AN ABSTRACT OF THE THESIS OF

Roberto A. Garcia III for the degree of Master of Science in Microbiology presented on May 28, 2014.

Title: Characterization of Novel Exopolysaccharide Producing Lactic Acid Bacteria.

Abstract approved:

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Janine E. Trempy

Lactic Acid Bacteria (LAB), an important group of organisms in modern food production, are known to secrete a unique compound called exopolysaccharide (EPS). EPS is economically important because it enhances functional properties in food and may confer beneficial health effects to consumers. Novel strains of *Lactococcus lactis*, *Streptococcus thermophilus*, and species of *Leuconostoc* were screened for production of unique thickening EPS in 1% fat milk and lactose free milk. Other important characteristics were investigated, such as pH tolerance and inhibitory compound biosynthesis.

A natural isolate, *L. lactis* subsp. *cremoris* Ropy 352 that produces a distinct ropy EPS was previously analyzed and two unique genes, *epsM* and *epsN*, vital for the expression of the ropy phenotype were discovered. In this study, sequence analysis of two novel LAB, *S.thermophilus* R-39 and *L. lactis* subsp. *cremoris* 18-1, were shown to harbor genes similar but not identical to *epsM* and/or *epsN*. Interestingly, said LAB strains do not exhibit the ropy phenotype, which suggests the substituted amino acids may alter the function of the epsM and epsN proteins.

Characterization of Novel Exopolysaccharide Producing Lactic Acid Bacteria

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Presented May 28, 2014

Commencement June 2014

Master of Science thesis of Roberto Garcia III presented on May 28, 2014

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Roberto A. Garcia III, Author

ACKNOWLEDGEMENTS

To give credit to all the people who have made my time here an exciting and stimulating experience would be close to impossible.

First and foremost, I would like to acknowledge and thank my major professor, advisor, mentor, friend, and most importantly adopted mother Dr. Janine Trempy. It was her passion for science and mentorship that brought me to this point in my education. Thank you for the generosity, advice, patience and privilege of working in your lab. I would also like to extend my gratitude and thanks to my committee members, Dr. Theo Dreher, Dr. Bruce Geller, and Dr. Adrian Gombart, for their time, commitment, and interest in my success.

I especially want to thank Dr. Karen Dierksen, whose vast experience and knowledge with Lactic Acid Bacteria have tremendously helped me with my project. She is truly a walking Wikipedia and Google search engine. I would not have been able to survive graduate school without her guidance and support.

I would like to thank present and past members of the Trempy lab: Farhad Mohammad, Holly Roach, Dylan Ledford, Sarah Michalski, and Collin Thompson for their assistance, friendships, and support.

In addition, I would like to extend my appreciation to everyone in the department; professors, staff, colleagues, and fellow graduate and undergraduate students who made my stay at Oregon State University memorable.

Finally, I want to thank my family: my mother, my father and siblings. Without their love and constant support I would not be who I am today. Thank you for always believing in me, serving as my inspiration and strength.

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For my Family

CHAPTER 1

INTRODUCTION

Fermented dairy products are produced and consumed in countries worldwide. Acidification of milk is one of the oldest techniques used to preserve and extend the shelf life of this product. Pinpointing the exact origin of the process is difficult. However, estimates suggest that such innovation occurred when humans decided to shift from primitive food gathering to food production about 10,000 years ago (70). Today, fermented dairy products for human consumption constitutes contribution from a myriad scientific disciplines, including microbiology, biology, engineering, chemistry, and biochemistry.

Different labels have been applied to the same fermented milk product dependent on the geographical region from where the product was produced. To reduce confusion, Robinson and Tamine (93) proposed a simple classification scheme based on the dominant microorganism in a particular product. A majority of fermented milk products fall under the classification, lactic fermentation, where mesophilic lactic acid bacteria (LAB) predominate. Products, such as cultured buttermilk and yogurt, are examples. Kefir is an example of a fermented milk product produced during yeast lactic fermentation, and the Scandinavian product, viili, is an example of mold (mould) lactic fermentation.

Production and consumption of dairy product, including fluid (non-fermented) milk and fermented milk (e.g cheese, butter, and yogurt), has increased throughout the years. Europe is still the main consumer of milk products (10). However, the

United States was the main producer of liquid cow milk in 2011, with roughly 89 million tons being produced. Historically, production and consumption of fermented milk products, such as yogurt, was mainly found in the Middle East and Eastern Europe regions. The increased consumption in other regions can be attributed to the following: (i) the invention of modern refrigeration allowed products to be preserved and distributed with ease; (ii) innovations such as the addition of fruits and sugars to traditional products made them more appealing and acceptable to consumer preference; (iii) the addition of value added components such as probiotics and dietary fiber plus new research into the health benefits made fermented milk product more marketable (92).

Recent innovations in dairy products involve using starter cultures that produce unique compounds called exopolysaccharides (EPS). EPS had been shown to enhance the quality of products by providing natural stabilizing and thickening properties. Lactic Acid Bacteria (LAB), which are starter cultures known to synthesize EPS, are exploited in the food industry, especially in production of fermented products.

