl-04. J Bacteriol 191(13):4144–4151. doi:10.1128/00155-09

Belanger AE, Hatfull GF (1999) Exponential-phase glycogen recycling is essential for growth of *Mycobacterium smegmatis*. J Bacteriol 181(21):6670–6678

Bercik P, Collins SM, Verdu EF (2012) Microbes and the gut-brain axis. Neurogastroenterol Motil 24(5):405–413. doi:10.1111/1365-2982.2012.01906

Bibel DJ (1988) Elie Metchnikoff's *Bacillus* of long life. ASM News 54:661–665

Breukink E, Wiedemann I, van Kraaij C, Kuipers OP, Sahl HG, de Kruijff B (1999) Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science 286(5448): 2361–2364. doi:10.1126/286.5448.2361

Bron PA, Marco M, Hoffer SM, Van Mullekom E, de Vos WM, Kleerebezem M (2004a) Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters in vitro and in situ in the gastrointestinal tract. J Bacteriol 186(23):7829–7835. doi:10.1128/186.23.7829-7835.2004

Bron PA, Grangette C, Mercenier A, de Vos WM, Kleerebezem M (2004b) Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. J Bacteriol 186(17):5721–5729. doi:10.1128/186.17.5721-5729.2004

Bron PA, Molenaar D, de Vos WM, Kleerezebem M (2006) DNA micro-array-based identification of bile-responsive genes in *Lactobacillus plantarum*. J Appl Microbiol 100(4):728–738. doi:10.1111/1365-2672.2006.02891

Bron PA, van Baarlen P, Kleerebezem M (2012) Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa. Nat Rev Microbiol 10(1):66–78. doi:10.1038/2690

Buck BL, Altermann E, Svingerud T, Klaenhammer TR (2005) Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol 71(12):8344–8351. doi:10.1128/71.12.8344-8351.2005

Buck BL, Azcarate-Peril MA, Klaenhammer TR (2009) Role of autoinducer-2 on the adhesion ability of *Lactobacillus acidophilus*. J Appl Microbiol 107(1):269–279. doi:10.1111/1365-2672.2009.04204

Chan RC, Reid G, Irvin RT, Bruce AW, Costerton JW (1985) Competitive exclusion of uropathogens from human uroepithelial cells by *Lactobacillus* whole cells and cell wall fragments. Infect Immun 47(1):84–89

Chauviere G, Coconnier MH, Kerneis S, Fourniat J, Servin AL (1992) Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. Microbiology 138(Pt 8):1689–1696

Claesson MJ, Li Y, Leahy S, Canchaya C, van Pijkeren JP, Cerdeno-Tarraga AM, Parkhill J, Flynn S, O'Sullivan GC, Collins JK, Higgins D, Shanahan F, Fitzgerald GF, van Sinderen D, O'Toole PW (2006) Multireplicon genome architecture of *Lactobacillus salivarius*. Proc Natl Acad Sci USA 103(17):6718–6723. doi:10.1073/0511060103

Cohendy M (1906a) Déscription d'un ferment lactique puissant capable de s'acclimater dans l'intestin d'homme. Comptes redus des séances et mémoirs de la société de biologie 60:558–560

Cohendy M (1906b) Essai de traitement de l'enterite muco-membraneuse aigue par l'acclimation d'un ferment lactique dans le gros intestine. Comptes redus des séances et mémoirs de la société de biologie 60:363

Corcoran BM, Stanton C, Fitzgerald GF, Ross RP (2007) Growth of probiotic lactobacilli in the presence of oleic acid enhances subsequent survival in gastric juice. Microbiology 153(1):291–299

Corr SC, O'Neill LA (2009) *Listeria monocytogenes* infection in the face of innate immunity. Cell Microbiol 11(5): 703–709. doi:10.1111/1462-5822.2009.01294.x

Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG (2007) Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. Proc Natl Acad Sci USA 104(18):7617–7621. doi:10.1073/0700440104

Cotter PD, Hill C, Ross RP (2005) Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 3(10): 777–788. doi:10.1038/1273

Cryan JF, Mahony SM (2011) The microbiome-gut-brain axis: from bowel to behavior. Neurogastroenterol Motil 23(3):187–192. doi:10.1111/j.1365-2982.2010.01664.x

Delves-Broughton J, Blackburn P, Evans RJ, Hugenholtz J (1996) Applications of the bacteriocin, nisin. Antonie Van Leeuwenhoek 69(2):193–202

Deshpande G, Rao S, Patole S, Bulsara M (2010) Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. Pediatrics 125(5):921–930. doi:10.1542/2009-1301

Dobson A, Cotter PD, Ross RP, Hill C (2012) Bacteriocin production: a probiotic trait? Appl Environ Microbiol 78(1):1–6. doi:10.1128/05576-11

Douglas GL, Klaenhammer TR (2010) Genomic evolution of domesticated microorganisms. Annu Rev Food Sci Technol 1:397–414. doi:10.1146/102308.124134

Engelbrektson A, Korzenik JR, Pittler A, Sanders ME, Klaenhammer TR, Leyer G, Kitts CL (2009) Probiotics to minimize the disruption of faecal microbiota in healthy subjects undergoing antibiotic therapy. J Med Microbiol 58(Pt 5):663–670. doi:10.1099/0.47615-0

FAO/WHO (2002) Guidelines for the Evaluation of Probiotics in Food. FAO/WHO, London, ON, Canada

Favier CF, Vaughan EE, De Vos WM, Akkermans AD (2002) Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol 68(1):219–226

Fayol-Messaoudi D, Berger CN, Coconnier-Polter MH, Le Lievin-Moal V, Servin AL (2005) pH-, Lactic acid-, and non-lactic acid-dependent activities of probiotic *Lactobacilli* against *Salmonella enterica* serovar *Typhimurium*. Appl Environ Microbiol 71(10):6008–6013. doi:10.1128/71.10.6008-6013.2005

Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM et al (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science 269(5223):496–512

Foligne B, Daniel C, Pot B (2013) Probiotics from research to market: the possibilities, risks and challenges. Curr Opin Microbiol 16(3):284–292. doi:10.1016/2013.06.008

Fozo EM, Kajfasz JK, Quivey RG (2004) Low pH-induced membrane fatty acid alterations in oral bacteria. FEMS Microbiol Lett 238:291–295. doi:10.1016/2004.07.047

Fujimura S, Watanabe A, Kimura K, Kaji M (2012) Probiotic mechanism of *Lactobacillus gasseri* OLL2716 strain

against *Helicobacter pylori*. J Clin Microbiol 50(3):1134–1136. doi:10.1128/06262-11

Gao XW, Mubasher M, Fang CY, Reifer C, Miller LE (2010) Dose-response efficacy of a proprietary probiotic formula of *Lactobacillus acidophilus* CL1285 and *Lactobacillus casei* LBC80R for antibiotic-associated diarrhea and *Clostridium difficile*-associated diarrhea prophylaxis in adult patients. Am J Gastroenterol 105(7):1636–1641. doi:10.1038/2010.11

Gasser F (1970) Electrophoretic characterization of lactic dehydrogenases in the genus *Lactobacillus*. Microbiology 62(2):223–239

Gasser F, Doudoroff M, Contopoulos R (1970) Purification and properties of NAD-dependent lactic dehydrogenases of different species of *lactobacillus*. Microbiology 62(2):241–250

Goh YJ, Klaenhammer TR (2010) Functional roles of aggregation-promoting-like factor in stress tolerance and adherence of *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol 76(15):5005–5012. doi:10.1128/00030-10

Goh YJ, Klaenhammer TR (2013) A functional glycogen biosynthesis pathway in *Lactobacillus acidophilus*: expression and analysis of the glg operon. Mol Microbiol 89(6):1187–1200. doi:10.1111/12338

Grangette C, Nutten S, Palumbo E, Morath S, Hermann C, Dewulf J, Pot B, Hartung T, Hols P, Mercenier A (2005) Enhanced antiinflammatory capacity of *Lactobacillus plantarum* mutant synthesizing modified teichoic acids. Proc Natl Acad Sci USA 102(29):10321–10326. doi:10.1073/s.0504084102

Gueimonde M, Garrigues C, van Sinderen D, de los Reyes-Gavilan CG, Margolles A (2009) Bile-inducible efflux transporter from *Bifidobacterium longum* NCC2705, conferring bile resistance. J Bacteriol 75(10):3153–3160. doi:10.1128/00172-09

Hammes WP, Vogel RF (1995) The genus *Lactobacillus*. In: Ludwig S (ed) The genera of lactic acid bacteria. Blackie Academic and Professional, Glasgow, pp 19–54

Hao Q, Lu Z, Dong BR, Huang CQ, Wu T (2011) Probiotics for preventing acute upper respiratory tract infections. Cochrane Database Syst Rev. doi:10.1002/14651858

He F, Ouwehan AC, Hashimoto H, Isolauri E, Benno Y, Salminen S (2001) Adhesion of *Bifidobacterium* spp. to human intestinal mucus. Microbiol Immunol 45(3):259–262

Hosono A (1992) Fermented milk in the orient in *Functions of Fermented Milk: Challenges for the Health Sciences*. Elsevier, Barking, pp 61–78

Hungin AP, Mulligan C, Pot B, Whorwell P, Agreus L, Fracasso P, Lionis C, Mendive J, de Philippart Foy JM, Rubin G, Winchester C, Wit N, European Society for Primary Care G (2013) Systematic review: probiotics in the management of lower gastrointestinal symptoms in clinical practice—an evidence-based international guide. Aliment Pharmacol Ther 38(8):864–886. doi:10.1111/12460

Johnson B, Selle K, O'Flaherty S, Goh YJ, Klaenhammer T (2013) Identification of extracellular surface-layer associated proteins in *Lactobacillus acidophilus* NCFM. Microbiology 159(Pt 11):2269–2282. doi:10.1099/0.070755-0

Kajikawa A, Nordone SK, Zhang L, Stoeker LL, LaVoy AS, Klaenhammer TR, Dean GA (2011) Dissimilar properties of two recombinant *Lactobacillus acidophilus* strains displaying *Salmonella* FliC with different anchoring motifs. Appl Environ Microbiol 77(18):6587–6596. doi:10.1128/05153-11

Kajikawa A, Zhang L, Long J, Nordone S, Stoeker L, LaVoy A, Bumgardner S, Klaenhammer T, Dean G (2012) Construction and immunological evaluation of dual cell surface display of HIV-1 gag and *Salmonella enterica* serovar *Typhimurium* FliC in *Lactobacillus acidophilus* for vaccine delivery. Clin Vaccine Immunol 19(9):1374–1381. doi:10.1128/00049-12

Kankainen M, Paulin L, Tynkkynen S, von Ossowski I, Reunanen J, Partanen P, Satokari R, Vesterlund S, Hendrickx AP, Lebeer S, De Keersmaecker SC, Vanderleyden J, Hamalainen T, Laukkanen S, Salovuori N, Ritari J, Alatalo E, Korpela R, Mattila-Sandholm T, Lassig A, Hatakka K, Kinnunen KT, Karjalainen H, Saxelin M, Laakso K, Surakka A, Palva A, Salusjarvi T, Auvinen P, de Vos WM (2009) Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human- mucus binding protein. Proc Natl Acad Sci USA 106(40):17193–17198. doi:10.1073/s.0908876106

Khazaie K, Zadeh M, Khan MW, Bere P, Gounari F, Dennis K, Blatner NR, Owen JL, Klaenhammer TR, Mohamadzadeh M (2012) Abating colon cancer polyposis by *Lactobacillus acidophilus* deficient in lipoteichoic acid. Proc Natl Acad Sci USA 109(26):10462–10467. doi:10.1073/s.1207230109

Klaenhammer TR (1993) Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol Rev 12(1–3):39–85

Klaenhammer T, Altermann E, Arigoni F, Bolotin A, Breidt F, Broadbent J, Cano R, Chaillou S, Deutscher J, Gasson M, van de Guchte M, Guzzo J, Hartke A, Hawkins T, Hols P, Hutkins R, Kleerebezem M, Kok J, Kuipers O, Lubbers M, Maguin E, McKay L, Mills D, Nauta A, Overbeek R, Pel H, Pridmore D, Saier M, van Sinderen D, Sorokin A, Steele J, O'Sullivan D, de Vos W, Weimer B, Zagorec M, Siezen R (2002) Discovering lactic acid bacteria by genomics. Antonie Van Leeuwenhoek 82(1–4):29–58

Klaenhammer TR, Kleerebezem M, Kopp MV, Rescigno M (2012) The impact of probiotics and prebiotics on the immune system. Nat Rev Immunol 12(10):728–734. doi:10.1038/3312

Kleerebezem M, Hols P, Bernard E, Rolain T, Zhou M, Siezen RJ, Bron PA (2010) The extracellular biology of the *Lactobacilli*. FEMS Microbiol Rev 34(2):199–230. doi:10.1111/1574-6976.2010.00208

Konstantinov SR, Smidt H, de Vos WM, Bruijns SC, Singh SK, Valence F, Molle D, Lortal S, Altermann E, Klaenhammer TR, van Kooyk Y (2008) S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. Proc Natl Acad Sci USA 105(49):19474–19479. doi:10.1073/s.0810305105

Lagesen K, Ussery DW, Wassenaar TM (2010) Genome update: the 1,000th genome—a cautionary tale. Microbiology 156(Pt 3):603–608. doi:10.1099/0.038257-0

Lebeer S, Claes IJ, Verhoeven TL, Shen C, Lambrichts I, Ceuppens JL, Vanderleyden J, De Keersmaecker SC (2008a) Impact of *luxS* and suppressor mutations on the gastrointestinal transit of *Lactobacillus rhamnosus* GG. Appl Environ Microbiol 74(15):4711–4718. doi:10.1128/00133-08

Lebeer S, Vanderleyden J, De Keersmaecker SC (2008b) Genes and molecules of lactobacilli supporting probiotic action. Microbiol Mol Biol Rev 72(4):728–764. doi:10.1128/00017-08

Lebeer S, Vanderleyden J, De Keersmaecker SC (2010) Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol 8(3):171–184. doi:10.1038/2297

Lee YK, Puong KY, Ouwehand AC, Salminen S (2003) Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. J Med Microbiol 52(10):925–930

Leyer GJ, Li S, Mubasher ME, Reifer C, Ouwehand AC (2009) Probiotic effects on cold and influenza-like symptom incidence and duration in children. Pediatrics 124(2): 172–179. doi:10.1542/2008-2666

Lukjancenko O, Ussery DW, Wassenaar TM (2012) Comparative genomics of *Bifidobacterium*, *Lactobacillus* and related probiotic genera. Microb Ecol 63(3):651–673. doi:10.1007/s00248-011-9948

Macfarlane GT, Blackett KL, Nakayama T, Steed H, Macfarlane S (2009) The gut microbiota in inflammatory bowel disease. Curr Pharm Des 15(13):1528–1536

Mattila-Sandholm T, Blum S, Collins JK, Crittenden R, de Vos W, Dunne C, Fondén R, Grenov G, Isolauri E, Kiely B, Marteau P, Morelli L, Ouwehand A, Reniero R, Saarela M, Salminen S, Saxelin M, Schiffrin E, Shanahan F, Vaughan E, von Wright A (1999) Probiotics: towards demonstrating efficacy. Trends Food Sci Technol 10:393–399

Maze A, Boel G, Zuniga M, Bourand A, Loux V, Yebra MJ, Monedero V, Correia K, Jacques N, Beaufils S, Poncet S, Joyet P, Milohanic E, Casaregola S, Auffray Y, Perez-Martinez G, Gibrat JF, Zagorec M, Francke C, Hartke A, Deutscher J (2010) Complete genome sequence of the probiotic *Lactobacillus casei* strain BL23. J Bacteriol 192(10):2647–2648. doi:10.1128/00076-10

Mennigen R, Bruewer M (2009) Effect of probiotics on intestinal barrier function. Ann N Y Acad Sci 1165:183–189. doi:10.1111/1749-6632.2009.04059

Metchnikoff E (1907) The prolongation of life: optomistic studies. William Heinemann, London

Mohamadzadeh M, Pfeiler EA, Brown JB, Zadeh M, Gramarossa M, Managlia E, Bere P, Sarraj B, Khan MW, Pakanati KC, Ansari MJ, O'Flaherty S, Barrett T, Klaenhammer TR (2011) Regulation of induced colonic inflammation by *Lactobacillus acidophilus* deficient in lipoteichoic acid. Proc Natl Acad Sci USA 108(Suppl 1):4623–4630. doi:10.1073/s1005066107

Morita H, Toh H, Oshima K, Murakami M, Taylor TD, Igimi S, Hattori M (2009) Complete genome sequence of the probiotic *Lactobacillus rhamnosus* ATCC 53103. J Bacteriol 191(24):7630–7631. doi:10.1128/01287-09

Nes IF, Bao Diep D, Sigve Havarstein L, Bente Brurberg M, Eijsink V, Holo H (1996) Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leeuwenhoek 70(2): 113–128

O'Connell Motherway M, Zomer A, Leahy SC, Reunanen J, Bottacini F, Claesson MJ, O'Brien F, Flynn K, Casey PG, Moreno-Munoz JA, Kearney B, Houston AM, O'Mahony C, Higgins DG, Shanahan F, Palva A, de Vos WM, Fitzgerald GF, Ventura M, O'Toole PW, van Sinderen D (2011) Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pilli as an essential and conserved host-colonization factor. Proc Natl Acad Sci USA 108(27):11217–11222. doi:10.1073/s1105380108

O'Flaherty SJ, Klaenhammer TR (2010a) The role and potential of probiotic bacteria in the gut, and the communication between gut microflora and the gut/host. Int Dairy J 20(4):262–268

O'Flaherty SJ, Klaenhammer TR (2010b) Functional and phenotypic characterization of a protein from *Lactobacillus acidophilus* involved in cell morphology, stress tolerance and adherence to intestinal cells. Microbiology 156(11): 3360–3367. doi:10.1099/0.043158-0

O'Flaherty S, Klaenhammer TR (2012) Influence of exposure time on gene expression by human intestinal epithelial cells exposed to *Lactobacillus acidophilus*. Appl Environ Microbiol 78(14):5028–5032. doi:10.1128/00504-12

Pfeiler EA, Klaenhammer TR (2009) Role of transporter proteins in bile tolerance of *Lactobacillus acidophilus*. Appl Environ Microbiol 75(18):6013–6016. doi:10.1128/00495-09

Pfeiler EA, Azcarate-Peril MA, Klaenhammer TR (2007) Characterization of a novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus acidophilus*. J Bacteriol 189(13):4624–4634. doi:10.1128/00337-07

Pieterse B, Leer RJ, Schuren FHJ, van der Werf MJ (2005) Unravelling the multiple effects of lactic acid stress on *Lactobacillus plantarum* by transcription profiling. Microbiology 151(12):3881–3894. doi:10.1099/0.28304-0

Plummer S, Weaver MA, Harris JC, Dee P, Hunter J (2004) *Clostridium difficile* pilot study: effects of probiotic supplementation on the incidence of *C. difficile* diarrhoea. Int Microbiol 7(1):59–62

Porter CK, Gormley R, Tribble DR, Cash BD, Riddle MS (2011) The Incidence and gastrointestinal infectious risk of functional gastrointestinal disorders in a healthy US adult population. Am J Gastroenterol 106(1):130–138. doi:10.1038/2010.371

Pot B, Ludwig W, Kersters K, Schleifer KH (1994) Taxonomy of lactic acid bacteria. In: Cocaign-Bousquet K (ed) Bacteriocins of lactic acid bacteria. Chapman and Hall, Glasgow, pp 13–90

Pridmore RD, Berger B, Desiere F, Vilanova D, Barretto C, Pittet AC, Zwahlen MC, Rouvet M, Altermann E, Barrangou R, Mollet B, Mercenier A, Klaenhammer T, Arigoni F, Schell MA (2004) The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. Proc Natl Acad Sci USA 101(8):2512–2517

Pridmore RD, Pittet AC, Praplan F, Cavadini C (2008) Hydrogen peroxide production by *Lactobacillus johnsonii* NCC 533 and its role in anti-*Salmonella* activity. FEMS Microbiol Lett 283(2):210–215. doi:10.1111/1574-6968.2008.01176

Rafter J, Bennett M, Caderni G, Clune Y, Hughes R, Karlsson PC, Klinder A, O'Riordan M, O'Sullivan GC, Pool-Zobel B, Rechkemmer G, Roller M, Rowland I, Salvadori M, Thijs H, Van Loo J, Watzl B, Collins JK (2007) Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. Am J Clin Nutr 85(2):488–496

Reid G, Chan RC, Bruce AW, Costerton JW (1985) Prevention of urinary tract infection in rats with an indigenous *Lactobacillus casei* strain. Infect Immun 49(2):320–324

Reid G, Younes JA, Van der Mei HC, Gloor GB, Knight R, Busscher HJ (2011) Microbiota restoration: natural and

supplemented recovery of human microbial communities. Nat Rev Microbiol 9(1):27–38. doi:10.1038/2473

Rettger LF (1915) The influence of milk feeding on mortality and growth, and on the character of the intestinal flora. J Exp Med 21(4):365–388

Rettger LF, Cheplin HA (1921) A treatise on the transformation of the intestinal flora, with special reference to implantation of *Bacillus acidophilus*. Yale University Press, New Haven

Ringel-Kulka T, Palsson OS, Maier D, Carroll I, Galanko JA, Leyer G, Ringel Y (2011) Probiotic bacteria *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* Bi-07 versus placebo for the symptoms of bloating in patients with functional bowel disorders: a double-blind study. J Clin Gastroenterol 45(6):518–525. doi:10.1097/0b013e31820ca4d6

Roberfroid M (2007) Prebiotics: the concept revisited. J Nutr 137(3 Suppl 2):830S–837S

Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, Wolvers D, Watzl B, Szajewska H, Stahl B, Guarner F, Respondek F, Whelan K, Coxam V, Davicco MJ, Leotoing L, Wittrant Y, Delzenne NM, Cani PD, Neyrinck AM, Meheust A (2010) Prebiotic effects: metabolic and health benefits. Br J Nutr 104(Suppl 2):S1–S63. doi:10.1017/S0007114510003363

Rousseaux C, Thuru X, Gelot A, Barnich N, Neut C, Dubuquoy L, Dubuquoy C, Merour E, Geboes K, Chamaillard M, Ouwehand A, Leyer G, Carcano D, Colombel JF, Ardid D, Desreumaux P (2007) *Lactobacillus acidophilus* modulates intestinal pain and induces opioid and cannabinoid receptors. Nat Med 13(1):35–37. doi:10.1038/1521

Ryan KA, O'Hara AM, van Pijkeren JP, Douillard FP, O'Toole PW (2009) *Lactobacillus salivarius* modulates cytokine induction and virulence factor gene expression in *Helicobacter pylori*. J Med Microbiol 58(Pt 8):996–1005. doi:10.1099/0.009407-0