CHAPTER 2

Literature Review

Lactic acid bacteria (LAB) are characterized by their ability to metabolize carbon sources, such as fermentable sugars, into lactic acid. This natural bacterial fermentation process has served a historic significance by providing fermented milk, cheese, pickled vegetables or meat products for human consumption. Because of this desired property, LAB continues to be a vital contributor to modern food production. LAB functional characteristics have been subject to modification to enhance desirable traits. In addition to lactic acid being the end product, some of the fermentable sugars are also converted into exopolysaccharides that can either be loosely attached to the cell wall or secreted into the environment. Bacterial exopolysaccharides, upon suspension in aqueous solution, affect the rheology of the solution by imparting thickening and gelling properties. Lactococcal strains have been used as starter cultures in fermented milk products, such as yogurt or viili, and have been known to produce exopolysaccharides that increase the desirability of these products' characteristics. Additionally, exopolysaccharides may contribute health benefits to the products consumed by humans. With more health conscious consumers demanding healthier and organic foods, naturally occurring food thickeners such as exopolysaccharides from non-pathogenic bacterial strains, can potentially replace other stabilizers currently used to thicken certain types of food and drink products.

Characterizing novel Lactococcal strains that produce unique exopolysaccharides may lead to the development of healthier consumable food and beverage products.

Definition of Lactic Acid Bacteria

Lactic acid bacteria (LAB) are a group of diverse organisms belonging to the phylum, Firmicutes. LAB have been exploited predominately in the food industry due to their desirable characteristics. LAB can be found in various environments, isolated from plants, food, and mucosal surfaces of both vertebrates and invertebrates. LAB are Gram-positive, cocci or rods, non-spore forming, catalase negative, and have low GC content. Generally, LAB are non-respiring, and their main pathway for carbohydrate metabolism is fermentation with lactic acid as the terminal product. LAB are used for preservation, textural, sensorial characteristics, and are sometimes added as nutritional value in a variety of food and agricultural products.

There are several genera that comprise this diverse group of microorganisms. In the food industry the most significant genera are considered to be: *Aerococcus*, *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus, Weissella*, and *Bifidobacterium* (81).

Lactic acid bacteria utilize two major types of sugar metabolism processes. Glycolysis, or homolactic fermentation, is a process where lactic acid alone is the end product. In contrast some LAB genera are able to produce products other than lactic acid, such as ethanol, acetate, and CO_2; this type of metabolism is called heterolactic fermentation. A majority of the members of the LAB use glycolysis as the major

fermentation pathway, except for Leuconstoc, Group III Lactobacilli, Oencocci, and Weissellas (81).

Characteristics of LAB

Genomics of Lactic acid bacteria

With the advent of more efficient and cheaper sequencing technology, complete genomes are now providing valuable insight about bacteria in terms of their physiological functions as well as evolutionary footprint. There are thirty-one complete LAB genomes available (86). A comparative analysis of published LAB genomes reveals there is significant genome reduction. In one study, comparing closely related *Lactobacillales* and *Bacilli*, the analysis revealed that *Lactobacillales* lack 600-1200 genes (53). The authors of this study suggested that environmental factors, such as available carbon source and the presence of other organisms, may play a vital role for this evolutionary loss of genes, especially for those LAB that found permanent stable niches in a high nutrient base food, such as dairy and vegetables. *Streptococcus thermophilus* contains a large number of pseudogenes, approximately 270, in which The pseudogenes were predicted to code for nonfunctional proteins involved in transport and metabolism of certain carbohydrates (54).

The presence of plasmids directly affects the bacterium's survival and metabolic strategies. Several LAB contain one or more plasmids that encode vital functions. In Lactococcal species harboring plasmids, these plasmids often contain genes coding for products involved in lactose utilization, proteolysis, bacteriophage

resistance, bacteriocin production, exopolysaccharide synthesis and stress response (26).

Acid Production (lactose metabolism)

The designation, Lactic Acid Bacteria (LAB), implies that lactic acid is a major end product of organisms from this group. However, depending on the environment, variations in the outcome of carbohydrate metabolism can occur. Lactic acid is a product of lactose fermentation, lactose being the most abundant sugar in milk. The decrease in pH due to acid production in milk causes milk proteins, called casein micelles, to aggregate due to contact between the hydrophobic nature and as a result milk coagulates. This is the basic principle behind cheese making. The use of LAB as starter cultures in fermentation is ideal due to their ability to metabolize a wide range of carbohydrates. Various genera process carbohydrates differently, and LAB have been divided into two metabolic classes dependent on whether they are homofermentative and utilize glycolysis to break down sugars or they are heterofermentative. The processes differ in the presence or absence of key catalytic enzymes, such as aldolase and phosphoketolase. However, both pathways share the commonality of formation of an electron acceptor, pyruvate. When pyruvate accepts two electrons from NADH, pyruvate is converted to lactic acid. Glycolysis results in two molecules of lactic acid and two ATP molecules, while the PPP results only in one molecule of lactic acid, ethanol, CO_2 and ATP per one molecule of glucose.

Proteolysis

Lactic acid bacteria are classified as fastidious because they require large amounts of exogenous amino acids and nutrients. In dairy, the proteolytic capability of this group of organisms allows them to ferment milk. LAB's proteolytic system can be divided into three components: (i) cell wall bound proteinase that initiates the breakdown of milk protein casein, (ii) peptide transporters that facilitates uptake of peptides into the cell, and (iii) intracellular peptidases that further degrades the peptides into smaller peptides or into amino acids (49). One characteristic that makes LAB an ideal starter culture in the dairy industry is their Cell-Envelope Proteinase or CEP (84). The majority of LAB contain CEP, responsible for degrading casein, the most dominant protein in milk, into smaller oligopeptides. Other bacterial species, which lack CEP function, import the smaller oligopeptides generated by the activity of LAB's CEP. Transporters that take up the oligopeptides into the cell are part of the oligopeptide permease system (Opp). Transportation of oligopeptides is crucial for normal cellular physiological functions, such as nutrient uptake and utilization, and other functions reliant on signaling, such as certain types of gene regulation (18). Peptidases inside the cell, further degrade peptides into shorter peptides and amino acids. The most well-studied protein degradation process is casein degradation by *Lactococcus lactis*. Successful degradation of milk proteins provides the cell with much needed substrate for normal growth and function. Through evolutionary fine-tuning, LAB have been designed and developed in ways to persist and maximize survivability in a dairy environment.

Aroma and Flavor Compounds

Lactic acid bacteria, due to their limited enzymatic repertoire, transform amino acids into aromatic compounds that can either be desirable or unacceptable in some food products. In products such as cheese, amino acid catabolism is essential to attain a desirable flavor profile (94). Aroma and flavor formation has been extensively studied in the past years. The first crucial step in aroma compound formation is amino acid transamination. The process is catalyzed by aminotransferases in which the α-ketoacid accepts an amine group. Evidence suggests that in LAB, the transamination step only occurs if α-ketoacid is present. In cheese products the addition of α-ketoglutarate to create a α-ketoacid has been shown to greatly increase the aroma profile intensity (3).

Probiotics

Probiotics have experienced an increase in interest due to their potential for added health "benefits" to consumable food and drink products. Probiotics lack a legal definition, but the internationally accepted definition of probiotics is "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (82). LAB represents a majority of the microbial flora in humans and has been studied extensively for their potential probiotic benefits. Several studies appear to support the claim that certain LAB genera have probiotic capabilities, especially those from genera including *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* (21). Consumption of specific LAB genera has been found to confer anti-tumoral properties or retard cancer formation, alleviate gastrointestinal tract diseases, lower blood cholesterol level, and enhance immunomodulating capacity (40, 41, 79, 28, 39).

Although health benefits of consuming probiotics have been demonstrated, the exact molecular and physiological mechanisms underlying such phenomenon are largely unknown.

Lactococcus Diversity

Historically, *Lactococcus* has played a vital role in milk fermentation from its use in food preservation to the production of dairy products such as cheese and yogurt. *Lactococcus* can be easily argued as one of the most economically important and exploited LAB in the industry. As a consequence, *Lactococcus* is the second best studied Gram positive bacterium after *Bacillus subtilus.*

Although the majority of the genus *Lactococcus* do not impose health threats to humans or other organisms, one species, *Lactococcus garvieae,* is known to be a fish pathogen. This organism was first isolated from a mastitis infected dairy cow and in recent years, *L. garvieae* has found a desirable niche in fishes. Presently, *L. garvieae* infection accounts for approximately 50% of production lost in the Mediterranean European trout industry (19).

Historically, *Lactococcus* has been divided into two subspecies of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris,* based on phenotypic characteristics. In applications in the dairy industry, *L lactis* subsp. *cremoris* is preferred over *L. lactis* subsp. *lactis* due to the high production of fruity off flavor compounds such as aldehyde, diacetyl, and pyruvic acid (34). Recently, a third subspecies of *L. lactis* was defined and named L. *lactis* subsp. *hordniae* (63). The phenotypes used to define subspecies are based on various growth conditions such as temperature, arginine utilization, pH tolerance, growth in the presence of sodium chloride, and ammonia

production. There is only one biovar, *L. lactis* subsp. *lactis* biovar. *dicaetylactis*, which is described capable of metabolizing citrate (38). A biovar or bio-variable is a strain that differentiate in a physiological or biochemical process in comparison to other strains. The enzyme, citrate lyase cleaves citrate into acetate and oxaloacetate, which is converted into pyruvate.

Genotypic analyses of *Lactococcus lactis* yield two subspecies as well, with the same *lactis* and *cremoris* designation, but genotypic classification does not always correspond to the phenotypic classification (91**).** *L. lactis* subspecies *cremoris* is almost exclusively isolated from dairy environments such as dairy farms, milk processing plants and cow pastures. Chromosomal diversity in *L. lactis* suggests the *cremoris* subspecies arose from adaptation to the rich dairy environment. *L. lactis* subspecies *cremoris* has a smaller chromosome compared to *L. lactis* subspecies *lactis*, a result of the loss of physiological functions not crucial in a dairy environment. Similar observations have been made with dairy strains of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, which a more condensed genome in comparison to their non-dairy counterparts (51). Presumably, through close association with other natural bacterial species in shared environments, *L. lactis* subspecies *cremoris* has acquired genes to utilize lactose as a carbon source and protect from bacteriophages, normally through uptake of plasmids by conjugation, transformation, or transduction, to fully establish its niche in a dairy environment (38). Dairy strains are very well equipped to survive in their dairy niche; it is thought impossible for them to survive other environments due to loss of essential genes.

Bacteriocin: Inhibition

Food safety has been a great concern of consumers and regulators, both in the United States and internationally. The CDC estimates approximately nine million cases of food borne illness occur annually in the US alone (68). With the invention of modern refrigeration, consumers demand minimal processed foods that are “fresh” and “natural.” From a manufacturing standpoint, both food safety and food quality are a priority. Depending on the nature of the food, food quality can be impacted by taste, color, consistency, and shelf life. A significant factor, impacting food quality, is the presence of spoiler organisms. Industry is attempting to control the impact of spoilage organisms by using bacteria or microbial derived components called antimicrobial peptides (AMPs) as natural sources of antimicrobial activity, which provides a commercially acceptable alternative than synthetic antimicrobials. LAB produces several types of inhibitory substances that can act as AMPs, including organic acids from sugar fermentation, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins. Bacteriocins, proteinaceous toxins capable of inhibiting growth of closely related bacteria and some food associated bacterial pathogens, can provide an alternative to antibiotics.