Sanders ME, in't Huis-Veld J (1999) Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labeling issues. Antonie Van Leeuwenhoek 76(1–4):293–315

Sanders ME, Guarner F, Guerrant R, Holt PR, Quigley EM, Sartor RB, Sherman PM, Mayer EA (2013) An update on the use and investigation of probiotics in health and disease. Gut 62(5):787–796. doi:10.1136/2012-302504

Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen MC, Desiere F, Bork P, Delley M, Pridmore RD, Arigoni F (2002) The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci USA 99(22):14422–14427. doi:10.1073/s212527599

Schnell N, Entian KD, Schneider U, Gotz F, Zahner H, Kellner R, Jung G (1988) Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulfide-rings. Nature 333:276–278. doi:10.1038/333276a0

Selle K, Klaenhammer TR (2013) Genomic and phenotypic evidence for probiotic influences of *Lactobacillus gasseri* on human health. FEMS Microbiol Rev 37(6):915–935. doi:10.1111/1574-6976.12021

Servin AL (2004) Antagonistic activities of *Lactobacilli* and *Bifidobacteria* against microbial pathogens. FEMS Microbiol Rev 28(4):405–440. doi:10.1016/2004.01.003

Shen YH, Nahas R (2009) Complementary and alternative medicine for treatment of irritable bowel syndrome. Can Fam Physician 55(2):143–148

Shortt C (1999) The probiotic century: historical and current perspectives. Trends Food Sci Technol 10:411–417

Smits HH, Engering A, van der Kleij D, de Jong EC, Schipper K, van Capel TMM, Zaat BAJ, Yazdanbakhsh M, Wierenga EA, van Kooyk Y, Kapsenberg ML (2005) Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell functiont through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. J Allergy Clin Immunol 115(6):1260–1267

St Amant DC, Valentin-Bon IE, Jerse AE (2002) Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* species that are commonly isolated from the female genital tract. Infect Immun 70(12):7169–7171

Stoeker L, Nordone S, Gunderson S, Zhang L, Kajikawa A, LaVoy A, Miller M, Klaenhammer TR, Dean GA (2011) Assessment of *Lactobacillus gasseri* as a candidate oral vaccine vector. Clin Vaccine Immunol 18(11):1834–1844. doi:10.1128/05277-11

Takeda K, Okumura K (2007) Effects of a fermented milk drink containing *Lactobacillus casei* strain Shirota on the human NK-cell activity. J Nutr 137(3 Suppl 2):791S–793S

Taranto MP, Fernandez Murga ML, Lorca G, de Valdez GF (2003) Bile salts and cholesterol induce changes in the lipid cell membrane of *Lactobacillus reuteri*. J Appl Microbiol 95(1):86–91. doi:10.1046/1365-2672.2003.01962

Tissier H (1900) Recherche sur la flore intestinale des nourissons (Etat normal et pathologique). PhD Thesis, Université de Médécine, Paris

Tuomola EM, Salminen SJ (1998) Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. Int J Food Microbiol 41(1):45–51

Turroni F, Ribbera A, Foroni E, van Sinderen D, Ventura M (2008) Human gut microbiota and bifidobacteria: from composition to functionality. Antonie Van Leeuwenhoek 94(1):35–50. doi:10.1007/s10482-008-9232-4

Turroni F, Taverniti V, Ruas-Madiedo P, Duranti S, Guglielmetti S, Lugli GA, Gioiosa L, Palanza P, Margolles A, van Sinderen D, Ventura M (2014) *Bifidobacterium bifidum* PRL2010 modulates the host innate immune response. Appl Environ Microbiol 80(2):730–740. doi:10.1128/03313-13

Ushiyama A, Tanaka K, Aiba Y, Shiba T, Takagi A, Mine T, Koga Y (2003) *Lactobacillus gasseri* OLL2716 as a probiotic in clarithromycin-resistant *Helicobacter pylori* infection. J Gastroenterol Hepatol 18(8):986–991

van Baarlen P, Troost FJ, van Hemert S, van der Meer C, de Vos WM, de Groot PJ, Hooiveld GJ, Brummer RJ, Kleerebezem M (2009) Differential NF-κB pathways induction by *Lactobacillus plantarum* in the duodenum of healthy humans correlating with immune tolerance. Proc Natl Acad Sci USA 106(7):2371–2376. doi:10.1073/0809919106

van Baarlen P, Troost F, van der Meer C, Hooiveld G, Boekschoten M, Brummer RJ, Kleerebezem M (2011) Human mucosal in vivo transcriptome responses to three *Lactobacilli* indicate how probiotics may modulate human cellular pathways. Proc Natl Acad Sci USA 108(Suppl 1):4562–4569. doi:10.1073/1000079107

van de Guchte M, Penaud S, Grimaldi C, Barbe V, Bryson K, Nicolas P, Robert C, Oztas S, Mangenot S, Couloux A, Loux V, Dervyn R, Bossy R, Bolotin A, Batto JM, Walunas T, Gibrat JF, Bessieres P, Weissenbach J, Ehrlich SD, Maguin E (2006) The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing evolution. Proc Natl Acad Sci USA 103(24):9274–9279. doi:10.1073/0603024103

van Pijkeren JP, Canchaya C, Ryan KA, Li Y, Claesson MJ, Sheil B, Steidler L, O'Mahony L, Fitzgerald GF, van Sinderen D, O'Toole PW (2006) Comparative and functional analysis of sortase-dependent proteins in the predicted secretome of *Lactobacillus salivarius* UCC118. Appl Environ Microbiol 72(6):4143–4153. doi:10.1128/03023-05

Velez MP, De Keersmaecker SC, Vanderleyden J (2007) Adherence factors of *Lactobacillus* in the human gastrointestinal tract. FEMS Microbiol Lett 276(2):140–148. doi:10.1111/1574-6968.2007.00908

Velraeds MM, van der Mei HC, Reid G, Busscher HJ (1996) Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. Appl Environ Microbiol 62(6):1958–1963

Ventura M, Canchaya C, Del Casale A, Dellaglio F, Neviani E, Fitzgerald GF, van Sinderen D (2006) Analysis of *bifidobacterial* evolution using a multilocus approach. Int J Syst Evol Microbiol 56(Pt 12):2783–2792. doi:10.1099/0.64233-0

Viljanen M, Kuitunen M, Haahtela T, Juntunen-Backman K, Korpela R, Savilahti E (2005) Probiotic effects on faecal inflammatory markers and on faecal IgA in food allergic atopic eczema/dermatitis syndrome infants. Pediatr Allergy Immunol 16(1):65–71. doi:10.1111/1399-3038.2005.00224

Wells JM, Mercenier A (2008) Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. Nat Rev Microbiol 6(5):349–362. doi:10.1038/1840

Whitehead K, Versalovic J, Roos S, Britton RA (2008) Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. Appl Environ Microbiol 74(6):1812–1819. doi:10.1128/02259-07

Wiedemann I, Breukink E, van Kraaij C, Kuipers OP, Bierbaum G, de Kruijff B, Sahl HG (2001) Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J Biol Chem 276:1772–1779. doi:10.1074/M006770200

Zhang ZY, Liu C, Zhu YZ, Zhong Y, Zhu YQ, Zheng HJ, Zhao GP, Wang SY, Guo XK (2009) Complete genome sequence of *Lactobacillus plantarum* JDM1. J Bacteriol 191(15):5020–5021. doi:10.1128/00587-09

Zhou M, Theunissen D, Wels M, Siezen RJ (2010) LAB-Secretome: a genome-scale comparative analysis of the predicted extracellular and surface-associated proteins of lactic acid bacteria. BMC Genom 11:651. doi:10.1186/1471-2164-11-651

***Appendix B***
Reprint from:

**Johnson BR, Selle KM, O'Flaherty S, Goh YJ, Klaenhammer TR**. 2013. Identification of extracellular surface-layer associated proteins in *Lactobacillus acidophilus* NCFM. *Microbiology* **159**: 2269-2282.

*Microbiology* (2013), **159**, 2269–2282 DOI 10.1099/mic.0.070755-0

# Identification of extracellular surface-layer associated proteins in *Lactobacillus acidophilus* NCFM

Brant Johnson,[1] Kurt Selle,[2] Sarah O'Flaherty,[2] Yong Jun Goh[2] and Todd Klaenhammer[1,2]

[1]Department of Microbiology, North Carolina State University, Raleigh, NC, USA

[2]Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, Raleigh, NC, USA

Correspondence
Todd Klaenhammer
trk@ncsu.edu

Received 21 June 2013
Accepted 29 August 2013

Bacterial surface (S-) layers are crystalline arrays of self-assembling, proteinaceous subunits called S-layer proteins (Slps), with molecular masses ranging from 40 to 200 kDa. The S-layer-forming bacterium *Lactobacillus acidophilus* NCFM expresses three major Slps: SlpA (46 kDa), SlpB (47 kDa) and SlpX (51 kDa). SlpA has a demonstrated role in adhesion to Caco-2 intestinal epithelial cells *in vitro*, and has been shown to modulate dendritic cell (DC) and T-cell functionalities with murine DCs. In this study, a modification of a standard lithium chloride S-layer extraction revealed 37 proteins were solubilized from the S-layer wash fraction. Of these, 30 have predicted cleavage sites for secretion, 24 are predicted to be extracellular, six are lipid-anchored, three have N-terminal hydrophobic membrane spanning regions and four are intracellular, potentially moonlighting proteins. Some of these proteins, designated S-layer associated proteins (SLAPs), may be loosely associated with or embedded within the bacterial S-layer complex. *Lba-1029*, a putative SLAP gene, was deleted from the chromosome of *L. acidophilus*. Phenotypic characterization of the deletion mutant demonstrated that the SLAP LBA1029 contributes to a pro-inflammatory TNF-α response from murine DCs. This study identified extracellular proteins and putative SLAPs of *L. acidophilus* NCFM using LC-MS/MS. SLAPs appear to impart important surface display features and immunological properties to microbes that are coated by S-layers.

## INTRODUCTION

Bacterial surface (S-) layers are crystalline arrays of self-assembling, proteinaceous subunits called S-layer proteins (Slps), with molecular masses ranging from 40 to 200 kDa (Sára & Sleytr, 2000). Present as the outermost component of the cell wall, S-layers are found in many species of eubacteria and archaea, and in varying environments. S-layer lattices are 5–25 nm thick and form oblique (p1, p2), square (p4) or hexagonal (p3, p6) symmetries, as observed by freeze-etched electron microscopy (Sleytr *et al.*, 2001). Further structural observations have revealed that S-layers are highly porous in nature, with pores occupying up to 70 % of the cell surface (Sleytr & Beveridge, 1999). Previous work comparing amino acid sequences from different S-layer-forming bacteria has shown that most S-layers are high in hydrophobic and acidic amino acids (Sára & Sleytr, 1996; Sleytr & Messner, 1983). Furthermore, the isoelectric points (pI) of many Slps are low, mostly found in the weakly acidic pH range (Sára & Sleytr, 2000). These sequence analyses have also revealed the presence of S-layer homologous (SLH) motifs on the N-terminal section of many Slps (Sára & Sleytr, 2000), which are responsible for tethering the S-layer to the secondary cell-wall polysaccharide (Brechtel & Bahl, 1999; Chauvaux *et al.*, 1999).

Lactic acid bacteria of the genus *Lactobacillus* are a diverse group of Gram-positive, anaerobic/microaerophilic, non-sporulating, low G+C content bacteria belonging to the phylum *Firmicutes* (Pot *et al.*, 1994). Biochemically, they are strictly fermentative; sugar fermentations result in either the sole production of lactic acid, or the production of lactic acid in conjunction with smaller amounts of carbon dioxide and ethanol/acetic acid (Hammes & Vogel, 1995; Pot *et al.*, 1994). There are several species of *Lactobacillus* that form S-layers, including mucosal-associated species (e.g. *Lactobacillus acidophilus*, *Lactobacillus*

Abbreviations: BSL, *Bacillus anthracis* S-layer protein; GRAVY, grand average of hydropathicity; (i)DC, (immature) dendritic cell; S-layer, surface layer; SLAP, S-layer associated protein; SLH, S-layer homologous; Slp, S-layer protein; SPase, signal peptidase.

Three supplementary figures are available with the online version of this paper.

*crispatus*, *Lactobacillus amylovorus* and *Lactobacillus gallinarum*) and dairy fermentation-associated species (e.g. *Lactobacillus helveticus* and *Lactobacillus kefiranofaciens*) (Åvall-Jääskeläinen & Palva, 2005; Hynönen & Palva, 2013). Compared with S-layers of other Gram-positive bacteria, those from *Lactobacillus* are biochemically unique. Specifically, S-layers from *Lactobacillus* do not possess SLH domains (Boot & Pouwels, 1996). Furthermore, their Slps are among the smallest known (25–71 kDa) and are highly basic with calculated pI values ranging from 9.35 to 10.4 (Åvall-Jääskeläinen & Palva, 2005).

*L. acidophilus* NCFM is a widely used probiotic microbe, found in both fermented dairy products and dietary supplements (Sanders & Klaenhammer, 2001). Utilizing a completely sequenced and annotated genome (Altermann *et al.*, 2005), *L. acidophilus* NCFM has been the subject of many investigations seeking to understand the mechanisms of probiotic functionality. *L. acidophilus* NCFM forms an S-layer composed principally of SlpA, with auxiliary components SlpB and SlpX (Altermann *et al.*, 2005; Goh *et al.*, 2009). Given its proximity to the cell surface, the S-layer is one of the first bacterial components to interact with the gastrointestinal surface of the human host. SlpA of *L. acidophilus* NCFM has demonstrated an important role in adhesion to the Caco-2 intestinal cell line (Buck *et al.*, 2005) and has been shown to modulate dendritic cell (DC) and T-cell functionality (Konstantinov *et al.*, 2008). Taken together, these studies highlight the potential role of S-layers in probiotic activities.

Transmission electron microscopy images of *L. acidophilus* reveal a cell envelope that is abundant with S-layer components. In fact, $\sim 5 \times 10^5$ Slp subunits are required to generate the S-layer of rod-shaped cells, such as *L. acidophilus* (Sleytr & Messner, 1983). Slps have been extracted from Gram-positive bacterial cell surfaces via treatment with high concentrations of salts [e.g. guanidine hydrochloride or lithium chloride (LiCl)], which disrupt hydrogen bonding between the S-layer and the secondary cell-wall polysaccharide (Sára & Sleytr, 2000). Specifically, LiCl treatments at 5 and 1 M concentrations have been used to isolate the Slps from many *Lactobacillus* species (Ashida *et al.*, 2011; Beganović *et al.*, 2011; Frece *et al.*, 2005; Goh *et al.*, 2009; Lortal *et al.*, 1992; Smit *et al.*, 2001; Taverniti *et al.*, 2013). Despite the dramatic presence of the S-layer and the highly expressed Slp subunits, it is notable that research on exoproteins associated with the S-layer is scarce. Although proteins associated with the S-layer have been observed in *L. acidophilus* (Smit *et al.*, 2001), there has been no work identifying these proteins using MS.

To identify these proteins in *L. acidophilus*, a LiCl S-layer extraction protocol noted above (Goh *et al.*, 2009; Lortal *et al.*, 1992) was modified to isolate proteins associated with the S-layer, while mostly excluding the major Slps. After protein identification through LC-MS/MS, 37 proteins were identified that may be associated with or embedded within the S-layer. Many of these proteins, designated S-layer associated proteins (SLAPs, Fig. 1), have unknown function and offer potential in advancing our understanding of the probiotic mechanism, cell envelope biology and immunomodulation in *L. acidophilus*. One of the predicted SLAPs, encoded by *lba-1029*, was deleted from the chromosome of *L. acidophilus* NCFM using a *upp*-based counterselective gene replacement system (Goh *et al.*, 2009). This study aimed to (i) develop a modified method for extracting SLAPs, (ii) identify the SLAPs found in *L. acidophilus* NCFM and (iii) functionally characterize the SLAP LBA1029.

## METHODS

**Bacterial strains and growth conditions.** Bacterial strains, plasmids and primers used in this study are reported in Table 1. *L. acidophilus* strains were propagated in de Man Rogosa Sharpe (MRS) broth (Difco) under aerobic conditions, statically or on MRS solid medium containing 1.5 % (w/v) agar (Difco) under anaerobic conditions at 37 °C, and at 42 °C where indicated. Recombinant strains were selected with 2 μg erythromycin $ml^{-1}$ (Sigma-Aldrich) and/or 5 μg chloramphenicol $ml^{-1}$ (Sigma-Aldrich). *Escherichia coli* strains were grown in brain heart infusion (Difco) medium at 37 °C with shaking for aeration. *E. coli* EC101 was grown in the presence of 40 μg kanamycin $ml^{-1}$ (Sigma-Aldrich) while NCK1911 and transformants were grown with 40 μg kanamycin $ml^{-1}$ and 150 μg erythromycin $ml^{-1}$. Counterselection of plasmid-free excision recombinants was performed using 5-fluorouracil-supplemented glucose semi-defined medium, as previously described (Goh *et al.*, 2009).

**LiCl extraction of SLAPs.** The extraction protocol for SLAPs was modified from a standard LiCl S-layer extraction protocol for *L. acidophilus* (Goh *et al.*, 2009; Lortal *et al.*, 1992). Bacterial cells were grown in 200 ml MRS to stationary phase (16 h), centrifuged at 2236 ***g*** for 10 min (4 °C), and washed twice with 25 ml cold PBS (Gibco), pH 7.4. Cells were agitated for 15 min at 4 °C following the addition of 5 M LiCl (Fisher Scientific). Supernatants, containing Slps and SLAPs, were harvested via centrifugation at 8994 ***g*** for 10 min (4 °C) and transferred to a 6000–8000 kDa Spectra/Por molecular porous membrane (Spectrum Laboratories) and dialysed against cold distilled water for 24 h, changing the water every 2 h for the first 8 h. The dialysed precipitate was harvested via centrifugation at 20 000 ***g*** for 30 min and agitated for a second time for 15 min with 1 M LiCl at 4 °C to disassociate the SLAPs from the Slps, which are insoluble in 1 M LiCl. Next, the suspension was centrifuged at 20 000 ***g*** for 10 min and the supernatants, containing the SLAPs, were again transferred to the 6000–8000 kDa Spectra/Por molecular porous membrane and dialysed against cold distilled water for 24 h. Finally, the precipitate was harvested via centrifugation at 20 000 ***g*** for 30 min and pellets were resuspended in 10 % (w/v) SDS (Fisher). Proteins were quantified via bicinchoninic acid assay kit (Thermo Scientific) and visualized via SDS-PAGE using precast 4–20 % Precise Tris-HEPES protein gels (Thermo Scientific). The gels were stained using AcquaStain (Bulldog Bio) according to the instructions of the manufacturer.

**Protein identification.** The SLAPs were electrophoresed for approximately 7 min in the resolving gel of the SDS-PAGE and excised using a sterile blade. The protein gel was submitted to the Genome Center Proteomics Core at the University of California, Davis, for MS-based protein identification. Briefly, proteins were reduced and alkylated according to the procedures of Shevchenko *et al.* (1996), and digested with sequencing-grade tryspin according to

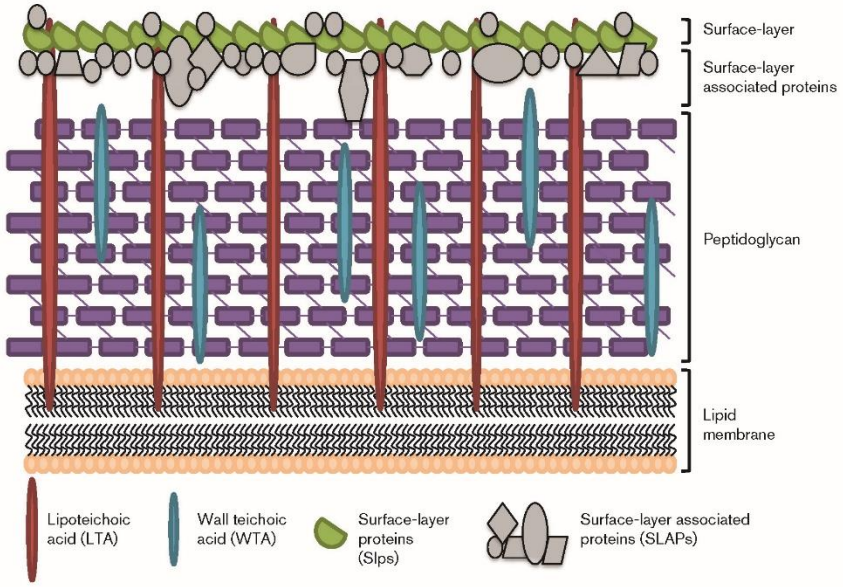


**Fig. 1.** Proposed schematic for the localization of SLAPs in *L. acidophilus* NCFM. The Gram-positive bacterial cell wall is comprised of a thick peptidoglycan layer (purple), stabilized by teichoic acids and tethered to the lipid membrane by lipoteichoic acid. The S-layer, composed of self-assembling Slps (green), is the outermost layer of the cell wall. SLAPs (grey) may be associated with this outermost S-layer.