The bacteriocins produced by LAB are classified into three major groups. Group I bacteriocins are called lantibiotics, due the presence of lanthionine bridges formed through post-translational modification of serine and threonine. Group II bacteriocins are unmodified heat-stable peptides. Group III bacteriocins include larger peptides that are more heat sensitive. A recently described fourth group, called complex bacteriocins, does not share characteristics with the other three groups of

bacteriocins. This group requires non-protein components such as lipids or carbohydrates for its functions and is not well understood (56).

Several types of bacteriocins are produced by *Lactococcus* species. Nisin, a lantibiotic, is one of the most highly studied bacteriocins. Nisin activity was first observed in the 1920's and was employed in an attempt to control mastitis in dairy cows. However, due to the lack of proper purification techniques and knowledge, isolation of nisin was initially unsuccessful and its potential was unrecognized until 50 years later. Nisin has been granted GRAS (Generally Recognized As Safe) status by the FDA and is allowed as a natural food preservative. In Europe it has also been approved as a preservative in many dairy products. However, consumers' attitude on the use of preservatives has shifted, and European countries are moving towards "cleaner" labels.

Two variations of nisin, A and Z, exist. Both compounds only differ in a single amino acid residue at position 27, which is a histidine in nisin A and an asparagine in nisin Z. Both forms are highly effective against Gram-positive bacteria. Nisin causes the bacterial cell membrane to be leaky.

Much controversy still exists between the difference of bacteriocin and antibiotics. A majority of the population have an unwarranted fear of bacteriocins because they view bacteriocins and antibiotics as the same. Two key differences exist between the compounds. Bacteriocins have a narrow host range; bacteriocins only restrict the activity of closely related organisms, while antibiotics have a wider spectrum of activity and host range (75) Secondly, bacteriocins are ribosomally synthesized and produced during the primary growth phase while antibiotics are

secondary metabolites (4). Consumers' regards antibiotics in food as undesirable due to the possible allergic ractions a portion of the population have. Additionally, with the rise of antibiotic resistance pathogenic bacteria, the use of antibiotic compounds are becoming unfavorable.

Lactococcal Bacteriophage

Bacteriophages are ubiquitous in nature, and are abundant and diversified microorganisms, outnumbering bacteria by an estimated ten-fold (9). Phage infection of *Lactococcus lactis* during production of cheese and yogurt is a common problem plaguing the food industry. One phage infection can cause severe economic problems. During the lytic cycle of the phage, off flavors can be imparted or incomplete fermentation can result. To obtain a uniform and high quality product, companies use bacteriophage resistant lactococcal starter cultures of selected strains at concentrations of at least 10^7 bacteria per ml of heat-treated milk (78). A rotation system employing starter cultures with resistance to different classes of bacteriophage was designed and first implemented by Dr. William Sandine at Oregon State University in 1989 (83). This method is known as " defined strain technique" and still in use today.

Bacteria have evolved several methods to resist phage infection. Labrie demonstrated that the production of extracellular matrix can protect a bacterium from phage infection by creating a physical barrier between the phage and the bacterial receptors the phage binds (46). However, to date, no clear evidence has been

generated to demonstrate that exopolysaccharide producing lactococcal strains are more resistant to phage infection.

A known mechanism that confers phage resistance that does not involve the EPS, is the Superinfection Exclusion System, or Sie system, that had been observed in both Gram-positive and Gram-negative bacteria. The very first Gram-positive Sie system was discovered from the lactococcal strain, UC_{509} (55). The Sie systems are composed of various proteins that block the entry of phage DNA into host cells (52). An example of a lactococcal Sie system is Sie_{2009}, where membrane associated Sie_{2009} proteins avert the DNA injection of certain phages but do not affect phage adsorption (Mahony 2008). *Lactococcus lactis* and *Streptococcus thermophilus* are the only known Gram-positive bacteria that can resist phage infection through a Sie system (25, 1)

Plasmids

Plasmids are extrachromosomal replicons that vary in size and copy number. Plasmids are capable of autonomous replication within the bacterial cell independent of the cell's chromosome. Many bacteria harbor plasmids. Plasmids differ from chromosomes in that they often carry genes coding for products non-essential for growth and metabolism, but can confer traits beneficial to the organism under certain conditions. Many virulence and pathogenic characteristics of enteric bacteria such as *Yersenia spp*, *Shigella spp*. and *Escherichia coli* are often due to plasmid encoded genes (77). Plasmids are easily transferred between bacteria by conjugation, transduction or transformation. Once inside the bacterial cell, plasmid DNA can

potentially recombine, through homologous recombination or transposition, to provide new genes to the cell's chromosomes. Plasmids in *Lactococci* have played a tremendous role, especially in the dairy industry. A majority of starter cultures employed by the dairy industry harbor plasmids encoding valuable characteristics such as lactose fermentation, proteinase activity, and phage resistance (31, 44, 64).

Bacterial Polysaccharides

Microbial polysaccharides are multifunctional and vary widely. The bacterial produced polysaccharides are classified as three types: intracellular polysaccharide, structural polysaccharide and extracellular polysaccharide (or exopolysaccharide, EPS) (74). Intracellular polysaccharides are generated within the cytoplasm and are used as an energy source. Structural polysaccharides are present in the bacterial cell wall, contributing to the peptidoglycan layer. Extracellular polysaccharides (EPS) are secreted by the bacterium into the external environment or loosely attached to the cell. In an event where EPS is attached loosely or covalently to the bacterial outer membrane, this characteristic is further classified as a capsule or capsular polysaccharide. However, when completely secreted into the environment, this characteristic is called slime (79). Bacterial EPS are long-chained polysaccharides consisting of repeating monosaccharides or hetersaccharides called homopolymeric and heteropolymeric respectively. Many LAB genera produce distinct types of polysaccharides.

Functions of Bacterial EPS

Many genera of the LAB have been identified as producers of exopolysaccharides. Although the exact function of EPS is still widely debated, researchers suggest that EPS, although not essential, is needed by the bacterial cell because EPS production exacts an energy and substrate cost to the cell. Bacterial polysaccharides play diverse biological roles in processes such as pathogenesis and symbiosis. Evidence suggests that EPS can be used as a form of protection from harsh environments; to prevent desiccation and phagocytosis, as well as protect against osmotic pressure (79**).** Although there may be only slight differences between capsular and slime EPS, the functions of these polysaccharides may be highly varied among different bacterial species. For some bacterial species, EPS acts as an adherent to surfaces, and for other bacterial species, EPS can be used as a carbohydrate source when presented with depletion of nutrients (14).

Exopolysaccharide Biosynthesis

Two types of bacterial EPS, homopolysaccharide (Homo-EPS) and heteropolysaccharide (Hetero-EPS), exist. They vary in chemical structure and method of biosynthesis. Homo-EPS is synthesized outside the cell by specific glycotransferases (GTF). In contrast, Hetero-EPS biosynthesis involves a much more complex process, as it is formed intracellularly.

Homo-EPS biosynthesis requires little energy expenditure due to the lack of active transport systems. The GTF enzymes are secreted outside the cell. Evidence suggests there are high yields of EPS when sucrose is the carbon source. Sucrose, in the environment, can undergo a natural hydrolysis and break down into glucose and

fructose. Under acidic conditions, such as in fermentation processes, sucrose hydrolysis is increased. LAB, such as *Leuconostoc*, have been found to have an increased yield of EPS and a corresponding increase (10-15 fold higher) in gene expression of the genes coding for GTF proteins when sucrose is present (61).

Hetero-EPS requires a much more elaborate biosynthesis process. Inside the bacterial cell substrates are critical commodities; some will be used in catabolic pathways while others will be used in anabolic pathways. Glucose-6-phosphate is the crucial component in determining whether the cell undergoes EPS synthesis or glycolysis to create lactate. The concentration of enzymes, such as phosphoglucomutase, can impact polysaccharide biosynthesis. The cell expresses GTF enzymes that link sugars to the growing EPS. The repeating sugar moiety is linked to a C55-isoprenoid-lipid carrier molecule attached to the cytoplasmic membrane (99). When biosynthesis is complete, researchers hypothesize that a flippase like protein inverts the finished EPS from the cytoplasmic side of the cell into the periplasmic side of the cell wall (47).

Probiotics: Characteristics due to Exopolysaccharide

Several *in vivo* studies have provided evidence suggesting EPS producing LAB confer health benefits to their host, such as lowering cholesterol and providing protection against cancer. Observations in rats fed milk containing EPS producing cultures showed a positive correlation with the amount of EPS produced, and thus consumed, and the amount of cholesterol left in the feces (96). One hypothesis proposes that EPS, due to its molecular similarities with dietary fiber, might provide the same benefits as dietary fiber with respect to cholesterol levels. Hypothetically,

EPS might bind to bile and get excreted out of the body, and to compensate for lowered bile levels, the body might use cholesterol to create more bile thereby lowering cholesterol levels. Some studies do suggest EPS can assimilate or hold onto cholesterol, making it unavailable for absorption (28).

Naturally occurring cultures of EPS producing *L. lactis* subsp *cremoris* KVS20, isolated from a Scandinavian fermented beverage called viili, first showed suppression of S-180 tumor in 1991 (41). However the exact mechanism of tumor suppression was unknown, and evidence did not prove that EPS from the bacterium was involved in tumor suppression. With the advancement of isolation methods and –omics analysis, new data suggests that EPS indeed plays a role in the probiotic characteristics of some organisms. Using a proteomic approach, researchers proposed possible pathways as to how EPS might inhibit the proliferation of HT-29 colon cancer cells. EPS has been found to induce an increase in the concentration of two major proteins, Beclin-1 and GRP78 (39), which leads cells to undergo autophagy. Autophagy is the process of controlled cell death where the lysosomal enzymes are released and the cell degrades itself. This finding could potentially lead to new cancer therapies in the form of novel drugs or food adjuncts. Despite the advances in our knowledge of EPS and its proposed anticancer properties, there is currently no experimental evidence for cancer suppression in human subjects and more *in vivo* testing is needed.

Hydrocolloids

Hydrocolloids are a diverse classification of long chain polymers characterized by their chemical composition and their capacity to trap water to create a viscous solution or to form organized structures creating a gel matrix. Chemically, hydrocolloids or "hydrophilic colloids" contain large numbers of hydroxyl (-OH) groups that increase hydrocolloids' affinity to bind or trap water (47). The food industry utilizes hydrocolloids in many food formulations to enhance or modify the properties and shelf life of the product. Hydrocolloids are used as food thickeners, gelling agents, stabilizers, and fat replacers. The global hydrocolloid market is estimated to be valued at $7 billion by 2018 (29). Hydrocolloids are extracted from natural sources such as seaweeds, plants, animals and microorganisms. Commonly used hydrocolloids can be separated into two major groups. The first group includes thickening hydrocolloids such as starch, xanthan, locust, guar, bean gums, gum Arabic and other cellulose derived products. The second group is the gelling type consisting of pectin, alginate, gelant, gellan, agar, and carrageenan. The processes of thickening and gel formation are fundamentally different and should not be confused. Thickening involves random non-specific entanglement of the polymer chains with solvents or substances in the environment (73). The intensity of the thickening phenomenon depends on which polymers are used, where highly branched or high molecular weight polymers will create a more viscous solution. Physical entanglement of the polymers in a concentrated system restricts the free movements of the molecules. Additionally, the concentration, the nature of the food system such as pH, salts, and temperature can also affect the process. Potato starch and xanthan

gum are the most commonly used thickening polymers due to their abundant supply and ease of extraction. Both are anionic in nature and cannot be used in the same system due to thermodynamic incompatibility (27).