**Table 1.** Strains, plasmids and primers used in this study

| Strain, plasmid or primer | Genotype or characteristics | Reference |
|---|---|---|
| ***L. acidophilus* strains** | | |
| NCFM (NCK56) | Human intestinal isolate | (Sanders & Klaenhammer, 2001) |
| NCK1909 | NCFM with chromosomal deletion of *upp*; background host for *upp*-based counterselective gene replacement system | (Goh et al., 2009) |
| NCK1910 | RepA$^-$, pWV01 integrated into the host chromosome. Cm$^r$ | (Goh et al., 2009) |
| NCK2258 | NCK1909 with chromosomal deletion of *lba-1029* | This study |
| ***E. coli* (EC101) strains** | | |
| NCK1911 | Host harbouring pTRK935, Kn$^r$ Em$^r$ | (Goh et al., 2009) |
| NCK2257 | Host harbouring pTRK1067, Kn$^r$ Em$^r$ | This study |
| **Plasmids** | | |
| pTRK669 | Ori (pWV01), Cm$^r$, RepA$^+$ thermosensitive | (Russell & Klaenhammer, 2001) |
| pTRK935 | pORI *upp*-based counterselective integration vector | (Goh et al., 2009) |
| pTRK1067 | pTRK935 with flanking regions of *lba-1029* cloned into *Bam*HI and *Sac*I restriction sites | This study |
| **Primers*** | | |
| 1*Bam*HI-F | GTAATAGGATCCGCAGAAATTAAGCCCGTTGT | This study |
| 2R | TGCAATTGTAGCCAAAATTAGTG | This study |
| 3Soe | TAATTTTGGCTACAATTGCACACACTGCTGTTTACGATCCA | This study |
| 4*Sac*I-R | TAAAGTAGAGCTCATCTTGCCCAATCGTGTAAA | This study |
| 1029up | CTTAATTCACTGGCCAAATC | This study |
| 1029dw | TCTGCTGACTTCTCTTGAGG | This study |

*Restriction sites are underlined.

the manufacturer's instructions (Promega). Peptides were dried down in a vacuum concentrator after digestion and then resolubilized in 2 % acetonitrile/0.1 % trifluoroacetic acid. Digested peptides were analysed by LC-MS/MS on a Thermo Scientific Q Exactive Orbitrap mass spectrometer in conjunction with a Paradigm MG4 HPLC machine and CTC Pal auto sampler (Michrom Bio Resources). The digested peptides were loaded onto a Michrom C18 trap and desalted before they were separated using a Michrom 200 μm × 150 mm Magic $C_{18}$AQ reversed-phase column. A flow rate of 2 μl $min^{-1}$ was used. Peptides were eluted using a 120 min gradient with 2 % acetonitrile to 35 % acetonitrile over 94 min, 35 % acetonitrile to 80 % acetonitrile for 10 min, 80 % acetonitrile for 2 min, and then a decrease from 80 to 5 % acetonitrile in 1 min. An MS survey scan was obtained for the *m/z* range 300–1600. MS/MS spectra were acquired using a top 15 method, where the top 15 ions in the MS spectra were subjected to high energy collisional dissociation. An isolation mass window of 2.0 *m/z* was used for the precursor ion selection, and a normalized collision energy of 27 % was used for fragmentation. A 5 s duration was used for the dynamic exclusion.

**Protein database searches.** Tandem mass spectra were extracted and charge state deconvoluted using MM File Conversion version 3. All MS/MS samples were analysed using X! Tandem (The GPM, www.thegpm.org/; version TORNADO). X! Tandem was set up to search the Uniprot *L. acidophilus* database (July 2012, 1859 entries), the cRAP database of common laboratory contaminants (www.thegpm.org/crap; 114 entries) plus an equal number of reverse protein sequences (assuming the digestion enzyme trypsin). X! Tandem was searched with a fragment ion mass tolerance of 20 p.p.m. and a parent ion tolerance of 20 p.p.m. The iodoacetamide derivative of cysteine was specified in X! Tandem as a fixed modification. Deamidation of asparagine and glutamine, oxidation of methionine and tryptophan, sulfone of methionine, tryptophan oxidation to formylkynurenin of tryptophan and acetylation of the N terminus were specified in X! Tandem as variable modifications. LocateP and the LAB-Secretome database were used to predict the secretion pathways of identified proteins (Zhou *et al.*, 2008, 2010). SignalP 4.1 was used to predict the signal peptidase cleavage site of each protein (Petersen *et al.*, 2011). Grand average of hydropathicity (GRAVY) scores were predicted using the GRAVY calculator (http://www.gravy-calculator.de).

**Criteria for protein identification.** Scaffold (version Scaffold_3.6.1, Proteome Software) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. X! Tandem identifications required scores of greater than 1.2 with a mass accuracy of 5 p.p.m. Protein identifications were accepted if they contained at least two identified peptides. Using the parameters above, the false discovery rate was calculated to be 1.1 % on the protein level and 0 % on the peptide level (Tabb *et al.*, 2008). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. For this study, only proteins with unique spectral counts of $\geqslant 10$ were considered significant.

**Molecular techniques.** Genomic DNA from *L. acidophilus* strains was isolated using a Zymo Research Fungal/Bacterial DNA MiniPrep kit (Zymo Research). Plasmid DNA from *E. coli* was isolated using a QIAprep Spin Miniprep kit (Qiagen). Restriction enzyme digestions and ligations were performed using Roche restriction enzymes (Roche Diagnostics) and T4 DNA ligase (New England Biolabs), respectively. PCR primers were designed based on the genomic sequence data and synthesized by Integrated DNA Technologies. PCRs were carried out in Bio-Rad MyCycler thermocyclers (Bio-Rad Laboratories) using Choice-*Taq* Blue DNA polymerase (Denville Scientific) for screening of recombinants and *PfuUltra* II fusion HS DNA polymerase (Agilent Technologies) for cloning purposes. PCR amplicons were analysed on 0.8 % agarose gels and purified using QIAquick Gel Extraction kits (Qiagen). DNA sequencing was performed by Davis Sequencing.

*E. coli* EC101 cells were made competent using a rubidium chloride competent cell protocol (Hanahan, 1983). *L. acidophilus* cells were prepared for electrotransformation using a modified penicillin treatment protocol (Goh *et al.*, 2009; Walker *et al.*, 1996; Wei *et al.*, 1995).

**Sequence analysis.** Identified protein sequences were compared against the non-redundant protein database using BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Further protein alignments were performed by comparing the deduced protein sequence with those from all of the protein databases of the following organisms: *L. helveticus*, *L. crispatus*, *L. amylovorus*, *Lactobacillus gasseri*, *Lactobacillus johnsonii* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. All BLAST analyses were performed with adjusted algorithms to display only alignments with an E-value of $\leqslant 1 \times 10^{-6}$. Rho-independent transcriptional terminators were predicted by TransTermHP (Kingsford *et al.*, 2007).

**Construction of an *L. acidophilus* Δ*lba-1029* mutant.** The *upp*-based counterselection gene replacement method, described previously (Goh *et al.*, 2009), was used as a strategy for creating an internal deletion of 1155 bp in *lba-1029* of NCK1909, a *upp*-deficient background strain of *L. acidophilus* NCFM. Using splicing by overlap extension PCR (Horton *et al.*, 1989), the 2 kb flanking regions of the deletion target were spliced with *Bam*HI restricted site added on the upstream end and *Sac*I on the downstream end. This construct was digested with *Bam*HI and *Sac*I, then ligated into the polylinker of the similarly digested integration plasmid pTRK935 and transformed into competent *E. coli* EC101. The resulting recombinant plasmid, pTRK1067, was transformed into *L. acidophilus* NCK1909 harbouring the helper plasmid pTRK669 (NCK1910). Single crossover integrants were screened as described previously (Goh *et al.*, 2009). Colonies with the Δ*lba-1029* genotype were screened among the double recombinants recovered on 5-fluorouracil glucose semi-defined medium agar plates. Deletion of *lba-1029* was confirmed by PCR and sequencing.

**Caco-2 intestinal epithelial cells adherence assay.** For cell adherence assays, the Caco-2 intestinal epithelial cell line (ATCC HTB-37; American Type Culture Collection) was used. Cell culture media and reagents were purchased from Gibco and the assay protocol was performed as described previously (Goh *et al.*, 2009). Caco-2 cultures were grown at 37 °C in a 95 % air/5 % $CO_2$ atmosphere. A minimal essential medium (MEM) supplemented with 1 mM sodium pyruvate, 20 % (v/v) heat-inactivated FBS, 0.1 mM non-essential amino acids, penicillin G (100 mg $ml^{-1}$), streptomycin sulfate (100 mg $ml^{-1}$) and amphotericin (0.25 mg $ml^{-1}$) was used. Monolayers for the adhesion assay were prepared in 12-well tissue culture plates by seeding approximately $6.5 \times 10^4$ cells per well in 2 ml cell culture medium. The culture medium was replaced every 2 days, while the monolayers were used for the assay 2 weeks post-confluence. On the day of the assay, monolayers were washed twice with 1 ml PBS before adding 1 ml MEM without antibiotics and incubating at 37 °C in a 5 % $CO_2$ incubator prior to adding bacterial cells. Overnight bacterial cultures (10 ml) were pelleted via centrifugation (3166 **g**, 10 min) at room temperature, washed and resuspended in PBS to a final concentration of ~$1 \times 10^8$ c.f.u. $ml^{-1}$. Next, 1 ml of the bacterial suspension was added to each well of the cell monolayer in triplicate and incubated at 37 °C in a 5 % $CO_2$ atmosphere for 1 h. Following incubation, monolayers were washed five times with 1 ml PBS and treated with 1 ml 0.05 % (v/v) Triton X-100. After 10 min at 37 °C, cell monolayers were disrupted via pipetting and transferred to microcentrifuge tubes. Finally, microbial cells from the monolayer were diluted and enumerated after plating onto MRS agar.

**Simulated gastric and small intestinal juice assays.** Simulated gastric juice and small intestinal juice were prepared as previously described (Goh & Klaenhammer, 2010). Overnight cultures were centrifuged, washed twice in PBS and resuspended in sterile distilled water. The cell suspension (1.2 ml) was mixed with 6 ml of freshly prepared simulated gastric juice [0.5 % (w/v) NaCl solution with 3 g pepsin $l^{-1}$ (Fisher Scientific), pH adjusted to 2.0 with HCl] or simulated small intestinal juice [0.5 % (w/v) NaCl solution containing 1 g pancreatin $l^{-1}$ (Sigma) and 3 g Oxgall $l^{-1}$ (Difco), pH adjusted to 8.0 with NaOH] and incubated at 37 °C. Viable cell counts were determined by plating onto MRS agar after 30 min, 1 h and 1.5 h in simulated gastric juice, and hourly for 5 h in simulated small intestinal juice.

**Bile tolerance assays.** Cells were tested for bile tolerance in two separate assays. First, cells were inoculated into a 96-well plate containing 200 µl per well of MRS, MRS + 0.3 % (w/v) Oxgall or MRS + 0.5 % Oxgall in triplicates. Growth curves were monitored over 24 h by measuring the absorbance ($OD_{600}$) using a FLUOStar Optima microtitre plate reader (BMG Labtech). Secondly, cells were measured in planktonic growth in 10 ml of each of the three media above. At each time point, $OD_{600}$ was measured and cells were plated for c.f.u. enumeration on MRS agar.

**Bacterial-DC co-incubation and cytokine measurement.** An *in vitro* DC co-incubation assay was performed based on a modification of previous protocols (Mohamadzadeh *et al.*, 2011; Stoeker *et al.*, 2011). Bone marrow-derived BALB/c murine immature DCs (iDCs) were acquired (Astarte-Biologics) and preserved in liquid nitrogen. On the day of the assay, iDCs were thawed in a 37 °C water bath and transferred to a 50 ml conical tube containing 100 µg of DNase I at a concentration of 1 mg $ml^{-1}$ (Stem Cell Technologies) to prevent clumping. RPMI 1640 medium with 10 % FBS was added to the DCs, which were subsequently centrifuged in a swing arm rotor (200 *g*) at room temperature for 10 min. An aliquot of cells was removed for enumeration of live cells using Trypan Blue (Sigma) and the Invitrogen Countess, according to the manufacturer's instructions. Viable cells were then diluted to a final concentration of $1 \times 10^6$ $ml^{-1}$ in the RPMI 1640 + 10 % FBS + 100 µg streptomycin $ml^{-1}$ and aliquoted (100 µl per well) into round bottom polypropylene 96-well plates and held in 5 % $CO_2$ at 37 °C. Bacterial strains grown to stationary phase (16 h) were harvested by centrifugation, washed, resuspended in PBS and then standardized to ~$1 \times 10^8$ c.f.u. $ml^{-1}$. A portion of these aliquots was set aside for dilution and enumerative plating on MRS agar. The standardized cell suspension was centrifuged and resuspended in RPMI 1640 + 10 % FBS + 100 ug streptomycin ml-1 and $1 \times 10^6$ cells were combined with $1 \times 10^5$ viable iDCs in each well, resulting in a final bacterial to DC ratio of 10 : 1. The bacterial cells and iDCs were co-incubated for 24 h in 5 % $CO_2$ at 37 °C, after which the suspension was centrifuged and the supernatant was harvested and stored at −80 °C for cytokine analysis.

Cytokine measurements for TNF-α, IL-6, IL-10 and IL-12 were quantified using Single-Analyte ELISArray kits (Qiagen), according to the manufacturer's instructions. Following cytokine quantification, the cytokine expression data were compared between the parent and mutant strains using univariate analysis of variance.

## RESULTS

### Identification of extracellular and putative SLAPs from modified LiCl extraction

Previous work characterizing the S-layer of lactobacilli used 5 M LiCl salt to extract Slps efficiently with less lethality than other denaturing salts, such as guanidine hydrochloride (Ashida *et al.*, 2011; Beganović *et al.*, 2011; Goh *et al.*, 2009; Lortal *et al.*, 1992; Smit *et al.*, 2001; Tavernitti *et al.*, 2013). To investigate the potential presence of SLAPs, a LiCl S-layer extraction protocol (Goh *et al.*, 2009; Lortal *et al.*, 1992) was modified. Cells were treated with 5 M LiCl, solubilizing all non-covalently bound Slp and proteins associated with the S-layer, and the proteins were extracted via dialysis. This fraction was then treated with 1 M LiCl to separate the Slp from the proteins associated with the S-layer. These supernatants, containing only the proteins associated with the S-layer, were centrifuged, dialysed and electrophoresed on SDS-PAGE (Fig. 2, lane 1). This subset of proteins may be associated with or embedded within the S-layer of *L. acidophilus* NCFM.

Proteins from the modified LiCl extraction protocol were identified using LC-MS/MS following trypsin digestion. After database searching, the identified proteins were presented using Scaffold proteome software. Proteomic data were processed by removing human contaminants (e.g. keratin), falsely identified mammalian proteins and any protein with a unique spectral count ⩽10, leaving 37 proteins of interest with molecular masses ranging from 10 to 78 kDa (Table 2). The newly identified SLAPs were ordered from the highest to lowest unique spectral counts. Interestingly, GRAVY values predicted that all proteins of interest were hydrophilic in nature. Secretion of proteins in Gram-positive bacteria is mediated through the Sec translocase system (Driessen & Nouwen, 2008). All proteins processed by the Sec translocase contain an N-terminal

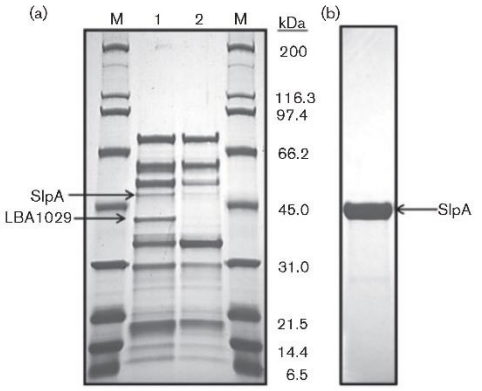


**Fig. 2.** (a) Putative SLAPs of *L. acidophilus* NCFM (NCK56) and NCK2258. Proteins were extracted using a series of washes in LiCl followed by dialyses in molecular porous membranes at 4 °C. The relative molecular masses (M) are labelled. Lane 1, SLAPs from *L. acidophilus* NCFM; lane 2, SLAPs from NCK2258, demonstrating the absence of the 43 kDa LBA1029. (b) Pure SlpA from *L. acidophilus* NCFM. Note the absence of other potential SLAPs/extracellular proteins using the standard protocol.

**Table 2.** Proteins extracted through modified exposure to LiCl

| ORF | Protein description | Predicted molecular mass (kDa) | SPase target | Amino acid coverage | GRAVY score | Predicted SPase cleavage site† | Unique spectral count‡ |
|---|---|---|---|---|---|---|---|
| **Extracellular proteins** | | | | | | | |
| LBA0695 | Putative bacterial Ig-like domain protein | 62 | SPI | 75 % (410/550) | −0.58 | VSA-AD (37–38) | 141 |
| LBA1029 | Putative S-layer protein | 43 | SPI | 78 % (300/385) | −0.41 | VQA-AT (37–38) | 95 |
| LBA0512 | SlpX | 54 | SPI | 65 % (324/499) | −0.58 | VQA-DT (30–31) | 68 |
| LBA1567 | Aminopeptidase | 57 | SPI | 67 % (339/505) | −0.59 | AQA-AA (27–28) | 66 |
| LBA0222 | Putative uncharacterized protein | 30 | SPI | 54 % (152/282) | −0.78 | AHA-KG (39–40) | 61 |
| LBA0191 | Putative fibronectin domain protein | 52 | SPI | 71 % (329/463) | −0.59 | VQA-GT (24–25) | 60 |
| LBA0864 | Putative uncharacterized protein | 55 | SPI | 64 % (316/497) | −0.52 | AQA-QH (25–26) | 52 |
| LBA1568 | Putative surface protein | 39 | SPI* | 66 % (233/353) | −0.38 | Ambiguous | 46 |
| LBA1539 | Putative uncharacterized protein | 19 | SPI | 71 % (122/171) | −0.29 | ANA-AS (28–29) | 41 |
| LBA1006 | Penicillin-binding protein | 41 | SPI | 76 % (276/364) | −0.46 | VHA-AY (26–27) | 36 |
| LBA0176 | *N*-Acetylmuramidase | 45 | SPI | 43 % (176/409) | −0.68 | VSA-AT (38–39) | 21 |
| LBA0494 | Putative surface exclusion protein | 40 | SPI | 47 % (168/355) | −0.53 | VQA-AS (32–33) | 18 |
| LBA0177 | Autolysin, amidase | 41 | SPI | 44 % (160/364) | −0.57 | VQA-DS (30–31) | 18 |
| LBA0046 | Putative uncharacterized protein | 13 | SPI | 53 % (62/118) | −0.29 | TQA-AS (30–31) | 12 |
| LBA1079 | Putative cell surface protein | 23 | SPI | 39 % (79/202) | −0.38 | VNA-TT (29–30) | 12 |
| **Extracellular proteins (predicted to be sec-attached)** | | | | | | | |
| LBA1578 | Putative serine protease | 78 | SPI | 84 % (583/694) | −0.62 | VKA-AD (34–35) | 202 |
| LBA0169 | SlpA | 47 | SPI | 63 % (278/444) | −0.25 | VSA-AT (31–32) | 65 |
| LBA0858 | Penicillin-binding protein | 42 | SPI | 67 % (248/369) | −0.43 | VNA-KV (30–31) | 43 |
| LBA1426 | Putative uncharacterized protein | 28 | SPI | 63 % (159/252) | −0.38 | VQA-AT (34–35) | 39 |
| LBA1690 | Putative surface exclusion protein | 31 | SPI | 74 % (207/280) | −0.65 | NQE-DN (30–31) | 35 |
| LBA1207 | Putative enterolysin A | 24 | SPI | 65 % (139/213) | −0.42 | VSA-DT (30–31) | 26 |
| LBA1661 | Putative membrane protein | 20 | SPI | 48 % (86/180) | −0.45 | VQA-AT (37–38) | 25 |
| LBA1227 | Putative uncharacterized protein | 21 | SPI | 60 % (109/182) | −0.59 | VNA-ST (33–34) | 23 |
| LBA1225 | Putative bacterial Ig-like domain protein | 57 | SPI | 19 % (95/501) | −0.51 | VLA-CS (27–28) | 10 |
| **Lipid-anchored proteins** | | | | | | | |
| LBA0197 | OppA – oligopeptide binding protein | 65 | SPII | 63 % (366/585) | −0.58 | ALA-AC (21–22) | 53 |
| LBA1641 | Glycerol-3-phosphate ABC transporter | 47 | SPII | 68 % (293/433) | −0.48 | SSS-SS (32–33) | 44 |
| LBA1588 | PrsA/PrtM – peptidylprolyl isomerase | 33 | SPII | 64 % (193/300) | −0.58 | STA-AS (33–34) | 38 |
| LBA0014 | Putative alkylphosphonate ABC transporter | 35 | SPII | 60 % (187/313) | −0.38 | TSA-SS (31–32) | 29 |
| LBA0585 | Glycerol-3-phosphate ABC transporter | 48 | SPII | 34 % (147/432) | −0.47 | NSS-ST (31–32) | 16 |
| LBA1497 | Putative uncharacterized protein | 36 | SPII | 37 % (125/336) | −0.77 | SQG-NS (26–27) | 11 |
| **N-terminally anchored proteins (no predicted cleavage site)** | | | | | | | |
| LBA0223 | CdpA – cell separation protein | 64 | SPI* | 62 % (372/599) | −0.53 | – | 66 |
| LBA0805 | Penicillin-binding protein | 79 | SPI* | 53 % (379/720) | −0.37 | – | 49 |
| LBA1010 | Putative secreted protein | 45 | SPI* | 29 % (115/401) | −0.58 | – | 14 |
| **Intracellular or moonlighting proteins** | | | | | | | |
| LBA0851 | LysA – diaminopimelate decarboxylase | 35 | – | 70 % (226/323) | −0.22 | – | 32 |

**Table 2.** cont.

| ORF | Protein description | Predicted molecular mass (kDa) | SPase target | Amino acid coverage | GRAVY score | Predicted SPase cleavage site† | Unique spectral count‡ |
|---|---|---|---|---|---|---|---|
| LBA0040 | Putative uncharacterized protein | 10 | – | 74 % (64/87) | −0.98 | – | 15 |
| LBA0297 | RpsC – 30S ribosomal protein | 25 | – | 44 % (99/224) | −0.54 | – | 14 |
| LBA0698 | Glyceraldehyde 3-P dehydrogenase | 36 | – | 30 % (102/338) | −0.09 | – | 10 |

*Proteins predicted to have an SPase type-I target with unknown cleavage site.
†The amino acid sequence of predicted SPase cleavage is displayed along with the sequence position in parenthesis.
‡The number of unique spectra for each protein is used as a semiquantitative measure of protein abundance. The proteins are ordered from highest to lowest unique spectral counts.

signal peptide sequence which, after translocation, is targeted by one of two signal peptidases (SPases). Type-I SPases recognize an AxAA cleavage site (van Roosmalen *et al.*, 2004), while type-II SPases recognize an L-x-x-C, or lipobox, cleavage site (Sutcliffe & Harrington, 2002). Using SignalP 4.1 (Petersen *et al.*, 2011), 30 of the 37 identified proteins have predicted cleavage sites through either the type-I SPase or the type-II SPase pathway.

With reference to LocateP and the LAB-Secretome database (Zhou *et al.*, 2008, 2010), proteins were sorted by their predicted subcellular locations. Twenty-four of the 37 proteins in the fraction are predicted to be extracellular with predicted type-I SPase-mediated N-terminal cleavage sites (Table 2). Notably, nine of these extracellular proteins are predicted, via hidden Markov models, to remain N-terminally associated with the cell membrane despite predicted cleavage sites. While these so-called 'sec-attached' proteins have been proteomically described in *Bacillus subtilis* (Tjalsma & van Dijl, 2005), there has been no work demonstrating the existence of such proteins in *Lactobacillus* species to date. In fact, the exact method by which such proteins avoid processing through SPase activity has not been elucidated (Dreisbach *et al.*, 2011; Tjalsma & van Dijl, 2005). Six of the remaining 13 proteins are predicted to be lipid-anchored proteins, mediated through type-II SPase activity; three are proteins predicted to be N-terminally anchored to the cell membrane due to the lack of predicted cleavage sites; and four are predicted to be intracellular or non-classically secreted proteins (Table 2).

To deduce which of the proteins in the LiCl-extracted fraction may be SLAPs, we compared the protein sequences against the deduced proteomes of closely related S-layer-forming (*L. helveticus*, *L. crispatus* and *L. amylovorus*) and non-S-layer-forming (*L. gasseri*, *L. johnsonii*, and *L. delbrueckii* subsp. *bulgaricus*) lactobacilli using BLASTP (Table 3). The majority of the extracellular proteins (18/24), including those described as potentially sec-attached, demonstrate high levels of sequence identity to the corresponding proteins in S-layer-forming lactobacilli, but with either weak sequence similarity or no orthologues in the non-S-layer-forming lactobacilli, whereas the lipid-anchored proteins and the intracellular proteins demonstrate equally high sequence identity in both the S-layer-forming and non-S-layer-forming species of *Lactobacillus* examined. There were eight proteins with highly inferred homology in the S-layer-forming species with no hits in all three non-S-layer-forming species. Furthermore, 19 proteins demonstrate a highly inferred homology in the S-layer-forming species with no hit in at least two of the non-S-layer-forming species. We propose that these 17 proteins (excluding SlpA and SlpX) are candidate SLAPs, given their absence in non-S-layer-forming *Lactobacillus* species that are closely related to *L. acidophilus*.

While both SlpX and SlpA were found to be present in the SLAP fraction, they appeared at a significantly lower

**Table 3.** Homology search of potential SLAPs to proteins in S-layer-forming and non-S-layer-forming lactobacilli

For each protein alignment, highest % identity score was presented for the six *Lactobacillus* species listed. BLAST analyses were set to display alignments with E-values $\leqslant 1 \times 10^{-6}$. —, no hits; no shading, 1-25%; light grey shading, 26-50%; dark grey shading, 51-75%; black shading, 76-100%.

| | | S-layer-forming lactobacilli | | | Non-S-layer-forming lactobacilli | | |
|---|---|---|---|---|---|---|---|
| ORF | Protein description | *L. helveticus* | *L. crispatus* | *L. amylovorus* | *L. gasseri* | *L. johnsonii* | *L. delbrueckii* |
| **Extracellular proteins** | | | | | | | |
| LBA0695* | Putative bacterial Ig-like domain protein | 434/543 (80 %) | 429/543 (79 %) | 455/543 (84 %) | – | – | 167/482 (35 %) |
| LBA1029* | Putative S-layer protein | 145/377 (38 %) | 134/371 (36 %) | 87/255 (34 %) | – | – | – |
| LBA0512 | SlpX | 385/511 (75 %) | 313/446 (70 %) | 314/517 (54 %) | – | – | 52/126 (41 %) |
| LBA1567* | Aminopeptidase | 414/505 (82 %) | 405/505 (80 %) | 407/505 (81 %) | – | – | – |
| LBA0222* | Putative uncharacterized protein | 2112/129 (86 %) | 190/285 (67 %) | 110/126 (87 %) | – | – | – |
| LBA0191* | Putative fibronectin domain protein | 379/464 (82 %) | 367/464 (79 %) | 134/387 (35 %) | – | – | – |
| LBA0864* | Putative uncharacterized protein | 196/474 (41 %) | 346/502 (69 %) | 401/496 (81 %) | – | – | 226/517 (44 %) |
| LBA1568* | Putative surface protein | 252/325 (78 %) | 252/325 (78 %) | 252/325 (78 %) | – | – | 42/133 (32 %) |
| LBA1539* | Putative uncharacterized protein | 111/176 (63 %) | 115/173 (66 %) | 115/175 (66 %) | – | – | – |
| LBA1006 | Penicillin-binding protein | 303/365 (83 %) | 304/368 (83 %) | 300/364 (82 %) | 75/305 (25 %) | 65/234 (28 %) | 152/336 (45 %) |
| LBA0176 | *N*-Acetylmuramidase | 324/409 (79 %) | 300/409 (73 %) | 334/410 (81 %) | 124/273 (45 %) | 123/272 (45 %) | 190/414 (46 %) |
| LBA0494 | Putative surface exclusion protein | 252/356 (71 %) | 238/355 (67 %) | 241/357 (68 %) | – | 26/79 (33 %) | 113/257 (44 %) |
| LBA0177* | Autolysin, amidase | 280/364 (77 %) | 271/365 (74 %) | 284/369 (77 %) | – | – | 166/385 (43 %) |
| LBA0046* | Putative uncharacterized protein | 81/101 (80 %) | 92/118 (78 %) | 90/118 (76 %) | – | – | – |
| LBA1079 | Putative cell surface protein | 76/165 (46 %) | 78/167 (47 %) | 164/202 (81 %) | 89/190 (47 %) | – | 89/234 (38 %) |
| **Extracellular proteins (predicted to be sec-attached)** | | | | | | | |
| LBA1578* | Putative serine protease | 135/438 (31 %) | 133/481 (28 %) | 130/420 (31 %) | – | – | – |
| LBA0169 | SlpA | 323/446 (72 %) | 250/454 (59 %) | 239/488 (49 %) | – | – | 53/131 (40 %) |
| LBA0858 | Penicillin-binding protein | 265/366 (72 %) | 260/369 (70 %) | 275/368 (75 %) | 77/276 (28 %) | 78/313 (25 %) | 159/338 (47 %) |
| LBA1426* | Putative uncharacterized protein | 154/263 (59 %) | 153/257 (60 %) | 148/260 (57 %) | – | – | – |
| LBA1690 | Putative surface exclusion protein | 82/90 (91 %) | 230/280 (82 %) | 232/282 (82 %) | – | 82/310 (26 %) | 103/283 (36 %) |
| LBA1207 | Putative enterolysin A | 117/147 (80 %) | 150/211 (71 %) | 130/184 (71 %) | 63/169 (78 %) | 65/170 (38 %) | 100/164 (61 %) |
| LBA1661 | Putative membrane protein | 68/140 (49 %) | 127/183 (69 %) | 138/182 (76 %) | 76/192 (40 %) | 80/184 (43 %) | – |
| LBA1227* | Putative uncharacterized protein | 99/140 (71 %) | 137/182 (75 %) | 70/96 (73 %) | – | – | 48/157 (31 %) |
| LBA1225* | Putative bacterial Ig-like domain protein | 189/294 (64 %) | 305/501 (61 %) | 195/510 (38 %) | – | – | 90/368 (24 %) |
| **Lipid-anchored proteins** | | | | | | | |
| LBA0197* | OppA – oligopeptide binding protein | 476/541 (88 %) | 484/539 (90 %) | 490/543 (90 %) | – | – | 332/543 (61 %) |
| LBA1641 | Glycerol-3-phosphate ABC transporter | 374/433 (86 %) | 390/433 (90 %) | 403/434 (93 %) | 310/433 (72 %) | 309/433 (71 %) | 357/433 (82 %) |
| LBA1588 | PrsA/PrtM – peptidylprolyl isomerase | 276/300 (92 %) | 278/296 (94 %) | 284/296 (96 %) | 214/296 (72 %) | 212/296 (72 %) | 167/298 (56 %) |
| LBA0014 | Putative alkylphosphonate ABC transporter | 278/308 (90 %) | 298/311 (96 %) | 300/314 (96 %) | 218/308 (71 %) | 220/308 (71 %) | 176/309 (57 %) |
| LBA0585 | Glycerol-3-phosphate ABC transporter | 383/433 (94 %) | 369/434 (85 %) | 404/434 (93 %) | 250/436 (57 %) | 291/432 (67 %) | 258/433 (60 %) |
| LBA1497* | Putative uncharacterized protein | 246/338 (73 %) | 250/341 (73 %) | 253/337 (75 %) | – | – | 90/368 (24 %) |

**Table 3.** cont.

| ORF | Protein description | S-layer-forming lactobacilli | | | Non-S-layer-forming lactobacilli | | |
|---|---|---|---|---|---|---|---|
| | | *L. helveticus* | *L. crispatus* | *L. amylovorus* | *L. gasseri* | *L. johnsonii* | *L. delbrueckii* |
| **N-terminally anchored proteins (no predicted cleavage site)** | | | | | | | |
| LBA0223* | CdpA – cell separation protein | 213/308 (69 %) | 337/602 (56 %) | 392/583 (67 %) | – | – | 74/232 (32 %) |
| LBA0805 | Penicillin-binding protein | 629/720 (87 %) | 609/720 (85 %) | 642/720 (89 %) | 449/722 (62 %) | 445/720 (62 %) | 434/722 (60 %) |
| LBA1010 | Putative secreted protein | 323/401 (81 %) | 328/401 (82 %) | 324/401 (81 %) | 193/390 (49 %) | 185/363 (51 %) | 190/405 (47 %) |
| **Intracellular or moonlighting proteins** | | | | | | | |
| LBA0851 | LysA – diaminopimelate decarboxylase | 353/432 (82 %) | 336/432 (78 %) | 366/432 (85 %) | – | 332/431 (77 %) | 282/431 (65 %) |
| LBA0040 | Putative uncharacterized protein | 74/87 (85 %) | – | 82/87 (94 %) | 52/85 (61 %) | – | 45/86 (52 %) |
| LBA0297 | RpsC – 30S ribosomal protein | 220/224 (98 %) | 221/234 (99 %) | 219/224 (98 %) | 192/223 (86 %) | 193/223 (87 %) | 189/224 (84 %) |
| LBA0698 | Glyceraldehyde 3-P dehydrogenase | 296/338 (88 %) | 299/338 (88 %) | 298/338 (88 %) | 314/338 (93 %) | 314/338 (93 %) | 317/338 (93 %) |

*Candidate SLAP.

concentration compared with the other proteins (Table 2). For reference of comparison, the standard LiCl extraction protocol was performed along with the modification presented in this study (Fig. 2b). It is clear that the method presented in this study recovered more proteins than the standard method. Of the 37 proteins identified, 21 are annotated as putative or uncharacterized proteins of unknown function. These include proteins with putative fibronectin-binding domains, putative surface proteins, bacterial Ig-like domain proteins, putative surface exclusion proteins, uncharacterized ABC transporters, a 78 kDa putative serine protease and a putative S-layer protein (LBA1029).

### Deletion of *lba-1029* from the chromosome of *L. acidophilus* NCFM

LBA1029 was observed to be a prevalent protein in the LiCl fraction. Examining annotated sequence data from *L. acidophilus* NCFM, we found that *lba-1029* encodes an uncharacterized protein, annotated as a putative S-layer protein. Notably, this protein was selected for functional analysis because of its apparent singularity to *L. acidophilus* compared with closely related S-layer- and non-S-layer-forming lactobacilli (Table 3). In fact, the deduced protein sequence of LBA1029 demonstrated low sequence identity to proteins in the dairy starter culture *L. helveticus* H10 (38 % sequence identity) and the vaginal commensal *L. crispatus* ST1 (36 %), both of which are S-layer-forming members of the Group A acidophilus complex. This 385 aa residue protein of unknown function has a predicted N-terminal signal peptide cleavage site between two alanine residues at positions 37 and 38. Furthermore, *lba-1029* is flanked by two hairpin terminators, suggesting monocistronic mRNA expression and control (Fig. 3a).

To assess the roles these putative SLAPs may play in cell function and immunomodulatory interaction, a Δ*lba-1029* strain was created and phenotypically characterized. The *lba-1029* gene was deleted from the chromosome using a *upp*-based counterselection gene replacement system (Fig. 3b). A colony containing the in-frame deletion of *lba-1029* (Δ*lba-1029*) was confirmed via sequencing and designated NCK2258. When the SLAPs of NCK2258 were profiled (Fig. 2, lane 2) and identified through LC-MS/MS, LBA1029 was not found in the fraction (data not shown), further confirming the absence of LBA1029 from the cell envelope of NCK2258.

### Phenotypic characterization of Δ*lba-1029* mutant as pertaining to probiotic functionality

Comparative analysis between NCK1909 (wild-type reference) and NCK2258 (Δ*lba-1029*) was used to characterize the function of LBA1029. NCK2258 showed no difference in growth in MRS medium or cell morphology under the light microscope compared with NCK1909. Likewise, mutant cells settled to the bottom of tubes in a similar

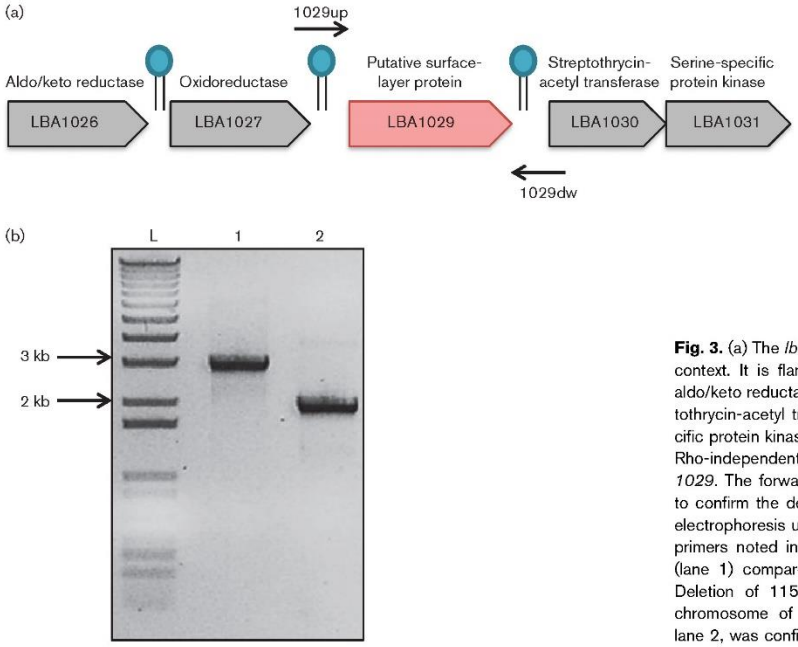


**Fig. 3.** (a) The *lba-1029* ORF in chromosomal context. It is flanked by genes encoding an aldo/keto reductase, an oxidoreductase, streptothrycin-acetyl transferase and a serine-specific protein kinase. Note the predicted hairpin Rho-independent terminators flanking *lba-1029*. The forward and reverse primers used to confirm the deletion are indicated. (b) Gel electrophoresis using the forward and reverse primers noted in (a) on WT reference strain (lane 1) compared with NCK2258 (lane 2). Deletion of 1155 bp from *lba-1029* in the chromosome of *L. acidophilus*, reflected in lane 2, was confirmed by sequencing.

fashion to NCK1909 when grown in planktonic culture, suggesting no difference in the aggregative properties between the two strains.

Assays for simulated gastric and small intestinal juices, adhesion to a Caco-2 epithelial cell line, and bile tolerance were also performed. Survival through the gastrointestinal tract was evaluated *in vitro* through exposure to simulated gastric juice and simulated small intestinal juice over 1.5 and 5 h, respectively. There was no significant difference in the survival rate between NCK2258 and the NCK1909 reference strain (Fig. S1, available in *Microbiology* Online). The Caco-2 epithelial cell line was employed for *in vitro* analysis of bacterial adherence to intestinal epithelia. Compared with the reference strain, NCK2258 showed insignificant changes in the adherence to Caco-2 (Fig. S2). Finally, NCK2258 and the NCK1909 reference strain were exposed to 0.3 and 0.5 % bile (Oxgall) to assay bile tolerance, but no difference was observed between the strains (Fig. S3).

### LBA1029 contributes to pro-inflammatory response through the induction of TNF-α

Previous work on the S-layer of *L. acidophilus* NCFM demonstrated a role of SlpA in modulating the host immune system (Konstantinov *et al.*, 2008). To test the immunomodulatory action of the LBA1029 protein, a bacterial/murine DC co-incubation assay was performed. After co-incubation for 24 h, the cytokines TNF-α, IL-6, IL-10 and IL-12 were quantified using ELISA. Both TNF-α and IL-6 were measured as an indicator of a general pro-inflammatory response. IL-10, however, was measured as a marker of an anti-inflammatory response via the down-regulation of a Th1 cell response. Conversely, IL-12 was measured as a pro-inflammatory response via the activation of Th1 cells. Three independent assays were performed in duplicate for each cytokine.

For each co-incubation, bacterial cells were diluted and exposed to DCs at a ratio of approximately 10 : 1. Notably, there was a significant decrease ($P$=0.006) in TNF-α production for DCs co-incubated with NCK2258 compared with the parental reference strain, NCK1909 (Fig. 4a). There was no significant difference between DCs co-incubated with NCK2258 and the NCK1909 reference with regard to IL-6, IL-10 and IL-12 production (Fig. 4b). Ultimately, NCK2258 demonstrated a 36 % reduction in TNF-α induction (Fig. 4b), suggesting that LBA1029 contributes to a pro-inflammatory response via the induction of TNF-α.

## DISCUSSION

This study utilized a method modified from a standard LiCl S-layer extraction to isolate proteins associated with

the primary S-layer fraction and identify extracellular proteins and putative SLAPs in *L. acidophilus* NCFM. Thity-seven proteins were identified and reported. One such protein, LBA1029, was eliminated via deletion of the gene from the chromosome. While the absence of LBA1029 did not seem to affect survival in simulated gastric juice, simulated small intestinal juice, bile tolerance or affinity to Caco-2 epithelial cells, it did demonstrate important capacity for immunomodulation through murine DCs. The 36 % reduction of TNF-α production by DCs co-incubated with the LBA1029 deficient strain compared with its parent strain suggests that the protein is pro-inflammatory through the TNF-α pathway.

Of the 37 reported proteins, 30 have predicted cleavage sites for secretion, of which 24 are predicted to be extracellular and six are lipid-anchored. Three of the remaining seven have an N-terminal transmembrane hydrophobic anchor (with no cleavage site) and the final four are intracellular, potentially non-classically secreted moonlighting proteins (Table 2). Both LocateP (Zhou *et al.* 2008) and the LAB-Secretome database (Zhou *et al.*, 2010) reported nine of the 24 extracellular proteins as 'sec-attached'. These sec-attached proteins, discovered in *Bacillus subtilis* (Tjalsma & van Dijl, 2005), are proteins that have predicted cleavage sites but avoid Sec-pathway SPase cleavage to remain N-terminally anchored to the cell membrane. The method by which these proteins avoid SPase activity for Sec processing has yet to be elucidated (Dreisbach *et al.*, 2011; Tjalsma & van Dijl, 2005). Notably, sec-attached proteins have not been proteomically characterized in any *Lactobacillus* species to date. The basis by which the LAB proteomes were analysed for the presence of sec-attached proteins used hidden Markov modelling comparing 63 cleaved proteins (extracellular) with 53 uncleaved proteins (sec-attached) from *B. subtilis* and other *Bacillus* orthologues (Zhou *et al.*, 2008). While this hidden Markov modelling scoring model is useful for processing large proteomic datasets, such as those from the LAB-Secretome database, it is important to acknowledge the possibility of incorrect predictions. For example, SlpA was predicted to be sec-attached despite the fact that it has been visually and biochemically characterized as the self-assembling constituent of the *L. acidophilus* S-layer (Boot *et al.*, 1995, 1996; Boot & Pouwels, 1996). Due to this incongruity and because these nine predicted sec-attached proteins were isolated using LiCl, we consider these proteins to be extracellular.

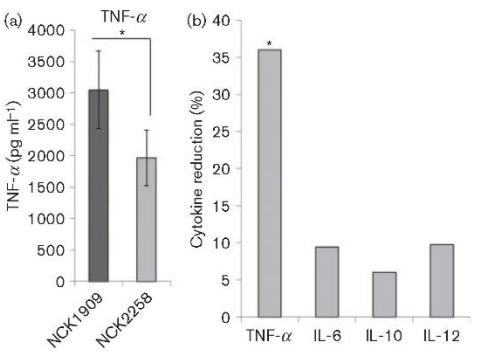


**Fig. 4.** (a) Induction of TNF-α in murine DCs after co-incubation with NCK1909 or NCK2258 as measured by ELISA. Three independent biological replicates were performed with each strain in duplicate, aiming for an approximate bacterial to DC ratio of 10 : 1. Using univariate ANOVA, the reduction in TNF-α induction between NCK1909 and NCK2258 was significant ($P$=0.006). Error bars show SD among the replicates. (b) Per cent cytokine reduction of NCK2258 compared with NCK1909 for the cytokines TNF-α, IL-6, IL-10 and IL-12. The induction of IL-6, IL-10 and IL-12 was not significantly different between NCK2258 and NCK1909.

Interestingly, four of the 37 proteins identified (Table 2) in the LiCl precipitate do not have predicted secretion pathways: a diaminopimelate decarboxylase, 30S ribosomal protein S3, an uncharacterized protein and a glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Because the cells were dialysed at stationary phase, it is possible that limited cell lysis may have begun by the time cells were treated with LiCl, allowing small amounts of intracellular and membrane-anchored proteins to be recovered non-specifically along with the S-layer. However, it is also possible that these intracellular proteins are non-classical, extracellular 'moonlighting' proteins (Jeffery, 1999). GAPDH, for example, is a moonlighting protein which mediates microbe–host interactions in lactic acid bacteria (Katakura *et al.*, 2010; Kinoshita *et al.*, 2008; Sánchez *et al.*, 2009). In fact, the GAPDH of *L. plantarum* LA 318 has demonstrated adherence capabilities to human colonic mucin (Kinoshita *et al.*, 2008).

Of particular interest in this study is the isolation and identification of possible SLAPs in the cell-surface proteome of *L. acidophilus* NCFM. Note that the *in vivo* localization or association of these SLAPs in relation to the S-layer has not been fully demonstrated, and is based on co-extraction following LiCl treatment. To select candidate SLAPs from the fraction of secreted proteins identified through LiCl extraction, we aligned the deduced protein sequences to the deduced proteomes of closely related S-layer- and non-S-layer-forming *Lactobacillus* species (Table 3). The analysis revealed that the 24 extracellular proteins had high sequence identity to the corresponding orthologues in S-layer-forming lactobacilli and either no or low sequence identity to the non-S-layer-forming lactobacilli. In contrast, the lipid-anchored proteins, many of which are ABC sugar transporters, and the intracellular proteins shared inferred homology in both S-layer- and non-S-layer-forming *Lactobacillus* species. Based on this *in silico* analysis, the proteins that are most likely SLAPs are the 17 extracellular proteins with no hits in two or three of the

non-S-layer-forming lactobacilli. Further work will be required to characterize the specific localization of these SLAPs and the Slp subunits with which they interact. Furthermore, there should be a distinction between the organizational definitions of SLAPs in the context of this study and the SLAP Pfam domain (PF03217) designated for bacterial S-layer proteins (Boot *et al.*, 1995).