Gels are defined as a form of matter intermediate between solid and liquid that present mechanical rigidity (97). Gelling is the phenomenon of cross-linking between the hydrocolloid polymers to form a three-dimension network. When two or more polymer molecules are associated with each other, "junction zones" result. The junction zones provide the three dimensional network. All hydrocolloids are capable of thickening but only few can form gels. It is common for food formulations to combine thickening and gelling polymers to enhance each polymers' desirable characteristic. Non-gelling agents such as xanthan gum are commonly used in parallel with locust bean gum or any galactomannan polysaccharide (polysaccharides consisting of a mannose backbone with galactose side groups). The synergistic effect creates a superior food property with higher elasticity. Similar to thickening polymers, properties of gelling hydrocolloids are also affected by food system characteristics such as concentration, pH, solvents, and temperatures.

Industry Application for Exopolysaccharide

EPS producing bacteria, such as the LAB, have played a major role in food, dairy, pharmaceutical, medical, chemical, and cosmetic industries. Advancements in technology have created ways to industrialize the production of naturally occurring EPS. LAB harboring GRAS status from the US Food and Drug Administration have great potential for commercialization. GRAS derived ingredients, such as bacterial EPS, can by-pass rigorous and time consuming toxicological testing required of

synthetic polymers. The commercial usefulness of EPS arises from the wide range of functions it supplies due to its hydrophilic chemical nature and efficient solubility in water. Exopolysaccharide produced by LAB in fermented milk product has been an avenue of great research. EPS plays a vital role in high quality yogurt and in solid fermented milk such as cheeses. In mozzarella cheese, when starter cultures included a EPS producing *Lactobacillus helvetics,* water retention was increased, creating a softer type of cheese (71,72). In the food industry, an EPS derived from *Xanthomonas campestris,* called xanthan gum, is probably the most successful EPS on the market today. Although there are documented pyrogenic properties associated with its use, xanthan gum is used as a food additive in food formulation and serves as a thickener in many products consumed by humans.

EPS derived from bacterial sources not only has found uses in the food industry but in the medical and pharmaceutical industries as well. Unlike chemically synthesized polymers, bacterial EPS are classified as natural and non-toxic. In medical and pharmaceutical uses, EPS has been found in applications such as tissue engineering, drug delivery, and wound dressing. A special type of structured EPS is required for certain medical application that require highly sulfated polysaccharides (SPSs). A majority of the mammalian cell receptors that play role in numerous growth factors in blood vessel formation or angiogenesis interact with SPS (50). In vitro studies, using EPS from the extremophilic bacterium, *Halomonas*, in combination with chitin to form nanoparticles for delivery of the drug 5-fluorouracil, demonstrated specific targeting of cancer cells and no toxic effect to normal cells.

Oil drilling is an essential and lucrative operation in the modern world. Tertiary oil recovery is an enhanced method of oil recovery, where trapped oil in hard to reach porous crevices of potential oil reservoirs is extracted by the use of special viscous fluids (drilling fluids) (32) made up of surfactants or hydrocolloids. Surfactants are chemical compounds that are surface-active, meaning they can reduce both surface and interfacial tension between phases of matter (solid, liquid, gas) and allow a homogenous mixture or even dispersion. Synthetic petroleum-based compounds currently meet the market demand for surfactants. However, man-made surfactants are often hazardous and toxic to the environment. With tightening environmental regulations, more oil companies are switching to natural or biosurfactants, one being bacterial derived EPS. Xanthan gum is a commonly used biopolymer in drilling fluids, altough it is limited by its salt and temperature sensitivity (69). Currently, a new EPS derived from *Sphingomonas paucimobilis* GS-1 that may potentially replace xanthan gum has been characterized. This EPS has been described to have superior rheological properties: 5.5x more viscous than xanthan gum, more stable and consistent at high temperature and pH (90°C and 9.00), and lastly the EPS is more pseudoplastic or is more resistant to shear thinning (47).

Despite the flourishing possibilities of EPS functionality, one hurdle that has been yet to be addressed is the high cost of production and relatively low yield. Much research is still needed, especially in the area of genetic mechanisms of EPS gene arrangement and expression, to understand and to optimize EPS biosynthesis.

Xanthan Gum Properties, Function and Structure

Xanthan gum is a natural metabolic by-product of the plant pathogen, *Xanthomonas camprestris* spp. (76). This bacterium is commonly found on cruciferous vegetables such as cabbage, cauliflower, and broccoli (76). In a large-scale production, xanthan gum is produced by batch fermentation (22). A series of purification steps, including centrifugation to remove bacterial cells and precipitation with the use of FDA approved isopropranol, are required prior to xanthan gum's use as a food ingredient (22). This natural polysaccharide is mainly used in the food industry, but also finds a purpose in pharmaceuticals and recently a greater demand with the oil industry.