To our knowledge, SLAPs have not been identified in any organism using the method of this study. However, in the S-layer-forming pathogen *Bacillus anthracis*, 22 *B. anthracis* S-layer proteins (BSLs) have been identified and characterized (Kern & Schneewind, 2008, 2010; Kern *et al.*, 2012; Lunderberg *et al.*, 2013). While these proteins are described as S-layer associated in recent publications (Kern *et al.*, 2012; Lunderberg *et al.*, 2013), these proteins were localized *in silico* based on N-terminal SLH domains and were originally designated Slps. In contrast, the SLAPs observed in the present study were found to be constituents of the S-layer after translational expression and secretion. An important distinction should therefore be made between the methodology of this study and the methodology for identifying BSLs of *B. anthracis*. Application of this study's methodology to *B. anthracis* may yield novel SLAPs that are not tethered to the S-layer through SLH domains.

The SLAPs in *L. acidophilus* NCFM offer potential in understanding cell envelope biology and function, as well as illuminating important factors pertaining to probiotic function. A cell division protein CdpA, which has previously been functionally characterized (Altermann *et al.*, 2004), was found to be a prevalent protein in the SLAP fraction. CdpA, which may be a SLAP due to its low sequence identity in the non-S-layer-forming lactobacilli (Table 3), has important roles in cell-wall processing during growth and cell–cell separation. This finding substantiates the prediction that certain SLAPs, especially the putative cell division proteins, aminopeptidases and penicillin-binding proteins, may play a role in cell growth, cell turnover and cell envelope function. A great deal of work has already been done pertaining to probiotic functions in *L. acidophilus* NCFM. This includes work on adherence factors (Buck *et al.*, 2005), probiotic sugar utilization (Barrangou *et al.*, 2003) and bacteriocin production (Dobson *et al.*, 2007). However, there are still many factors of the probiotic mechanism that have not been fully elucidated. Because of their localization to the cell surface, the SLAPs identified in this study are candidate mediators of probiotic function. Furthermore, access to microarray data pertaining to acid tolerance (Azcarate-Peril *et al.*, 2004), bile tolerance (Pfeiler *et al.*, 2007) and oligosaccharide utilization (Barrangou *et al.*, 2003), could offer insight into the role that these extracellular proteins may play in probiotic survival, persistence and immunomodulation in the host gastrointestinal tract.

Understanding the role of intestinal microbiota in gut homeostasis has been regarded with great interest given the prevalence of inflammatory bowel disease, such as ulcerative colitis, and Crohn's disease (MacDonald & Monteleone, 2005). Recent work regarding cell surface components of *L. acidophilus* NCFM, such as lipoteichoic acid and SlpA, has demonstrated regulation of colonic inflammation and T-cell functionality, respectively (Konstantinov *et al.*, 2008; Mohamadzadeh *et al.*, 2011). Given their localization to the outermost layer of the cell envelope, SLAPs are ideal candidates for studying the immunomodulatory interaction between *L. acidophilus* and the gut immune system. The findings of this study support this observation, given that LBA1029 exhibited an effect on immunomodulatory properties through the induction of TNF-α. Exploring the SLAPs of *L. acidophilus*, as well as those of other S-layer-forming commensal bacteria, will be important in understanding the full context of the interaction between gut epithelial cells, gut immune system and intestinal microbiota.

Beyond *L. acidophilus*, this study highlights further potential in understanding S-layer and cell envelope function of other S-layer-forming bacteria and archaea. In certain S-layer-forming pathogens, such as *B. anthracis* (Kern & Schneewind, 2010), *Rickettsia* species (Walker *et al.*, 2003), *Aeromonas salmonicida* (Kay *et al.*, 1984), *Campylobacter fetus* (Blaser *et al.*, 1988) and *Clostridium difficile* (Calabi *et al.*, 2002), S-layer and cell surface components are important pathogenicity factors. Studying potential SLAPs with the cell surface proteome in these and other S-layer-forming pathogens may offer further insight into pathogenicity. The hyperthermophilic archaea that form S-layers, such as *Methanococcus* and *Methanothermus* species, are of certain biotechnological interest. In particular, the hexagonal S-layers of these archaea have been regarded for their potential uses in nanotechnology because of their heat stability and the ability to self-assemble (Sleytr & Sára, 1997). Examining potential SLAPs in these and other extremophilic archaea could explicate cell envelope function, as well as potentiate the discovery of important thermostable proteins for use in biotechnological applications.

Overall, the characterization of SLAPs significantly expands the opportunities to understand the probiotic activities and immunomodulatory actions of *L. acidophilus* NCFM. Likewise, SLAPs may be of particular interest in other members of the genus *Lactobacillus*. Comparing the SLAPs of *L. acidophilus* with the proteomes of other S-layer-forming lactobacilli, such as the dairy starter *L. helveticus* and the vaginal commensal *L. crispatus*, could provide key ecological and evolutionary insights. Novel proteinases or proteases could be identified as SLAPs in the cell surface proteome of *L. helveticus* that may be important in the cheese ripening process. Moreover, SLAPs could be important factors of adherence and retention for *L. crispatus* in the vaginal mucosa. Ultimately, the proteins identified in this study are potentially novel extracellular proteins, some of which may be associated with the bacterial S-layer. These proteins afford the possibility to

functionally characterize bacterial S-layers and will provide important insights into the architecture and physiology of bacterial cell surfaces.

## ACKNOWLEDGEMENTS

This work was supported in part by funding from the North Carolina Agriculture Foundation (Raleigh, NC) and DuPont Nutrition & Health (Madison, WI). Our thanks are extended to Dr Brett Phiney and Michelle Salemi at the University of California Davis Proteomics Core Facility for LC-MS/MS services. We are grateful to Rosemary Sanozky-Dawes for technical assistance in preparing Caco-2 epithelial cells. Finally, we thank Dr Dahlia Nielsen for insightful statistical discussion and Kiersten Johnson for assistance with statistical analyses.

## REFERENCES

**Altermann, E., Buck, L. B., Cano, R. & Klaenhammer, T. R. (2004).** Identification and phenotypic characterization of the cell-division protein CdpA. *Gene* **342**, 189–197.

**Altermann, E., Russell, W. M., Azcarate-Peril, M. A., Barrangou, R., Buck, B. L., McAuliffe, O., Souther, N., Dobson, A., Duong, T. & other authors (2005).** Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *Proc Natl Acad Sci U S A* **102**, 3906–3912.

**Ashida, N., Yanagihara, S., Shinoda, T. & Yamamoto, N. (2011).** Characterization of adhesive molecule with affinity to Caco-2 cells in *Lactobacillus acidophilus* by proteome analysis. *J Biosci Bioeng* **112**, 333–337.

**Åvall-Jääskeläinen, S. & Palva, A. (2005).** *Lactobacillus* surface layers and their applications. *FEMS Microbiol Rev* **29**, 511–529.

**Azcarate-Peril, M. A., Altermann, E., Hoover-Fitzula, R. L., Cano, R. J. & Klaenhammer, T. R. (2004).** Identification and inactivation of genetic loci involved with *Lactobacillus acidophilus* acid tolerance. *Appl Environ Microbiol* **70**, 5315–5322.

**Barrangou, R., Altermann, E., Hutkins, R., Cano, R. & Klaenhammer, T. R. (2003).** Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by *Lactobacillus acidophilus*. *Proc Natl Acad Sci U S A* **100**, 8957–8962.

**Beganović, J., Frece, J., Kos, B., Leboš Pavunc, A., Habjanič, K. & Sušković, J. (2011).** Functionality of the S-layer protein from the probiotic strain *Lactobacillus helveticus* M92. *Antonie van Leeuwenhoek* **100**, 43–53.

**Blaser, M. J., Smith, P. F., Repine, J. E. & Joiner, K. A. (1988).** Pathogenesis of *Campylobacter fetus* infections. Failure of encapsulated *Campylobacter fetus* to bind C3b explains serum and phagocytosis resistance. *J Clin Invest* **81**, 1434–1444.

**Boot, H. J. & Pouwels, P. H. (1996).** Expression, secretion and antigenic variation of bacterial S-layer proteins. *Mol Microbiol* **21**, 1117–1123.

**Boot, H. J., Kolen, C. P. & Pouwels, P. H. (1995).** Identification, cloning, and nucleotide sequence of a silent S-layer protein gene of *Lactobacillus acidophilus* ATCC 4356 which has extensive similarity with the S-layer protein gene of this species. *J Bacteriol* **177**, 7222–7230.

**Boot, H. J., Kolen, C. P., Pot, B., Kersters, K. & Pouwels, P. H. (1996).** The presence of two S-layer-protein-encoding genes is conserved among species related to *Lactobacillus acidophilus*. *Microbiology* **142**, 2375–2384.

**Brechtel, E. & Bahl, H. (1999).** In *Thermoanaerobacterium thermosulfurigenes* EM1 S-layer homology domains do not attach to peptidoglycan. *J Bacteriol* **181**, 5017–5023.

**Buck, B. L., Altermann, E., Svingerud, T. & Klaenhammer, T. R. (2005).** Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. *Appl Environ Microbiol* **71**, 8344–8351.

**Calabi, E., Calabi, F., Phillips, A. D. & Fairweather, N. F. (2002).** Binding of *Clostridium difficile* surface layer proteins to gastrointestinal tissues. *Infect Immun* **70**, 5770–5778.

**Chauvaux, S., Matuschek, M. & Beguin, P. (1999).** Distinct affinity of binding sites for S-layer homologous domains in *Clostridium thermocellum* and *Bacillus anthracis* cell envelopes. *J Bacteriol* **181**, 2455–2458.

**Dobson, A. E., Sanozky-Dawes, R. B. & Klaenhammer, T. R. (2007).** Identification of an operon and inducing peptide involved in the production of lactacin B by *Lactobacillus acidophilus*. *J Appl Microbiol* **103**, 1766–1778.

**Dreisbach, A., van Dijl, J. M. & Buist, G. (2011).** The cell surface proteome of *Staphylococcus aureus*. *Proteomics* **11**, 3154–3168.

**Driessen, A. J. M. & Nouwen, N. (2008).** Protein translocation across the bacterial cytoplasmic membrane. *Annu Rev Biochem* **77**, 643–667.

**Frece, J., Kos, B., Svetec, I. K., Zgaga, Z., Mrsa, V. & Susković, J. (2005).** Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. *J Appl Microbiol* **98**, 285–292.

**Goh, Y. J. & Klaenhammer, T. R. (2010).** Functional roles of aggregation-promoting-like factor in stress tolerance and adherence of *Lactobacillus acidophilus* NCFM. *Appl Environ Microbiol* **76**, 5005–5012.

**Goh, Y. J., Azcárate-Peril, M. A., O'Flaherty, S., Durmaz, E., Valence, F., Jardin, J., Lortal, S. & Klaenhammer, T. R. (2009).** Development and application of a *upp*-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. *Appl Environ Microbiol* **75**, 3093–3105.

**Hammes, W. P. & Vogel, R. F. (1995).** The genus *Lactobacillus*. In *The Genera of Lactic Acid Bacteria*, pp. 19–54. Edited by B. J. B. Wood & W. H. Holzapfel. Glasgow: Blackie Academic & Professional.

**Hanahan, D. (1983).** Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.

**Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. (1989).** Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61–68.

**Hynönen, U. & Palva, A. (2013).** *Lactobacillus* surface layer proteins: structure, function and applications. *Appl Microbiol Biotechnol* **97**, 5225–5243.

**Jeffery, C. J. (1999).** Moonlighting proteins. *Trends Biochem Sci* **24**, 8–11.

**Katakura, Y., Sano, R., Hashimoto, T., Ninomiya, K. & Shioya, S. (2010).** Lactic acid bacteria display on the cell surface cytosolic proteins that recognize yeast mannan. *Appl Microbiol Biotechnol* **86**, 319–326.

**Kay, W. W., Phipps, B. M., Ishiguro, E. E., Olafson, R. W. & Trust, T. J. (1984).** Surface layer virulence A-proteins from *Aeromonas salmonicida* strains. *Can J Biochem Cell Biol* **62**, 1064–1071.

**Kern, J. W. & Schneewind, O. (2008).** BslA, a pXO1-encoded adhesin of *Bacillus anthracis*. *Mol Microbiol* **68**, 504–515.

**Kern, J. & Schneewind, O. (2010).** BslA, the S-layer adhesin of *B. anthracis*, is a virulence factor for anthrax pathogenesis. *Mol Microbiol* **75**, 324–332.

**Kern, V. J., Kern, J. W., Theriot, J. A., Schneewind, O. & Missiakas, D. (2012).** Surface-layer (S-layer) proteins sap and EA1 govern the binding of the S-layer-associated protein BslO at the cell septa of *Bacillus anthracis*. *J Bacteriol* **194**, 3833–3840.

**Kingsford, C. L., Ayanbule, K. & Salzberg, S. L. (2007).** Rapid, accurate, computational discovery of Rho-independent transcription terminators illuminates their relationship to DNA uptake. *Genome Biol* **8**, R22.

**Kinoshita, H., Uchida, H., Kawai, Y., Kawasaki, T., Wakahara, N., Matsuo, H., Watanabe, M., Kitazawa, H., Ohnuma, S. & other authors (2008).** Cell surface *Lactobacillus plantarum* LA 318 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) adheres to human colonic mucin. *J Appl Microbiol* **104**, 1667–1674.

**Konstantinov, S. R., Smidt, H., de Vos, W. M., Bruijns, S. C., Singh, S. K., Valence, F., Molle, D., Lortal, S., Altermann, E. & other authors (2008).** S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. *Proc Natl Acad Sci U S A* **105**, 19474–19479.

**Lortal, S., Vanheijenoort, J., Gruber, K. & Sleytr, U. B. (1992).** S-layer of *Lactobacillus helveticus* ATCC 12046: isolation, chemical characterization and re-formation after extraction with lithium chloride. *J Gen Microbiol* **138**, 611–618.

**Lunderberg, J. M., Nguyen-Mau, S. M., Richter, G. S., Wang, Y. T., Dworkin, J., Missiakas, D. M. & Schneewind, O. (2013).** *Bacillus anthracis* acetyltransferases PatA1 and PatA2 modify the secondary cell wall polysaccharide and affect the assembly of S-layer proteins. *J Bacteriol* **195**, 977–989.

**MacDonald, T. T. & Monteleone, G. (2005).** Immunity, inflammation, and allergy in the gut. *Science* **307**, 1920–1925.

**Mohamadzadeh, M., Pfeiler, E. A., Brown, J. B., Zadeh, M., Gramarossa, M., Managlia, E., Bere, P., Sarraj, B., Khan, M. W. & other authors (2011).** Regulation of induced colonic inflammation by *Lactobacillus acidophilus* deficient in lipoteichoic acid. *Proc Natl Acad Sci U S A* **108** (Suppl. 1), 4623–4630.

**Petersen, T. N., Brunak, S., von Heijne, G. & Nielsen, H. (2011).** SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* **8**, 785–786.

**Pfeiler, E. A., Azcarate-Peril, M. A. & Klaenhammer, T. R. (2007).** Characterization of a novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus acidophilus*. *J Bacteriol* **189**, 4624–4634.

**Pot, B., Ludwig, W., Kersters, K. & Schleifer, K. H. (1994).** Taxonomy of lactic acid bacteria. In *Bacteriocins of Lactic Acid Bacteria*, pp. 13–90. Edited by L. de Vuyst & E. J. Vandamme. Glasgow: Chapman & Hall.

**Russell, W. M. & Klaenhammer, T. R. (2001).** Efficient system for directed integration into the Lactobacillus acidophilus and Lactobacillus gasseri chromosomes via homologous recombination. *Appl Environ Microb*, **67**, 4361–4364.

**Sánchez, B., Schmitter, J. M. & Urdaci, M. C. (2009).** Identification of novel proteins secreted by *Lactobacillus plantarum* that bind to mucin and fibronectin. *J Mol Microbiol Biotechnol* **17**, 158–162.

**Sanders, M. E. & Klaenhammer, T. R. (2001).** Invited review: the scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic. *J Dairy Sci* **84**, 319–331.

**Sára, M. & Sleytr, U. B. (1996).** Crystalline bacterial cell surface layers (S-layers): from cell structure to biomimetics. *Prog Biophys Mol Biol* **65**, 83–111.

**Sára, M. & Sleytr, U. B. (2000).** S-Layer proteins. *J Bacteriol* **182**, 859–868.

**Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. (1996).** Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* **68**, 850–858.

**Sleytr, U. B. & Beveridge, T. J. (1999).** Bacterial S-layers. *Trends Microbiol* **7**, 253–260.

**Sleytr, U. B. & Messner, P. (1983).** Crystalline surface layers on bacteria. *Annu Rev Microbiol* **37**, 311–339.

**Sleytr, U. B. & Sára, M. (1997).** Bacterial and archaeal S-layer proteins: structure-function relationships and their biotechnological applications. *Trends Biotechnol* **15**, 20–26.

**Sleytr, U. B., Sára, M., Pum, D. & Schuster, B. (2001).** Characterization and use of crystalline bacterial cell surface layers. *Prog Surf Sci* **68**, 231–278.

**Smit, E., Oling, F., Demel, R., Martinez, B. & Pouwels, P. H. (2001).** The S-layer protein of *Lactobacillus acidophilus* ATCC 4356: identification and characterisation of domains responsible for S-protein assembly and cell wall binding. *J Mol Biol* **305**, 245–257.

**Stoeker, L., Nordone, S., Gunderson, S., Zhang, L., Kajikawa, A., LaVoy, A., Miller, M., Klaenhammer, T. R. & Dean, G. A. (2011).** Assessment of *Lactobacillus gasseri* as a candidate oral vaccine vector. *Clin Vaccine Immunol* **18**, 1834–1844.

**Sutcliffe, I. C. & Harrington, D. J. (2002).** Pattern searches for the identification of putative lipoprotein genes in Gram-positive bacterial genomes. *Microbiology* **148**, 2065–2077.

**Tabb, D. L., Ma, Z. Q., Martin, D. B., Ham, A. J. L. & Chambers, M. C. (2008).** DirecTag: accurate sequence tags from peptide MS/MS through statistical scoring. *J Proteome Res* **7**, 3838–3846.

**Taverniti, V., Stuknyte, M., Minuzzo, M., Arioli, S., De Noni, I., Scabiosi, C., Cordova, Z. M., Junttila, I., Hämäläinen, S. & other authors (2013).** S-layer protein mediates the stimulatory effect of *Lactobacillus helveticus* MIMLh5 on innate immunity. *Appl Environ Microbiol* **79**, 1221–1231.

**Tjalsma, H. & van Dijl, J. M. (2005).** Proteomics-based consensus prediction of protein retention in a bacterial membrane. *Proteomics* **5**, 4472–4482.

**van Roosmalen, M. L., Geukens, N., Jongbloed, J. D. H., Tjalsma, H., Dubois, J. Y. F., Bron, S., van Dijl, J. M. & Anné, J. (2004).** Type I signal peptidases of Gram-positive bacteria. *Biochim Biophys Acta* **1694**, 279–297.

**Walker, D. C., Aoyama, K. & Klaenhammer, T. R. (1996).** Electrotransformation of *Lactobacillus acidophilus* group A1. *FEMS Microbiol Lett* **138**, 233–237.

**Walker, D. H., Valbuena, G. A. & Olano, J. P. (2003).** Pathogenic mechanisms of diseases caused by *Rickettsia*. *Ann N Y Acad Sci* **990**, 1–11.

**Wei, M. Q., Rush, C. M., Norman, J. M., Hafner, L. M., Epping, R. J. & Timms, P. (1995).** An improved method for the transformation of *Lactobacillus* strains using electroporation. *J Microbiol Methods* **21**, 97–109.

**Zhou, M., Boekhorst, J., Francke, C. & Siezen, R. J. (2008).** LocateP: genome-scale subcellular-location predictor for bacterial proteins. *BMC Bioinformatics* **9**, 173.

**Zhou, M., Theunissen, D., Wels, M. & Siezen, R. J. (2010).** LAB-Secretome: a genome-scale comparative analysis of the predicted extracellular and surface-associated proteins of lactic acid bacteria. *BMC Genomics* **11**, 651.

Edited by: D. Mills

***Appendix C***

Reprint from:

**Johnson BR, Hymes J, Sanozky-Dawes R, DeCrescenzo Henriksen E, Barrangou R, Klaenhammer TR**. 2016. Conserved S-layer associated proteins revealed by exoproteomic survey of S-layer forming lactobacilli. *Applied and Environmental Microbiology* **82**: 134-145.

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

# Conserved S-Layer-Associated Proteins Revealed by Exoproteomic Survey of S-Layer-Forming Lactobacilli

Brant R. Johnson,[a,b] Jeffrey Hymes,[b] Rosemary Sanozky-Dawes,[b] Emily DeCrescenzo Henriksen,[b] Rodolphe Barrangou,[a,b] Todd R. Klaenhammer[a,b]

Graduate Program in Microbiology, College of Agriculture and Life Sciences, North Carolina State University, Raleigh, North Carolina, USA[a]; Department of Food, Bioprocessing, and Nutrition Sciences, North Carolina State University, Raleigh, North Carolina, USA[b]

**The *Lactobacillus acidophilus* homology group comprises Gram-positive species that include *L. acidophilus*, *L. helveticus*, *L. crispatus*, *L. amylovorus*, *L. gallinarum*, *L. delbrueckii* subsp. *bulgaricus*, *L. gasseri*, and *L. johnsonii*. While these bacteria are closely related, they have varied ecological lifestyles as dairy and food fermenters, allochthonous probiotics, or autochthonous commensals of the host gastrointestinal tract. Bacterial cell surface components play a critical role in the molecular dialogue between bacteria and interaction signaling with the intestinal mucosa. Notably, the *L. acidophilus* complex is distinguished in two clades by the presence or absence of S-layers, which are semiporous crystalline arrays of self-assembling proteinaceous subunits found as the outermost layer of the bacterial cell wall. In this study, S-layer-associated proteins (SLAPs) in the exoproteomes of various S-layer-forming *Lactobacillus* species were proteomically identified, genomically compared, and transcriptionally analyzed. Four gene regions encoding six putative SLAPs were conserved in the S-layer-forming *Lactobacillus* species but not identified in the extracts of the closely related progenitor, *L. delbrueckii* subsp. *bulgaricus*, which does not produce an S-layer. Therefore, the presence or absence of an S-layer has a clear impact on the exoproteomic composition of *Lactobacillus* species. This proteomic complexity and differences in the cell surface properties between S-layer- and non-S-layer-forming lactobacilli reveal the potential for SLAPs to mediate intimate probiotic interactions and signaling with the host intestinal mucosa.**

Bacterial cell surface proteins play a critical role in the molecular dialogue between bacteria and their interaction with the host. For beneficial microbes, such as probiotics, these proteins mediate health-promoting functions through gastrointestinal adhesion, competitive exclusion of pathogens, enhancement of intestinal barrier function, and activation of gut mucosal immunity (1, 2). Probiotics are defined by the FAO/WHO as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (3). Some beneficial actions of these organisms are strain specific and can be harnessed to treat or reduce the risk of multiple maladies, including acute infectious diarrhea, irritable bowel syndrome, vaginal infections, ulcerative colitis, lactose maldigestion, and necrotizing enterocolitis (4). In fact, the efficacy of probiotic treatment depends largely on the various cell surface components that mediate this specificity (5). Therefore, the characterization of effector cell surface ligands and their health-promoting interactions with the host is of increasing scientific and medical interest.

Some of the most prevalent and well-studied probiotics are lactobacilli, many of which are members of the *Lactobacillus acidophilus* homology group (6). The *L. acidophilus* group is a clade of homologous Gram-positive *Lactobacillus* species that includes *L. acidophilus*, *L. helveticus*, *L. crispatus*, *L. amylovorus*, *L. gallinarum*, *L. delbrueckii* subsp. *bulgaricus*, *L. gasseri*, and *L. johnsonii* (7–11). Although these bacteria are closely related phylogenetically, they have varied ecological lifestyles ranging from dairy and food fermentations to allochthonous probiotics or autochthonous commensals of the host gastrointestinal and urogenital tracts. Biochemically, they are obligately homofermentative; they almost exclusively ferment sugar (>85%) to lactate via the Embden-Meyerhof-Parnas pathway. Early taxonomic descriptions were based on the metabolic end products of fermentation, resulting in a seemingly indistinguishable group of microbes, which were all called *L. acidophilus* (10). However, DNA-DNA hybridization studies revealed the heterogeneity in the group (11, 12). Since then, genome sequencing and comparative genomic analyses have clearly established and solidified the current description of the *L. acidophilus* group (13, 14). Notably, these closely related strains can be dichotomized based on their ability to create surface (S)-layer protein arrays as the outermost constituent of the cell wall (15).

Bacterial S-layers are semiporous proteinaceous crystalline arrays composed of self-assembling (glyco)protein subunits called S-layer proteins (SLPs) (15). They can be found in both Gram-positive and Gram-negative bacteria and species of *Archaea* but are not ubiquitous in all microorganisms. When present, S-layers form two-dimensional lattices on the outermost layer of the cell, which are tethered through noncovalent interactions with the cell wall (15). S-layers from various species of the *L. acidophilus* homology group have been characterized for their roles in intestinal adhesion, competitive exclusion of pathogens, and immunomodulation of the gastrointestinal mucosa. *In vitro* studies using

**Received** 11 June 2015 **Accepted** 13 October 2015

**Accepted manuscript posted online** 16 October 2015

**Citation** Johnson BR, Hymes J, Sanozky-Dawes R, Henriksen ED, Barrangou R, Klaenhammer TR. 2016. Conserved S-layer-associated proteins revealed by exoproteomic survey of S-layer-forming lactobacilli. Appl Environ Microbiol 82:134–145. doi:10.1128/AEM.01968-15.

**Editor:** H. Nojiri

Address correspondence to Todd R. Klaenhammer, klaenhammer@ncsu.edu.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01968-15.

TABLE 1 Strains used in this study

| Organism (strain)[a] | Study designation | Source[b] | Origin | S-layer | Reference |
|---|---|---|---|---|---|
| *L. acidophilus* (NCFM) | NCK56 | | Human intestinal isolate | + | 47 |
| *L. helveticus* (1846) | NCK230 | NCDO | Dairy isolate | + | 48 |
| ***L. helveticus*** (481-C) | NCK246 | NCDO | Dairy isolate | + | 49 |
| *L. helveticus* | NCK338 | NCDO | Dairy isolate | + | 50 |
| ***L. helveticus*** (CNRZ32) | NCK936 | CNRZ | Industrial cheese starter culture | + | 51 |
| ***L. helveticus*** (ATCC 15009) | NCK1088 | ATCC | Dairy isolate | + | 52 |
| ***L. crispatus*** (ATCC 33820) | NCK777 | ATCC | Human isolate | + | 53, 54 |
| ***L. crispatus*** | NCK953 | | Chicken isolate | + | |
| ***L. crispatus*** (CZ6) | NCK1351 | | Human endoscopy isolate | + | 55 |
| ***L. amylovorus*** (ATCC 33620) | NCK776 | ATCC | Cattle feces | + | 56, 57 |
| *L. gallinarum* (ATCC 33199) | NCK778 | ATCC | Chicken isolate | + | 58 |
| *L. gallinarum* | NCK1560 | | Chicken isolate | + | |
| ***L. delbrueckii* subsp. *bulgaricus*** | NCK1561 | | Dairy isolate | − | |
| *L. gasseri* (ATCC 33323) | NCK334 | ATCC | Human isolate | − | 59 |
| *L. johnsonii* (ATCC 33200) | NCK779 | ATCC | Human isolate | − | 58 |
| *L. reuteri* (ATCC 23272)[c] | NCK702 | ATCC | Human feces | − | 11 |
| ***L. casei*** (ATCC 393)[c] | NCK125 | ATCC | Dairy isolate | − | 60 |

[a] Proteins from organisms indicated in bold were proteomically identified using LC-MS/MS.
[b] NCDO, National Collection of Dairy Organisms; ATCC, American Type Culture Collection; CNRZ, Centre National de Recherches Zootechniques.
[c] Species outside the *L. acidophilus* homology group.

intestinal epithelial cell lines suggest that the S-layer is a major factor in intestinal adhesion for *L. acidophilus* (16, 17), *L. crispatus* (18–20), *L. helveticus* (21), and *L. amylovorus* (22). In fact, this adhesion has been shown to competitively exclude enteropathogenic bacteria by both *L. crispatus* (23) and *L. helveticus* (24, 25). Compelling studies have begun to reveal the mechanisms of gastrointestinal immunomodulation. For example, SlpA, the primary constituent of the S-layer in *L. acidophilus* NCFM, was found to bind to dendritic cell (DC) orthologous C-type lectin receptors (CLR), DC-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN) (26), and a specific intracellular adhesion molecule-3-grabbing nonintegrin homolog-related 3 (SIGNR-3) (27). This SlpA-CLR interaction exerts regulatory signals, which have been reported to mitigate inflammatory disease states and promote the maintenance of healthy intestinal barrier function (27). Similar experiments have aimed to elucidate the roles of the S-layer in modulating gastrointestinal immunity for *L. crispatus* (28), *L. helveticus* (29), and *L. amylovorus* (22).

The S-layer-forming species of the *L. acidophilus* homology group form S-layers composed of a dominant protein constituent, SlpA/Slp1 (~46 kDa), and the minor constituents SlpB/Slp2 (~47 kDa) and SlpX (~51 kDa) (30). Recent evidence, however, suggests that the S-layer may not be as monomorphic as previously proposed. In *L. acidophilus* NCFM, proteomic analysis revealed the presence of 37 noncovalently bound extracellular S-layer-associated proteins (SLAPs), 23 of which are putative/uncharacterized proteins of unknown function (31). In this study, the noncovalent exoproteomes of various S-layer- and non-S-layer-forming *Lactobacillus* strains were proteomically identified, genomically compared, and transcriptionally analyzed. These data reveal both the conservation and variability of SLAPs across lactobacilli and their potential to mediate intimate interactions with the intestinal mucosa.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains used in this study are reported in Table 1. *Lactobacillus* strains were propagated statically at 37°C under ambient atmospheric conditions in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Inc., Detroit, MI).

**DiversiLab analysis of strains.** *L. crispatus* and *L. helveticus* strains were typed using the repetitive extragenic palindromic-PCR (Rep-PCR)-based DiversiLab typing system (bioMérieux, Durham, NC). DNA from the *Lactobacillus* strains was extracted using a Mo Bio UltraClean microbial DNA isolation kit (Mo Bio, Carlsbad, CA) and quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). The DNA was then normalized to 20 ng $\mu l^{-1}$ with UltraPure distilled water (Invitrogen, Carlsbad, CA). Rep-PCR was performed in preparation for typing using the *Lactobacillus* DiversiLab kit (bioMérieux). DNA amplification was performed in a Bio-Rad MyCycler thermal cycler (Bio-Rad, Hercules, CA), programmed for 2 min at 94°C (initial denaturation) and 35 cycles of 30 s at 94°C (denaturation), 30 s at 55°C (annealing), and 90 s at 70°C (extension), followed by a final extension cycle of 3 min at 70°C using AmpliTaq DNA polymerase from Applied Biosystems (Carlsbad, CA). The reaction mixture was pipetted into the DiversiLab system chip along with the DiversiLab DNA reagents and supplies (bioMérieux), according to the manufacturer's protocol. The chip samples were analyzed using the DiversiLab software version 3.4, and the similarity of the strains was determined by comparing the resulting electropherogram/bar codes.

**Extraction of extracellular noncovalently bound cell surface proteins.** Noncovalently bound cell surface proteins, including S-layer proteins and S-layer-associated proteins, were extracted from the *Lactobacillus* strains using LiCl denaturing salt, as described previously (31). Briefly, cells were grown in 200 ml of MRS broth to stationary phase (16 h), centrifuged at 2,236 × *g* for 10 min (4°C), and washed twice with 25 ml of cold phosphate-buffered saline (PBS) (Gibco) (pH 7.4). The cells were agitated for 15 min at 4°C following the addition of 5 M LiCl (Fisher Scientific). Supernatants containing SLPs and SLAPs were harvested via centrifugation at 8,994 × *g* for 10 min (4°C), transferred to a 6,000- to 8,000-kDa Spectra/Por molecular porous membrane (Spectrum Laboratories), and dialyzed against cold distilled water for 24 h. The precipitate was harvested at 20,000 × *g* for 30 min and agitated for a second time with 1 M LiCl at 4°C for 15 min to disassociate the SLAPs from the SLPs. The suspension was then centrifuged at 20,000 × *g* for 10 min, and the SLAP supernatants were separated from the SLP pellet, transferred to the 6,000- to 8,000-kDa Spectra/Por molecular porous membrane, and dialyzed against cold distilled water for 24 h. Finally, the precipitate was harvested

via centrifugation at 20,000 × *g* for 30 min to pellet the SLAPs. Both SLP and SLAP pellets were resuspended in 10% (wt/vol) SDS (Fisher). Proteins were quantified via a bicinchoninic acid assay kit (Thermo Scientific) and visualized via SDS-PAGE using precast 4% to 20% Precise Tris-HEPES protein gels (Thermo Scientific). The gels were stained using AquaStain (Bulldog Bio), according to the manufacturer's instructions. SLAP extractions were performed with two biological replicates for each strain and visualized through SDS-PAGE to confirm that the resultant banding patterns were reproducible.

**Proteomic identification and analysis.** SLAPs extracted from the various *Lactobacillus* species were identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) from the Genome Center Proteomics Core at the University of California, Davis, CA, as described previously (31). Proteomic screenings were performed once per strain and used as a tool for selecting candidate SLAPs within each strain. Tandem mass spectra were extracted and the charge state deconvoluted using MM File Conversion version 3. All MS/MS samples were analyzed using X! Tandem (Tornado version; The GPM [www.thegpm.org/]). UniProt searches were performed using proteome databases for the respective proteins isolated from *L. acidophilus* NCFM, *L. helveticus* CNRZ32, *L. crispatus* ST1, and *L. amylovorus* GRL1112. X! Tandem was searched with a fragment ion mass tolerance and parent ion tolerance of 20 ppm. The iodoacetamide derivative of cysteine was specified in X! Tandem as a fixed modification. The deamination of asparagine and glutamine, oxidation of methionine and tryptophan, sulfonation of methionine, tryptophan oxidation to formylkynurenine of tryptophan, and acetylation of the N terminus were specified in X! Tandem as variable modifications. Scaffold (version Scaffold_3.6.1; Proteome Software) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. X! Tandem identifications required scores of >1.2 with a mass accuracy of 5 ppm. Protein identifications were accepted if they contained at least two identified peptides. Using the parameters described above, the false-discovery rate was calculated to be 1.1% at the protein level and 0% at the peptide level. Proteins that contained similar peptides and that could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. For this study, only proteins with unique spectral counts of >20 were considered significant. For all analyses, total spectral counts were utilized as a semiquantitative indicator of protein abundance (32). Two-way clustering of total spectral counts was performed using JMP Genomics (version 5; SAS). Protein domains were identified for analysis using the Pfam protein family database (33).

**Genomic *in silico* analyses.** Genomic analysis was performed on genomes curated from the genome library of the National Center for Biotechnology Information (NCBI [http://www.ncbi.nlm.nih.gov/genome/]), including *L. acidophilus* NCFM (GenBank accession no. NC_006814.3), *L. helveticus* CNRZ32 (GenBank accession no. NC_021744.1), *L. amylovorus* GRL1112 (GenBank accession no. NC_014724.1), *L. crispatus* ST1 (GenBank accession no. NC_014106.1), *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (GenBank accession no. NC_008054.1), and *L. casei* ATCC 334 (GenBank accession no. NC_008526.1). Identified genes were compared using the BLASTn and BLASTp features of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). SignalP 4.1 was used to predict the signal peptidase cleavage site of each identified protein (34). Genomes were uploaded to Geneious 8.0.5 (35) for comparative genomic and promoter analyses of the identified SLAP genes. The genetic context of SLAP genes was examined using the chromosomal graphical interface in Geneious 8.0.5. *In silico* promoter elements were identified in the upstream intergenic regions of SLAP genes using PromoterWise (http://www.ebi.ac.uk/Tools/psa/promoterwise/). To identify conserved promoter elements between the various SLAP genes, genome-wide sequence motifs of the putative −10 and −35 regions were scanned against the four S layer-forming genomes using Geneious 8.0.5, with a variable spacer length of 16 to 23 nucleotides (nt) between the −10 and −35 regions.

**RNA extraction, sequencing, and transcriptional analysis.** Cells were grown to mid-log phase (8 h) and flash-frozen for RNA extraction and sequencing. RNA was extracted using the Zymo Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA) and analyzed for quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Library preparation and RNA sequencing were performed at the High-Throughput Sequencing and Genotyping Unit of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign, IL. For each sample, rRNA was removed with the Ribo-Zero bacterial kit (Illumina, San Diego, CA), followed by library preparation with the TruSeq stranded RNA sample preparation kit (Illumina). Single-read RNA sequencing was performed using an Illumina HiSeq 2500 ultrahigh-throughput sequencing system) with a read length of 180 nt. Raw sequencing reads were assessed for quality using FastQC version 0.11.3 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and processed using Geneious 8.0.5 (35). Briefly, after the adaptor sequences were trimmed, the raw reads were quality trimmed to remove sequence reads with an error probability limit of 0.001 (Phred score, 30) and filtered to remove reads <20 nt. These quality trimmed and filtered sequences were then mapped to the reference genomes of the S-layer-forming *Lactobacillus* spp. using Bowtie 2 (36), with default settings within Geneious 8.0.5 (35). The sequencing coverage depths were calculated to be 767×, 730×, 727×, and 665× for *L. acidophilus* NCFM strain NCK56, *L. amylovorus* ATCC 33620 strain NCK776, *L. crispatus* ATCC 33820 strain NCK777, and *L. helveticus* CNRZ32 strain NCK938, respectively. Transcriptional analyses were based on the normalized transcripts per million (TPM) calculation within Geneious 8.0.5 (35).

## RESULTS

**Proteomic identification of noncovalently bound extracellular proteins in S-layer- and non-S-layer-forming lactobacilli.** Based on the previous identification of S-layer-associated proteins (SLAPs) in *L. acidophilus* NCFM (31), we performed exoproteome screenings on multiple S-layer- and non-S-layer-forming strains of *Lactobacillus*. Thus, five S-layer- and five non-S-layer-forming *Lactobacillus* species were analyzed (Fig. 1). Seventeen strains were tested in total, comprising 12 S-layer- and 5 non-S-layer-producing lactobacilli (Table 1). Notably, 15 of the strains are members of the closely related *L. acidophilus* homology group.

Electrophoresis of SLAP extractions revealed a surprisingly diverse array of protein banding patterns in the S-layer-forming species and a notable absence of proteins in the non-S-layer-forming species (Fig. 2). SLAP extractions were performed on two biological replicates, and the SDS-PAGE banding patterns of the SLAPs extracted from each strain did not differ in the major banding patterns between replicates. Further, the LiCl extract of *L. acidophilus* demonstrated a banding profile similar to that of the SLAPs identified previously (28) (Fig. 2, lane 1). Proteins from the other S-layer-forming strains, including *L. crispatus*, *L. amylovorus*, *L. gallinarum*, and *L. helveticus*, were not only distinct from *L. acidophilus* but also from one another. Moreover, there was also heterogeneity in the protein banding between various strains within each species. In the five *L. helveticus* strains, there were distinctive differences between the various dairy isolates NCK936, NCK338, NCK230, NCK246, and NCK1088 (Fig. 2A, lanes 2 and 6 to 9). The three *L. crispatus* strains were also discrete from one another (Fig. 2A, lanes 3, 10, and 11). Rep-PCR-based DiversiLab strain typing was performed on the five *L. helveticus* and three *L. crispatus* strains to examine genomic similarities (Fig. 2B and C). The five *L. helveticus* strains clustered into two groups with >93% and >98% similarity (Fig. 2B), and the *L. crispatus* strains were >85% similar (Fig. 2C). Remarkably, the *L. helveticus* strains

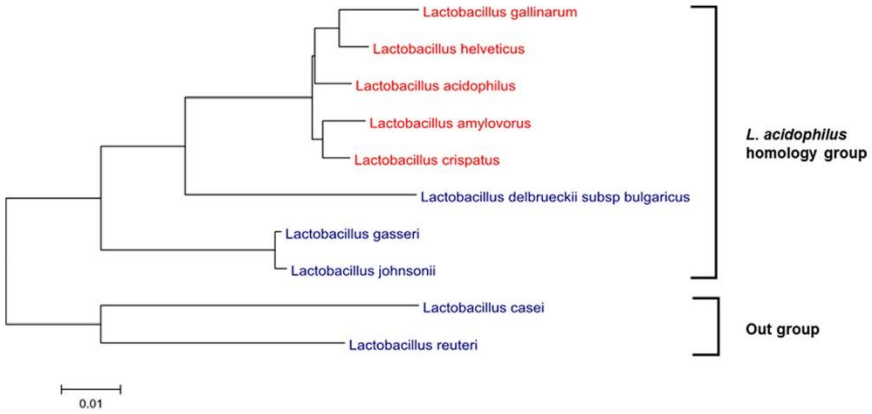


**FIG 1** 16S rRNA dendrogram of the S-layer-forming (red) and non-S-layer-forming (blue) species of the *L. acidophilus* homology group. The tree is rooted by the non-S-layer-forming species *L. casei* and *L. reuteri*, which are not members of the *L. acidophilus* homology group.

NCK338 and NCK230, and NCK1088 and NCK936, distinctly varied in terms of the isolated extracellular proteins (Fig. 2A) despite >98% and >95% similarity between the Rep-PCR typing patterns (Fig. 2B). A similar trend was observed among the *L. crispatus* strains. Thus, there was no correlation between the genotype clustering and the exoproteome profiles revealed by SDS-PAGE.

There were very few proteins isolated from the non-S-layer-forming species of *Lactobacillus*, as observed in the gel lanes of the SDS-PAGE (Fig. 2, lanes 13 to 17). *L. johnsonii* and *L. gasseri* of the *L. acidophilus* homology group exhibited no discernible proteins in the gel lanes (Fig. 2, lanes 15 and 17). *L. delbrueckii* subsp. *bulgaricus*, the non-S-layer-producing strain, which is the most closely related and progenitor to the other S-layer-forming members of the *L. acidophilus* homology group (Fig. 1), showed only a small number of proteins isolated from the LiCl extract (Fig. 2, lane 13). Distantly related *L. casei*, devoid of any S-layer, also exhibited few proteins (Fig. 2, lane 14). To identify the electrophoresed proteins, lanes with visible proteins in the gel were sent for proteomic identification (Table 1, in bold).

Of the 12 S-layer-forming strains, seven were selected for proteomic identification, including three *L. helveticus* strains, three *L. crispatus* strains, and one *L. amylovorus* strain (Table 1, underlined). Notably, *L. gallinarum* was not selected for analysis, as there are no publically available genomes or proteomes published for this species to date. From the five non-S-layer-forming species tested, only *L. delbrueckii* subsp. *bulgaricus* and *L. casei* were selected from proteomic screening, as they were the only non-S-layer-forming species in which proteins were isolated from the

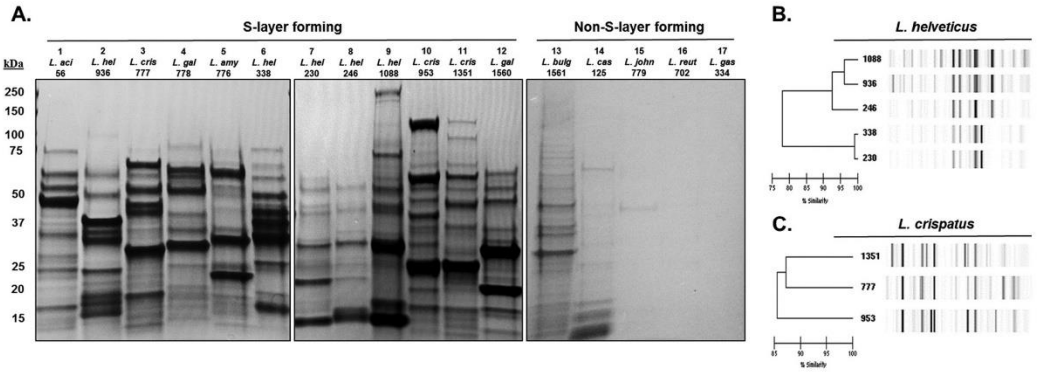


**FIG 2** Noncovalently bound exoproteomes were extracted using LiCl and electrophoresed on SDS-PAGE gels. These gels are representative of protein extractions from two biological replicates of each strain. (A) The S layer forming strains of the *L. acidophilus* (*L. aci*) homology group presented a diverse array of proteins in the LiCl extracts, including many anticipated S-layer-associated proteins (SLAPs). In contrast, the non-S-layer-forming species harbored very few proteins in the cell surface extracts. Five strains of *L. helveticus* (*L. hel*) (B) and three strains of *L. crispatus* (*L. cris*) (C) were typed using the Rep-PCR-based DiversiLab typing system. *L. gal*, *L. gallinarum*; *L. amy*, *L. amylovorus*; *L. bulg*, *L. delbrueckii* subsp. *bulgaricus*; *L. cas*, *L. casei*; *L. john*, *L. johnsonii*; *L. reut*, *L. reuteri*; *L. gas*, *L. gasseri*.