Understanding the chemical structure of xantham gum is vital in explaining its ideal characteristics. Xanthan gum is a heteropolysaccharide that is composed of a β-D-glucose backbone, mainly consisting of repeats of two glucose units, two mannose units, and one glucuronic acid unit. The backbone composition is similar to cellulose (22). The general shape of the molecule is linear with some branching as indicated (Figure 1), however, it behaves like a linear polymer (5). The branched nature of xanthan gum makes it very soluble in water, even in cold water (11).

Overall, xanthan gum is anionic due to the negative charged carboxyl group side chain. The anionic nature of xanthan creates a viscous solution when mixed with water. The use of xanthan gum in product formulation, especially in sauce products, such as pasta sauce, increases viscosity even with small concentrations. The linear like property of the xanthan gum allows it to arrange into a loose-linked structure. This effectively increases low shear-rate viscosity. When subjected to high shear-rate,

such as chewing or mixing, the linked structure will undergo disentanglement. Eventually, xanthan molecules align with each other in accordance to the direction of the shear stress, exhibiting shear thinning or decrease in viscosity.

Xanthan gums are capable of creating a thick viscous sauce on noodles where no shear stress is introduced. However once exposed to chewing (increased shear rate) xanthan gum loses viscosity. Additionally, xanthan gum is particularly insensitive to high concentrations of salts and heat in food systems (59). Such properties are also beneficial when processing many food products, when shear-thinning behavior occurs as it is pumped from the production line into packaging.

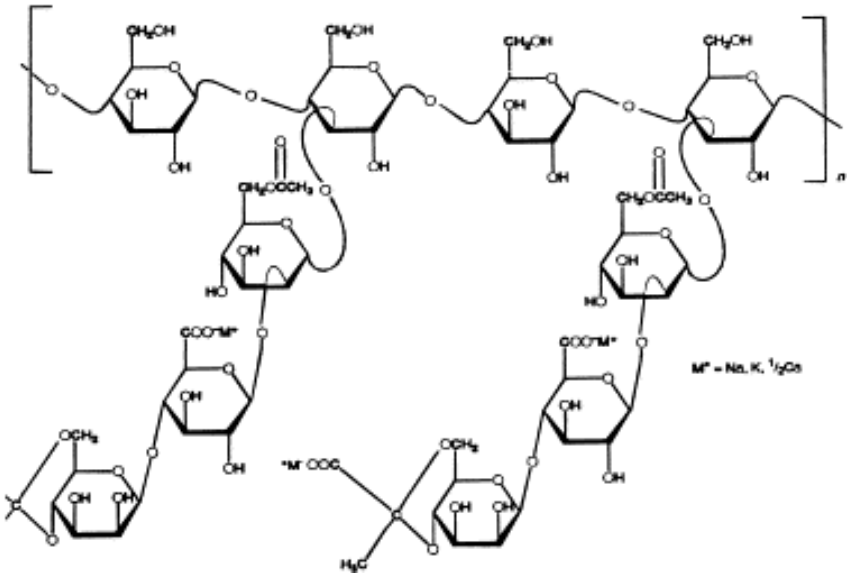

Figure 1. Structure of exopolysaccharide xanthan gum, produced by *X. campestris*
Adapted from Garcia-Ochoa et al (22).

Exopolysaccharide Gene Cluster in LAB

The interest in EPS producing LAB is fueled by unique properties that can potentially be economically valuable in various industries. To better understand EPS, research has moved to more in-depth genetic and biochemical studies to unravel the

complexity of the biosynthesis of EPS in hopes to find ways to maximize EPS production and possible create specific chemically structured EPS with desired characteristics. In recent years several EPS gene clusters from both Gram-negative and Gram-positive bacteria have been identified (37). The genetic locus encoding enzymes for the production of EPS in LAB was first found in *Streptococcus thermophilus* Sfi6 (88). Commonly in LAB, *eps* gene clusters are found on large plasmids; however, in some instances they can also be integrated in the chromosome through a transpositional event. *L. lactis* subsp. *cremoris* SMQ-461 is an example of an LAB that harbors its *eps* biosynthesis cluster genes in its chromosome (12). In general, chromosomal derived *eps* gene clusters have more genes involved in glycotransferase activity as compared to *eps* gene clusters found on plasmids. LAB *eps* gene clusters are highly conserved in the arrangement and regulation of the *eps* genes needed for the biosynthesis and polymerization of the repeating sugar unit, and the export of the EPS.

In efforts to understand the EPS gene cluster, mutational analysis of certain genes within the cluster was done to observe the effect on either the structure or production of EPS. *eps*A is a regulator of the *eps* operon, and the sequence and function of *eps*A is well conserved among other EPS producing LAB. Recent studies in *L. lactis* NIZO B40 suggest that *eps*C gene product is not as crucial as the *epsA* and *epsB* gene products. Mutations in either *epsA* and *epsB* completely inhibited ability to produce EPS in *L. lactis* NIZO B40, while mutations in *epsC* merely decreased the amount of EPS produced (62). This result might also suggest the functionality of the *eps*C gene product is duplicated through the activity of another gene product. The

first of many glycosyltransferase (GTF) genes, *epsD* is located adjacent to the *epsABC* genes. Eps D, also known as the primary GTF, functions to link the first sugar residue to the lipid carrier. Deletion of the *epsD* gene prevents the formation of EPS in *L.lactis* NIZOB40.