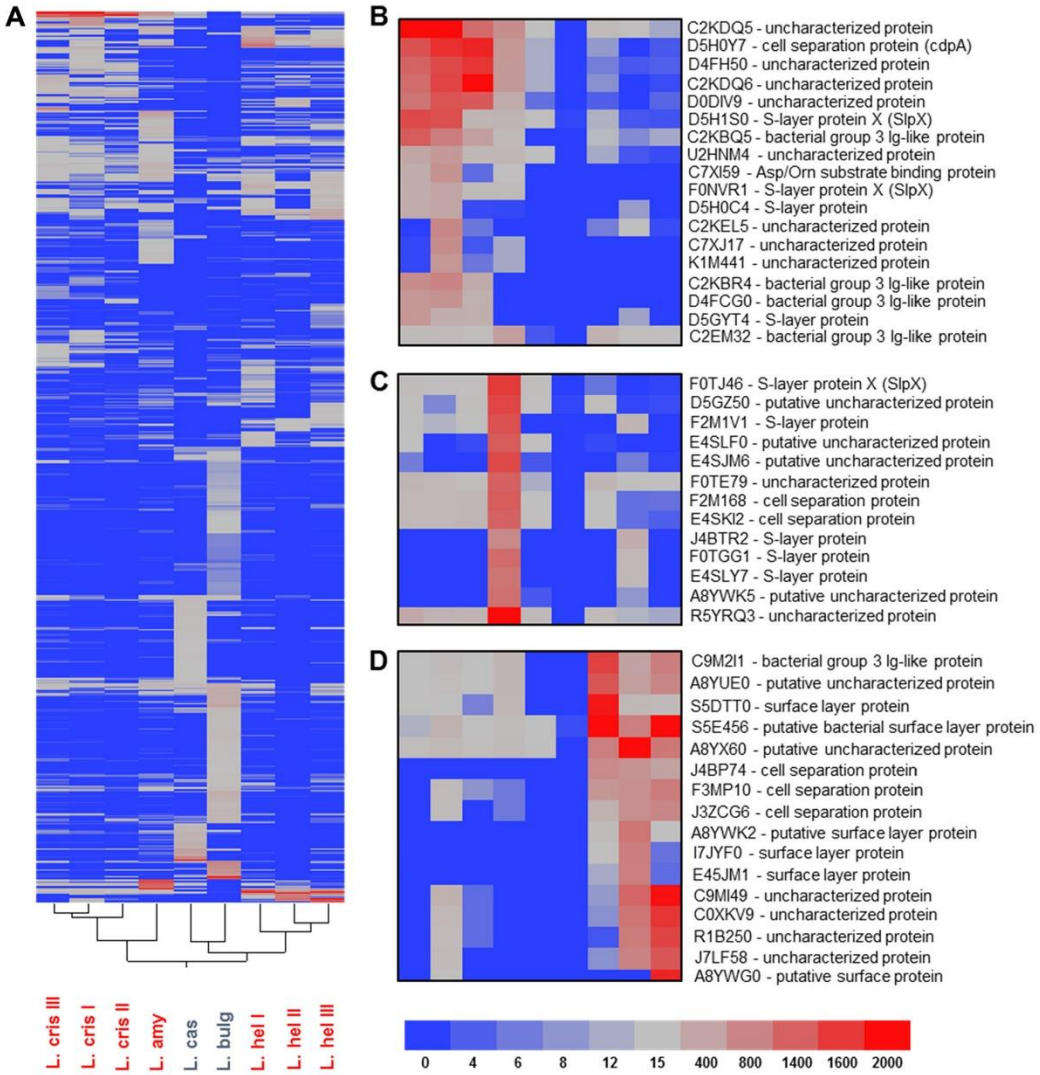


**FIG 3** (A) A total of 2,929 proteins were identified from the S-layer-forming strains (red) of *L. crispatus*, *L. amylovorus*, and *L. helveticus* and the non-S-layer-forming strains (blue) of *L. delbrueckii* subsp. *bulgaricus* and *L. casei*. Two-way clustering was performed on the identified proteins based on their similarity between strains and visualized using a red blue heat map. The colors in the heat map represent the spectral counts of the identified proteins (semiquantitative measure of protein abundance), with red being the most present (400 to 1,000 total spectral counts), gray being somewhat present (12 to 400 total spectral counts), and blue being low or no presence (0 to 12 total spectral counts). Regarding the S-layer-forming strains, there were three main clusters of proteins: SLAPs specific to *L. crispatus* (B), *L. amylovorus* (C), and *L. helveticus* (D). These three clusters have been noted with the corresponding UniProt and protein annotations of the identified proteins.

SLAP extraction (Table 1, underlined). Proteins were identified from the LiCl extracts of the seven S-layer- and two non-S-layer-forming *Lactobacillus* species using liquid chromatography-tandem mass spectrometry (see Table S1 in the supplemental material). Two-way clustering was performed based on the total spectral counts of identified proteins and visualized using a two-way clustering heat map (Fig. 3A). The proteins identified in the two non-S-layer-forming strains, *L. casei* and *L. delbrueckii* subsp.

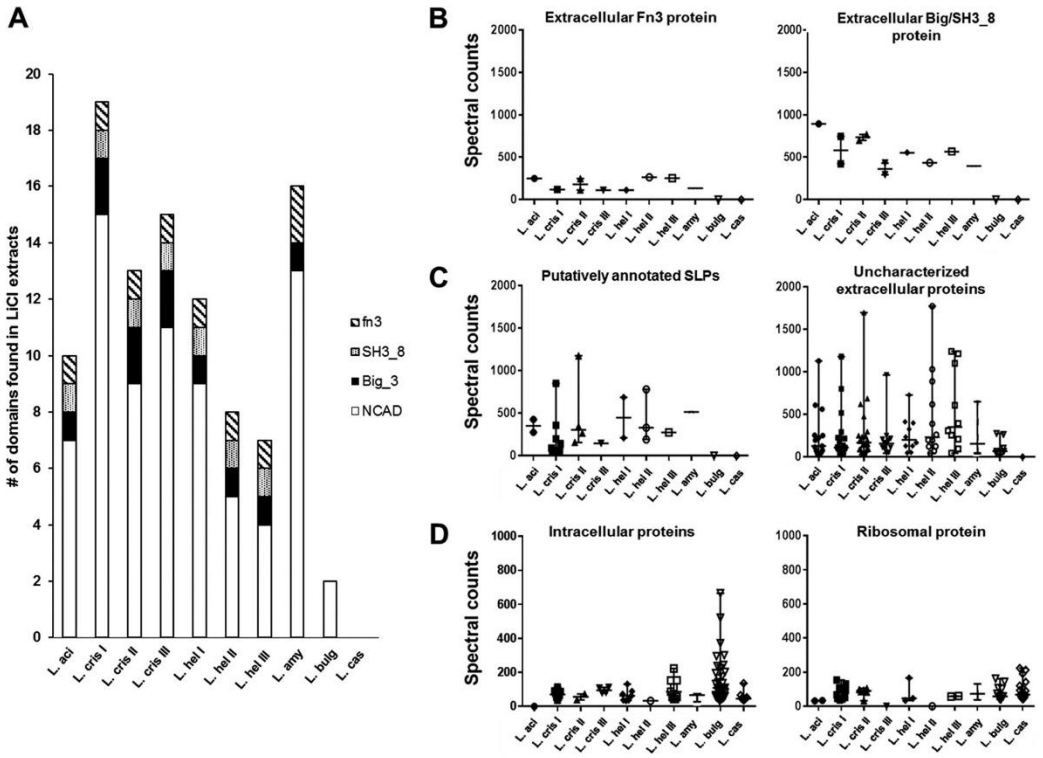


**FIG 4** (A) Four protein domains found consistently in the proteins identified within the LiCl extracts: NCAD (white), BIg_3 (black), SH3_8 (dots), and fn3 (diagonal lines). Dot plots were created using the semiquantitative total spectral counts from the identified proteins of each strain. Plotted are the extracellular Fn3 proteins and extracellular BIg_3/SH3_8 proteins (B), putative annotated S-layer proteins and uncharacterized extracellular proteins (C), and intracellular and ribosomal proteins (D). The proteins in panel C contain the NCAD, while the proteins in panel D do not.

*bulgaricus*, are unambiguously distinct from the other seven S-layer-forming strains. Furthermore, almost all of the proteins identified in the non-S-layer-forming strains were predicted intracellular proteins, likely presented extracellularly as the result of cell death occurring at stationary phase. With regard to the S-layer-forming *Lactobacillus* species, there were three main groupings of proteins identified: SLAPs specific to *L. crispatus* (Fig. 3B), SLAPs specific to *L. amylovorus* (Fig. 3C), and SLAPs specific to *L. helveticus* (Fig. 3D). Surprisingly, although each group had distinctive homologies, the same types of proteins were observed in each group. In fact, these proteins, which included multiple putative uncharacterized proteins, cell surface proteases, and group 3 bacterial Ig-like domain proteins, were the same types of proteins identified as SLAPs in *L. acidophilus* NCFM (see Table S2 in the supplemental material). Notably, these putative SLAPs were not found in the non-S-layer-producing strains analyzed, which were *L. casei* and *L. delbrueckii* subsp. *bulgaricus*.

**Functional exoproteomic analysis of S-layer- and non-S-layer-forming lactobacilli.** After proteomic identification, selected putative SLAPs and noncovalently bound extracellular proteins were functionally analyzed based on predicted protein domains. Four predominant protein domains were found consistently in the S-layer-forming species tested (Fig. 4A), including SLAP (PF03217), Big_3 (PF07523), SH3_8 (PF13457), and fn3 (PF00041). We propose that the SLAP (PF03217) domain, responsible for the noncovalent attachment of SLP and other extracellular proteins in lactobacilli, be redesignated the noncovalent attachment domain (NCAD). This domain designation prevents confusion with the abbreviation for S-layer-associated proteins, SLAPs. Notably, the NCAD was the most abundant protein domain identified in the extracellular fractions tested (Fig. 4A). Other domains associated with bacterial extracellular proteins, including group 3 bacterial Ig-like domains (Big_3), SH3-like domains (SH3_8), and fibronectin type III domains (fn3), were found in the proteomic analysis of the S-layer-forming species but were absent from the non-S-layer-forming species (Fig. 4A). Notably, only two NCAD-containing proteins were identified within the exoproteome of *L. delbrueckii* subsp. *bulgaricus*, while none of these domains were identified in the exoproteome of the non-S-layer-forming *L. casei*.

Identified proteins were functionally categorized based on pu-

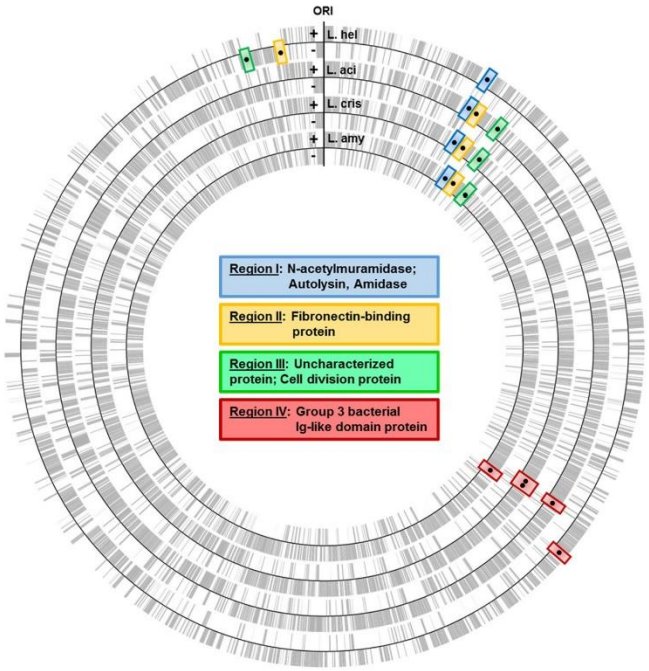


**FIG 5** All open reading frames (ORFs) from the positive (+) and negative (−) strands of *L. helveticus* CNRZ32, *L. acidophilus* NCFM, *L. crispatus* ST1, and *L. amylovorus* GRL1112 were mapped onto circular chromosomes with an annotated origin of replication (Ori). Four conserved SLAP gene regions were identified based on position between strains. Blue, region I; yellow, region II; green, region III; red, region IV.

tative domains and placed into one of six groupings: extracellular fn3 domain proteins and extracellular BIg3/SH3_8 proteins (Fig. 4B), putatively annotated SLPs and uncharacterized extracellular proteins (Fig. 4C), and intracellular proteins and ribosomal proteins (Fig. 4D). The distribution of the proteins within these functional groupings was plotted for each of the strains using the semiquantitative total spectral counts identified through the LC-MS/MS survey (Fig. 4B to D). Group 3 bacterial Ig-like domain proteins, which contain the Big_3 and SH3_8 domains, were only found in the SLAP fractions of the S-layer-forming lactobacilli (Fig. 4B). Similarly, uncharacterized proteins putatively annotated as SLPs and fibronectin-binding proteins were found solely in the S-layer-forming species of *Lactobacillus* (Fig. 4B and C). There was an increase in both the occurrence and abundance of NCAD-containing uncharacterized extracellular proteins in the SLAP fractions from the S-layer strains compared to the non-S-layer strains (Fig. 4C). Furthermore, there was an increase in the presence of intracellular proteins, including ribosomal proteins, in the non-S-layer strains (Fig. 4D), as measured by total spectral counts. These data reveal a pattern of noncovalently bound proteins identified in S-layer species of *Lactobacillus* compared to non-S-layer-forming lactobacilli.

**Genomic characterization of genes corresponding to the extracellular S-layer-associated proteins.** The putative SLAPs identified in this study, along with the previously identified SLAPs of *L. acidophilus* NCFM, were curated to the genomes of *L. acidophilus* NCFM, *L. helveticus* CNRZ32, *L. amylovorus* GRL 1112, and *L. crispatus* ST1 (see Table S2 in the supplemental material). By visualizing the corresponding genes on the four genomes, four conserved genetic regions containing six genes were consistently observed (Fig. 5). Two cell division-related genes, including an *N*-acetylmuramidase and autolysin, are found in region I. Region II is composed of genes encoding fn3 domain-containing fibronectin-binding proteins. Region III also contains two cell division-related genes, including the gene encoding cell division protein A (*cdpA*) (33). Finally, region IV includes genes encoding group 3 bacterial Ig-like proteins, which contain the domains Big_3 and SH3_8. The relative positions of the four gene regions were conserved among the four genomes, with the exception of regions II and III in *L. helveticus*, which were translocated to the minus strand leading away from the origin of replication (Fig. 5).

In addition, the genetic context of each region was examined within the four strains. Notably, there was synteny observed between the four chromosomal regions of each organism (Fig. 6). Although region I was the least syntenic overall, it is noteworthy that the *N*-acetylmuramidase and autolysin/amidase genes were positioned directly downstream of the genes encoding the primary S-layer protein, *slpA* and *slpB*. Conversely, region II exhibited increased conservation of genetic loci near the SLAP gene encoding a fibronectin-binding protein, including genes for a

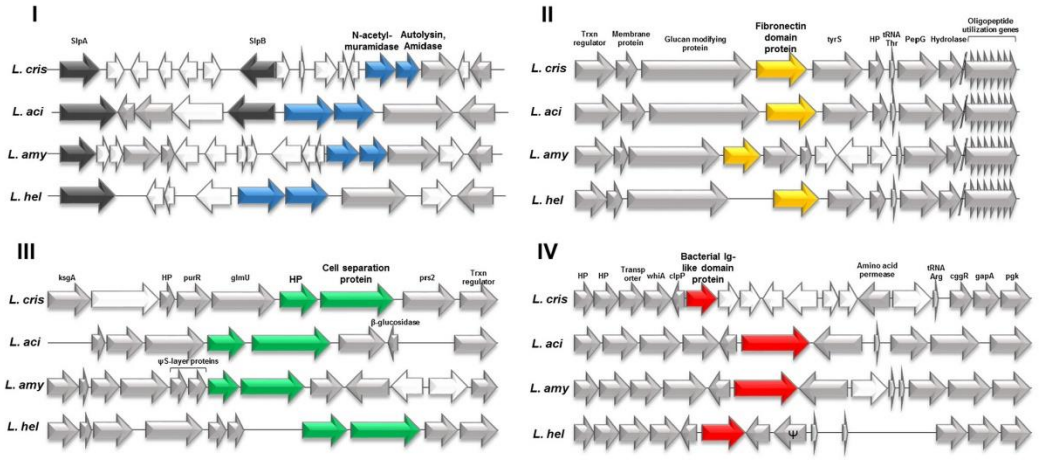


**FIG 6** Genomic context of the five SLAP gene regions among the four strains of *Lactobacillus*: *L. crispatus* ST1, *L. acidophilus* NCFM, *L. amylovorus* GRL1112 and *L. helveticus* CNRZ32. Arrows represent genes. Gray arrows represent conserved synteny between the four strains, while white arrows represent divergence. Colored arrows represent the SLAP gene regions as follows: blue, region I; yellow, region II; green, region III; red, region IV. HP, hypothetical protein; Trxn, transcriptional.

high-molecular-weight glucan-modifying protein, a tyrosine-tRNA synthetase, and an oligopeptide utilization gene cluster. Region III was also syntenic surrounding the putative SLAP genes, with genes encoding the *pur* operon repressor gene *purR* and the cell division gene *glmU*. Last, region IV containing the gene encoding the putative SLAP with a group 3 bacterial Ig-like domain was directly downstream of the endopeptidase gene, *clpP*, and upstream of the glycolysis genes *gapA* and *pgk*.

**RNA sequencing and transcriptional analysis of the S-layer-forming *Lactobacillus* species.** Whole-transcriptome profiling through deep RNA sequencing (RNA-seq) was employed to examine the global expression of the putative SLAP gene regions in *L. acidophilus*, *L. helveticus*, *L. crispatus*, and *L. amylovorus*. While expression was similar between the four strains in each gene region (Fig. 7, bar graphs), the gene regions were themselves expressed at different levels (Fig. 7, line graphs). Both regions I and II had expression levels between 100 and 500 TPM, while regions III and IV had expression levels of >1,000 TPM (Fig. 7). These data also confirmed the monocistronic expression of region IV and the predicted polycistronic expression of the *N*-acetylmuramidase and autolysin of region I. Conversely, the cell division genes in region III appeared to be monocistronically expressed. Surprisingly, the gene encoding a fibronectin-binding protein of region II was found to be polycistronically expressed, along with a tyrosyl-tRNA synthetase gene, *tyrS*. Finally, *in silico* promoter identification and analysis suggested that the *N*-acetylmuramidase gene and the group 3 bacterial Ig-like domain gene were under the constitutive transcriptional control of a putative $\sigma^{70}$ (*rpoD*)-like promoter with a TANAAT −10 region consensus motif and an NTGTNT −35 region consensus motif (see Fig. S1 in the supplemental material). This promoter was found upstream of numerous housekeeping genes, including *ftsA*, *ldhD*, *secA*, and *eno* (see Fig. S1 in the supplemental material).

## DISCUSSION

Previous work has shown that the S-layers are more complex than previously understood. SLAPs were first identified in *L. acidophilus* NCFM and were hypothesized to scaffold to the cell wall with the S-layer (31). Additionally, a recent proteomic cell-shaving study in the S-layer-forming food bacterium *Propionibacterium freudenreichii* characterized various cell surface proteins, including putative SLAPs, for their anti-inflammatory immunomodulatory capacity (37). In the present study, we demonstrate that the presence or absence of an S-layer has a clear and direct impact on the exoproteomic composition of *Lactobacillus* species (Fig. 2). In S-layer-forming species of the *L. acidophilus* homology group, numerous noncovalently bound proteins were identified, which may be associated with the S-layer. In contrast, the few proteins that were isolated with LiCl treatment in the non-S-layer-forming strains were mostly intracellular proteins. These observations substantiate the aforementioned studies, lending credence to the existence of SLAPs as an integral component of the complex S-layer.

There were four protein domains found consistently within the putative SLAPs: BIg_3 (PF07523), SH3_8 (PF13457), fn3 (PF00041), and NCAD (PF03217). NCAD are predicted to be responsible for the noncovalent attachment of S-layer proteins to the cell wall in *Lactobacillus* species (38). Notably, there are extracellular proteins within the annotated proteome of *L. delbrueckii* subsp. *bulgaricus* that contain the NCAD. Similarly, the fn3 domain, an Ig-fold domain found in fibronectin-binding proteins, was also within the predicted proteomes of the non-S-layer-forming species *L. gasseri* and *L. johnsonii*. In both of these examples, the domains were ubiquitously identified in the noncovalently bound exoproteome fractions of the S-layer-forming strains but were not apparent in the exoproteomes extracted from the non-S-layer-forming

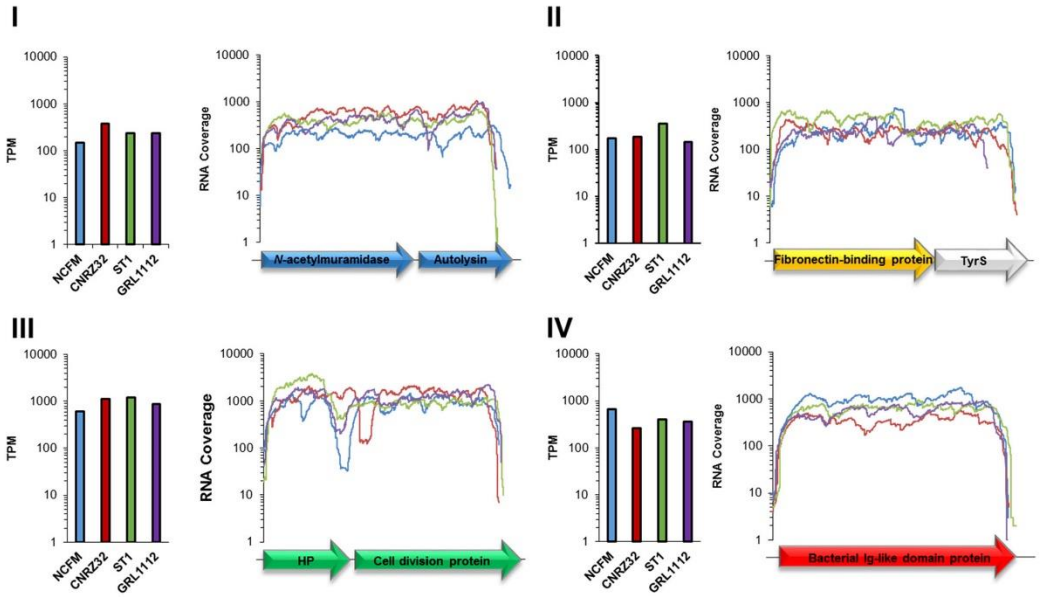


FIG 7 Transcription levels of the four conserved SLAP genomic regions were measured through RNA sequencing. (I to IV) Illustrated expression shown in each region: blue, region I; yellow, region II; green, region III; red, region IV. The bar graphs for each panel present the normalized TPM, while the line graphs present RNA coverage across each gene from the SLAP regions in *L. acidophilus* NCFM (light blue), *L. helveticus* CNRZ32 (dark red), *L. crispatus* ST1 (light green), and *L. amylovorus* GRL1112 (purple).

strains. These observations suggest that the S-layer may be an important scaffold for extracellular proteins with NCAD.

From the numerous putative SLAPs, six were found to be conserved among the four S-layer-forming strains, *L. acidophilus*, *L. crispatus*, *L. amylovorus*, and *L. helveticus*, into four genomic regions. These four genomic regions include genes encoding the cell division protein CdpA, an *N*-acetylmuramidase, an uncharacterized fibronectin-binding protein, and an uncharacterized group 3 bacterial Ig-like domain protein. The cell division protein CdpA was first functionally described in *L. acidophilus* NCFM (39). Specifically, phenotypic analysis of a *cdpA* knockout strain revealed a strain with increased chain length, aberrant cell morphology, decreased resistance to environmental stressors, and decreased adhesion to Caco-2 epithelial cells (39). The direct mechanisms regarding the function of CdpA and the aforementioned phenotypes were unclear but were thought to be a pleiotropic response to the modified cell wall structure. Notably, the results of the current study offer further insight into this mechanism. First, the protein has two of the NCAD, suggesting localization to the cell wall along with the S-layer. Second, CdpA is one of the most prevalent SLAPs in the S-layer-forming strains but is not found in any non-S-layer-forming *Lactobacillus* species. It is possible that CdpA is a structural intermediary between the cell wall and the S-layer and other SLAPs during cell division. There is evidence for this in the original study in which the *cdpA*-deficient strain was treated with guanidine HCl, and the extracted extracellular SLAPs and SLPs were reduced compared to those of the parent strain (39). These observations indicate that CdpA may be an important component of S-layer structure and function.

The conserved SLAP gene regions were organized into four regions, which demonstrated remarkable conservation in genome position within the overall chromosome architectures (Fig. 5). Strand location of genes on the bacterial chromosome is an important factor for codon usage, which correlates with gene expression (40–42). Moreover, genes of low-G+C-content Gram-positive bacteria illustrate a strand bias for the positive and negative leading strands diverging from the origin of replication (43, 44). The conserved SLAP genes reflect this bias, as they were all found on the leading strands of the positive and negative strands of the chromosomes (Fig. 5).

The transcription of these genes, as measured by RNA sequencing, was similar among the four strains, albeit their rates of transcription were not uniform throughout all four gene regions (Fig. 7). In fact, the genes encoding the *N*-acetylmuramidase and group 3 bacterial Ig-like domain protein appeared to be under the control of a putative $\sigma^{70}$(*rpoD*)-like promoter. The −10 region followed the TANAAT consensus described by Pribnow (45), while the −35 region followed an NTGTNT consensus. These motifs are similar to the $\sigma^{70}$-like promoters of housekeeping genes identified in *Lactobacillus plantarum* (46). Housekeeping genes, such as *ftsA*, *ldhD*, *secA*, and *eno*, were identified as genes under similar transcriptional control.

Taken together, the genomic architecture and transcription data suggest that the conserved SLAPs found in the S-layer-form-

ing strains of *Lactobacillus* are housekeeping genes expressed at constitutive levels. Given their conservation, we conclude that they likely participate in various essential cell processes, such as cell wall hydrolysis, maintenance of cell shape, protein turnover, and cell adhesion. It is notable that genes encoding SLAPs with rudimentary function, such as *cdpA* and the *N*-acetylmuramidase gene, are absent in non-S-layer-forming strains. There also remain the two uncharacterized proteins, the fibronectin-binding protein and the group 3 bacterial Ig-like domain proteins, which have yet to be functionally characterized and are functionally associated with S-layer-forming strains.

Given the extracellular localization of these proteins, the SLAPs identified in this study may have unexplored, potentially important roles in probiotic-host interactions and signaling. Among the conserved SLAPs explored, both the fibronectin-binding protein and the group 3 bacterial Ig-like domain protein have Ig-like folds within their respective amino acid tertiary structures, which may be involved in cell-to-cell adhesion or cell-to-host adhesion. Furthermore, all of these proteins, regardless of their cellular function, are accessible for intimate interactions with the gut epithelium and mucosal immune system (31, 37). In this study, all proteomic and genomic comparisons made for *L. helveticus*, *L. crispatus*, and *L. amylovorus* were made with only one respective genome for each species (*L. helveticus* CNRZ32, *L. crispatus* ST1, and *L. amylovorus* GRL1112). A more complete picture could be made if the genomes of each strain tested were utilized as proteomic and genomic references.

Despite being prevalent among all bacterial types, little is known about the evolutionary function of S-layers. Here, we present the S-layer as a scaffold for numerous noncovalently attached secreted proteins. These S-layer-associated proteins are conserved among S-layer-forming species and absent in non-S-layer-forming species. It is unambiguously clear that the noncovalent exoproteomes of the S-layer-forming strains are more diverse and dynamic than those of the non-S-layer-forming strains. The understanding of these exoproteins opens new avenues for the functional characterization of the S-layer and the health-promoting mechanisms of probiotic-host signaling and cross talk.

## ACKNOWLEDGMENTS

This work was supported through funding from the North Carolina Agriculture Foundation (Raleigh, NC) and DuPont Nutrition & Health (Madison, WI).

We thank Sarah O'Flaherty and Yong Jun Goh for insightful discussion and critical reading of the manuscript. We acknowledge Brett Phinney and Michelle Salemi at the University of California, Davis Proteomics Core Facility for LC MS/MS services, and Alvaro G. Hernandez at the University of Illinois at Urbana-Champaign Roy J. Carver Biotechnology Center for RNA sequencing services.

## FUNDING INFORMATION

NC Agricultural Foundation provided funding to Todd R. Klaenhammer under grant number 6-60780. DuPont/Danisco Nutrition and Health provided funding to Todd R. Klaenhammer under grant number 5-51509.

## REFERENCES

1. Lebeer S, Vanderleyden J, De Keersmaecker SCJ. 2010. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol 8:171–184. http://dx.doi.org/10.1038/nrmicro2297.
2. Johnson BR, Klaenhammer TR. 2014. Impact of genomics on the field of probiotic research: historical perspectives to modern paradigms. Antonie Van Leeuwenhoek 106:141–156. http://dx.doi.org/10.1007/s10482-014-0171-y.
3. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME. 2014. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11:506–514.
4. Sanders ME, Guarner F, Guerrant R, Holt PR, Quigley EMM, Sartor RB, Sherman PM, Mayer EA. 2013. An update on the use and investigation of probiotics in health and disease. Gut 62:787–796. http://dx.doi.org/10.1136/gutjnl-2012-302504.
5. Bron PA, Tomita S, Mercenier A, Kleerebezem M. 2013. Cell surface-associated compounds of probiotic lactobacilli sustain the strain-specificity dogma. Curr Opin Microbiol 16:262–269. http://dx.doi.org/10.1016/j.mib.2013.06.001.
6. Klein G, Pack A, Bonaparte C, Reuter G. 1998. Taxonomy and physiology of probiotic lactic acid bacteria. Int J Food Microbiol 41:103–125. http://dx.doi.org/10.1016/S0168-1605(98)00049-X.
7. Schleifer KH, Ludwig W. 1995. Phylogeny of the genus *Lactobacillus* and related genera. Syst Appl Microbiol 18:461–467. http://dx.doi.org/10.1016/S0723-2020(11)80404-2.
8. Hammes WP, Hertel C. 2003. The genera *Lactobacillus* and *Carnobacterium*. *In* Dworkin M (ed), The prokaryotes: an evolving electronic resource for the microbiological community, 3rd ed. Springer-Verlag, New York, NY.
9. Dellaglio F, Felis GE. 2005. Taxonomy of *Lactobacilli* and *Bifidobacteria*, p 25–50. *In* Tannock GW (ed), Probiotics and prebiotics: scientific aspects. Caister Academic Press, Dorset, United Kingdom.
10. Hammes WP, Vogel RF. 1995. The genus *Lactobacillus*, p 19–54. *In* Wood BJB, Holzapfel WH (ed), The genera of lactic acid bacteria. Blackie Academic & Professional, Glasgow, Scotland.
11. Kandler O, Stetter K-O, Köhl R. 1980. *Lactobacillus reuteri* sp. nov., a new species of heterofermentative lactobacilli. Zentralbl Bakteriol Hyg Abt I Orig Reihe C 1:264–269.
12. Johnson JL, Phelps CF, Cummins CS, London J, Gasser F. 1980. Taxonomy of the *Lactobacillus acidophilus* Group. Int J Syst Bacteriol 30:53–68. http://dx.doi.org/10.1099/00207713-30-1-53.
13. Felis GE, Dellaglio F. 2007. Taxonomy of *Lactobacilli* and *Bifidobacteria*. Curr Issues Intest Microbiol 8:44–61.
14. Berger B, Pridmore RD, Barretto C, Delmas-Julien F, Schreiber K, Arigoni F, Brüssow H. 2007. Similarity and differences in the *Lactobacillus acidophilus* group identified by polyphasic analysis and comparative genomics. J Bacteriol 189:1311–1321. http://dx.doi.org/10.1128/JB.01393-06.
15. Hynönen U, Palva A. 2013. *Lactobacillus* surface layer proteins: structure, function and applications. Appl Microbiol Biotechnol 97:5225–5243. http://dx.doi.org/10.1007/s00253-013-4962-2.
16. Buck BL, Altermann E, Svingerud T, Klaenhammer TR. 2005. Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol 71:8344–8351. http://dx.doi.org/10.1128/AEM.71.12.8344-8351.2005.
17. Frece J, Kos B, Svetec IK, Zgaga Z, Mrsa V, Susković J. 2005. Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. J Appl Microbiol 98:285–292. http://dx.doi.org/10.1111/j.1365-2672.2004.02473.x.
18. Sillanpää J, Martínez B, Antikainen J, Toba T, Kalkkinen N, Tankka S, Lounatmaa K, Keränen J, Höök M, Westerlund-Wikström B, Pouwels PH, Korhonen TK. 2000. Characterization of the collagen-binding S-layer protein CbsA of *Lactobacillus crispatus*. J Bacteriol 182:6440–6450. http://dx.doi.org/10.1128/JB.182.22.6440-6450.2000.
19. Antikainen J, Anton L, Sillanpää J, Korhonen TK. 2002. Domains in the S-layer protein CbsA of *Lactobacillus crispatus* involved in adherence to collagens, laminin and lipoteichoic acids and in self-assembly. Mol Microbiol 46:381–394. http://dx.doi.org/10.1046/j.1365-2958.2002.03180.x.
20. Sun Z, Kong J, Hu S, Kong W, Lu W, Liu W. 2013. Characterization of a S-layer protein from *Lactobacillus crispatus* K313 and the domains responsible for binding to cell wall and adherence to collagen. Appl Microbiol Biotechnol 97:1941–1952. http://dx.doi.org/10.1007/s00253-012-4044-x.
21. Beganović J, Frece J, Kos B, Leboš Pavunc A, Habjanić K, Sušković J. 2011. Functionality of the S-layer protein from the probiotic strain *Lactobacillus helveticus* M92. Antonie Van Leeuwenhoek 100:43–53. http://dx.doi.org/10.1007/s10482-011-9563-4.

22. Hynönen U, Kant R, Lähteinen T, Pietilä TE, Beganović J, Smidt H, Uroić K, Avall-Jääskeläinen S, Palva A. 2014. Functional characterization of probiotic surface layer protein-carrying *Lactobacillus amylovorus* strains. BMC Microbiol 14:199. http://dx.doi.org/10.1186/1471-2180-14-199.
23. Chen X, Xu J, Shuai J, Chen J, Zhang Z, Fang W. 2007. The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* O157:H7 and *Salmonella* Typhimurium. Int J Food Microbiol 115:307–312. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.11.007.
24. Johnson-Henry KC, Hagen KE, Gordonpour M, Tompkins TA, Sherman PM. 2007. Surface-layer protein extracts from *Lactobacillus helveticus* inhibit enterohaemorrhagic *Escherichia coli* O157:H7 adhesion to epithelial cells. Cell Microbiol 9:356–367. http://dx.doi.org/10.1111/j.1462-5822.2006.00791.x.
25. Wine E, Gareau MG, Johnson-Henry K, Sherman PM. 2009. Strain-specific probiotic (*Lactobacillus helveticus*) inhibition of *Campylobacter jejuni* invasion of human intestinal epithelial cells. FEMS Microbiol Lett 300:146–152. http://dx.doi.org/10.1111/j.1574-6968.2009.01781.x.
26. Konstantinov SR, Smidt H, de Vos WM, Bruijns SCM, Singh SK, Valence F, Molle D, Lortal S, Altermann E, Klaenhammer TR, van Kooyk Y. 2008. S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. Proc Natl Acad Sci U S A 105:19474–19479. http://dx.doi.org/10.1073/pnas.0810305105.
27. Lightfoot YL, Selle K, Yang T, Goh YJ, Sahay B, Zadeh M, Owen JL, Colliou N, Li E, Johannssen T, Lepenies B, Klaenhammer TR, Mohamadzadeh M. 2015. SIGNR3-dependent immune regulation by *Lactobacillus acidophilus* surface layer protein A in colitis. EMBO J 34:881–895. http://dx.doi.org/10.15252/embj.201490296.
28. Abramov V, Khlebnikov V, Kosarev I, Bairamova G, Vasilenko R, Suzina N, Machulin A, Sakulin V, Kulikova N, Vasilenko N, Karlyshev A, Uversky V, Chikindas ML, Melnikov V. 2014. Probiotic properties of *Lactobacillus crispatus* 2,029: homeostatic interaction with cervicovaginal epithelial cells and antagonistic activity to genitourinary pathogens. Probiotics Antimicrob Proteins 6:165–176. http://dx.doi.org/10.1007/s12602-014-9164-4.
29. Taverniti V, Stuknyte M, Minuzzo M, Arioli S, De Noni I, Scabiosi C, Cordova ZM, Junttila I, Hämäläinen S, Turpeinen H, Mora D, Karp M, Pesu M, Guglielmetti S. 2013. S-layer protein mediates the stimulatory effect of *Lactobacillus helveticus* MIMLh5 on innate immunity. Appl Environ Microbiol 79:1221–1231. http://dx.doi.org/10.1128/AEM.03056-12.
30. Goh YJ, Azcarate-Peril MA, O'Flaherty S, Durmaz E, Valence F, Jardin J, Lortal S, Klaenhammer TR. 2009. Development and application of a *upp* based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol 75:3093–3105. http://dx.doi.org/10.1128/AEM.02502-08.
31. Johnson B, Selle K, O'Flaherty S, Goh YJ, Klaenhammer T. 2013. Identification of extracellular surface-layer associated proteins in *Lactobacillus acidophilus* NCFM. Microbiology 159:2269–2282. http://dx.doi.org/10.1099/mic.0.070755-0.
32. Liu H, Sadygov RG, Yates JR, III. 2003. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem 76:4193–4201.
33. Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, Heger A, Hetherington K, Holm L, Mistry J, Sonnhammer ELL, Tate J, Punta M. 2014. Pfam: the protein families database. Nucleic Acids Res 42:D222–D230. http://dx.doi.org/10.1093/nar/gkt1223.
34. Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8:785–786. http://dx.doi.org/10.1038/nmeth.1701.
35. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649. http://dx.doi.org/10.1093/bioinformatics/bts199.
36. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359. http://dx.doi.org/10.1038/nmeth.1923.
37. Le Maréchal C, Peton V, Plé C, Vroland C, Jardin J, Briard-Bion V, Durant G, Chuat V, Loux V, Foligné B, Deutsch S-M, Falentin H, Jan G. 2015. Surface proteins of *Propionibacterium freudenreichii* are involved in its anti-inflammatory properties. J Proteomics 113:447–461. http://dx.doi.org/10.1016/j.jprot.2014.07.018.
38. Boot HJ, Kolen CP, Pouwels PH. 1995. Identification, cloning, and nucleotide sequence of a silent S-layer protein gene of *Lactobacillus acidophilus* ATCC 4356 which has extensive similarity with the S-layer protein gene of this species. J Bacteriol 177:7222–7230.
39. Altermann E, Buck LB, Cano R, Klaenhammer TR. 2004. Identification and phenotypic characterization of the cell-division protein CdpA. Gene 342:189–197. http://dx.doi.org/10.1016/j.gene.2004.08.004.
40. McInerney JO. 1998. Replicational and transcriptional selection on codon usage in *Borrelia burgdorferi*. Proc Natl Acad Sci U S A 95:10698–10703. http://dx.doi.org/10.1073/pnas.95.18.10698.
41. Lafay B, Lloyd AT, McLean MJ, Devine KM, Sharp PM, Wolfe KH. 1999. Proteome composition and codon usage in spirochaetes: species-specific and DNA strand-specific mutational biases. Nucleic Acids Res 27:1642–1649. http://dx.doi.org/10.1093/nar/27.7.1642.
42. Grocock RJ, Sharp PM. 2002. Synonymous codon usage in *Pseudomonas aeruginosa* PAO1. Gene 289:131–139. http://dx.doi.org/10.1016/S0378-1119(02)00503-6.
43. Karlin S, Theriot J, Mrázek J. 2004. Comparative analysis of gene expression among low G+C Gram-positive genomes. Proc Natl Acad Sci U S A 101:6182–6187. http://dx.doi.org/10.1073/pnas.0401504101.
44. Rocha EPC. 2004. The replication-related organization of bacterial genomes. Microbiology 150:1609–1627. http://dx.doi.org/10.1099/mic.0.26974-0.
45. Pribnow D. 1975. Nucleotide sequence of an RNA polymerase binding site at an early T7 promoter. Proc Natl Acad Sci U S A 72:784–788. http://dx.doi.org/10.1073/pnas.72.3.784.
46. Todt TJ, Wels M, Bongers RS, Siezen RS, van Hijum SAFT, Kleerebezem M. 2012. Genome-wide prediction and validation of sigma70 promoters in *Lactobacillus plantarum* WCFS1. PLoS One 7:e45097. http://dx.doi.org/10.1371/journal.pone.0045097.
47. Altermann E, Russell WM, Azcarate-Peril MA, Barrangou R, Buck BL, McAuliffe O, Souther N, Dobson A, Duong T, Callanan M, Lick S, Hamrick A, Cano R, Klaenhammer TR. 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. Proc Natl Acad Sci U S A 102:3906–3912. http://dx.doi.org/10.1073/pnas.0409188102.
48. Joerger MC, Klaenhammer TR. 1990. Cloning, expression, and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin J. J Bacteriol 172:6339–6347.
49. Joerger MC, Klaenhammer TR. 1986. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J Bacteriol 167:439–446.
50. Barefoot SF, Klaenhammer TR. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl Environ Microbiol 45:1808–1815.
51. Broadbent JR, Hughes JE, Welker DL, Tompkins TA, Steele JL. 2013. Complete genome sequence for *Lactobacillus helveticus* CNRZ 32, an industrial cheese starter and cheese flavor adjunct. Genome Announc 1(4):e00590-13. http://dx.doi.org/10.1128/genomeA.00590-13.
52. Ventura M, Callegari ML, Morelli L. 1999. Surface layer variations affecting phage adsorption on seven *Lactobacillus helveticus* strains. Ann Microbiol Enzymol 49:45–53.
53. Cato EP, Moore WEC, Johnson JL. 1983. Synonymy of strains of "*Lactobacillus acidophilus*" group A2 (Johnson et al. 1980) with the type strain of *Lactobacillus crispatus* (Brygoo and Aladame 1953) Moore and Holdeman 1970. Int J Syst Bacteriol 33:426–428. http://dx.doi.org/10.1099/00207713-33-2-426.
54. Ojala T, Kuparinen V, Koskinen JP, Alatalo E, Holm L, Auvinen P, Edelman S, Westerlund-Wikström B, Korhonen TK, Paulin L, Kankainen M. 2010. Genome sequence of *Lactobacillus crispatus* ST1. J Bacteriol 192:3547–3548. http://dx.doi.org/10.1128/JB.00399-10.
55. Kullen MJ, Sanozky-Dawes RB, Crowell DC, Klaenhammer TR. 2000. Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. J Appl Microbiol 89:511–516. http://dx.doi.org/10.1046/j.1365-2672.2000.01146.x.
56. Nakamura LK. 1981. *Lactobacillus amylovorus*, a new starch-hydrolyzing species from cattle waste-corn fermentations. Int J Syst Bacteriol 31:56–63. http://dx.doi.org/10.1099/00207713-31-1-56.
57. Kant R, Paulin L, Alatalo E, de Vos WM, Palva A. 2011. Genome

sequence of *Lactobacillus amylovorus* GRL1112. J Bacteriol 193:789–790. http://dx.doi.org/10.1128/JB.01365-10.

58. Fujisawa T, Benno Y, Yaeshima T, Mitsuoka T. 1992. Taxonomic study of the *Lactobacillus acidophilus* group, with recognition of *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and synonymy of *Lactobacillus acidophilus* group A3 (Johnson et al. 1980) with the type strain of *Lactobacillus amylovorus* (Nakamura 1981). Int J Syst Bacteriol 42:487–491. http://dx.doi.org/10.1099/00207713-42-3-487.
59. Azcarate-Peril MA, Altermann E, Goh YJ, Tallon R, Sanozky-Dawes RB, Pfeiler EA, O'Flaherty S, Buck BL, Dobson A, Duong T, Miller MJ, Barrangou R, Klaenhammer TR. 2008. Analysis of the genome sequence of *Lactobacillus gasseri* ATCC 33323 reveals the molecular basis of an autochthonous intestinal organism. Appl Environ Microbiol 74:4610–4625. http://dx.doi.org/10.1128/AEM.00054-08.
60. Judicial Commission of the International Committee on Systematics of Bacteria. 2008. The type strain of *Lactobacillus casei* is ATCC 393, ATCC 334 cannot serve as the type because it represents a different taxon, the name *Lactobacillus paracasei* and its subspecies names are not rejected and the revival of the name "*Lactobacillus zeae*" contravenes rules 51b (1) and (2) of the International Code of Nomenclature of Bacteria. Opinion 82. Int J Syst Evol Microbiol 58:1764–1765. http://dx.doi.org/10.1099/ijs.0.2008/005330-0.