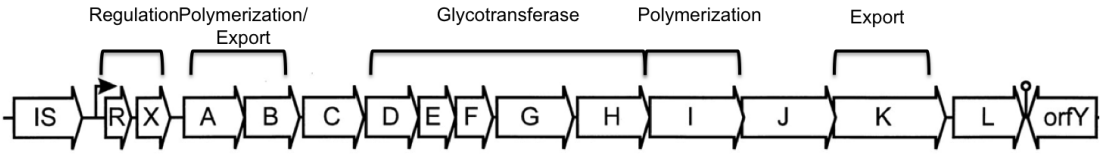

Figure 2. Plasmid pNZ4000, *eps* gene cluster of *L. lactis* NIZO B40. Adapted from Karrenberg et al (62).

In addition to the conserved mosaic arrangement of *eps* genes, *L. lactococcus* subsp. *cremoris* Ropy 352 was found to harbor a large 22kb plasmid containing these *eps* gene, named pEPS352 (42). This *eps* gene cluster is arranged in an operon similar to other *eps* gene cluster motif from other plasmid born EPS biosynthesis gene (Figure 3), and this operon contains *eps* genes R ,-X, -A, -B, -C, -D, -E, -F, -O, -P. Downstream from the last *eps* gene, *epsP* (10,696bp) of the *eps* operon, two more unique glycosyltransferases -N and -M were discovered. An insertion mutation in the *epsN* gene inhibited its function and as a result inhibited the production of EPS in Ropy 352. The mutational study suggests that *epsN* a glycosyltransferase enzyme provides crucial step in the biosynthesis of the to the EPS produced by the Ropy 352 strain despite the position, being a significant distance away from the main *eps* gene cluster (42).

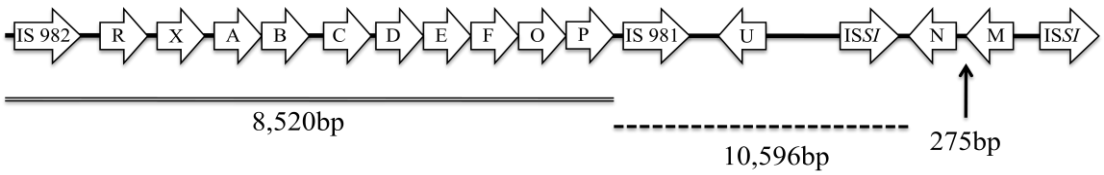

Figure 3. Plasmid pEPS352, *eps* gene cluster of *L. lactis* Ropy 352. Adapted from Knoshaug et al (42).

EPS gene clusters in almost all Gram-positive bacteria follow a similar conserved orientationof genes dedicated to regulation, chain length determination, biosynthesis of sugar linkage, polymerization and transport of finished product. Comparison of known EPS clusters available among LAB suggests there are common mechanisms of EPS synthesis, and that some bacterial strains, such as *L. lactis* subp. *cremoris* Ropy 352, have unique putative GTFs that make the EPS different. Such GTFs can be considered as methods of modifying structure of the EPS to engineer a designer EPS for specific applications.

Research Objectives

Previous research efforts have revealed that Lactic Acid Bacteria (LAB) are capable of producing exopolysacchrides (EPS), and that strains, such as *L. lactis* subsp. *cremoris* Ropy 352, are capable of drastically changing the viscosity of milk. Previously, mutational analysis revealed two unique *eps* genes, *epsM* and *epsN* whose gene product are crucial for the eps phenotype. *epsM* and *epsN* are located downstream from the major *eps* operon and appeared to have been placed adjacent to (but not part of) the major *eps* operon through a transpositional event. The first objective is to identify new strains of LAB that produce EPS and compare their EPS phenotype to the phenotype of Ropy 352. The approaches used to address this objective include phenotypically describing and comparing LAB strains on whey agar plates, in 1% milk, and in lactose free milk. The second objective is to test for antimicrobial activity, and to phenotypically describe if there is a link between the EPS phenotype and inhibitory compounds. The third objective is to further characterize EPS strains for pH sensitivity. The last objective is to characterize EPS producing LAB strains genotypically for the presence of *epsM* and *epsN* gene sequence through polymerase chain reaction. Lactic acid bacterial strains that do not produce the unique ropy EPS are postulated to not harbor the two unique genes. The outcome of this research effort will increase the understanding of EPS in Lactic acid bacteria. Strains characterized can potentially serve as starter cultures in fermented products, especially in the dairy industry.

Chapter 3

Materials and Methods

Bacterial Strains and Growth Media

All bacterial strains used in this work and their sources or derivative are listed in Table 1. Lactococcal glycerol stocks were maintained at -80°C in 11% nonfat dry milk (NFM) containing 20% glycerol. *Lactococcus* cultures were streaked for isolation using 7% whey agar (100) or grown in M17 liquid medium supplemented with 5% lactose (95). All lactococcal strains were grown at 30°C and without aeration if grown in broth. Whey media was prepared in two parts. First, 70 grams (g) of sweet whey powder (Tillamook County Creamery, Tillamook OR. & Bob's Red Mill, Milwaukie, OR) was dissolved in 500 milliliters (mL) of double distilled water (ddH_2O) and mixed until whey powder was evenly distributed. The whey solution was then centrifuged for one hour at 7000 rpm to remove any un-dissolved whey. The whey supernatant was fortified with 19 g of β- Sodium glycerophosphate, 5 g of yeast extracts and the volume brought up to 600 mL, prior to autoclaving for 10 minutes. In a separate flask 15 g of agar was combined with 400 mL of ddH_2O and autoclaved for 25 minutes. Both flasks were combined after cooling to 55°C and the mixture was poured aseptically intp sterile petri plates. M17 medium was prepared by mixing 37.25 g of Difco^{TM} M17 broth in 950 mL of ddH_2O before autoclaving for 15 minutes. When cooled to 55°C, 50 mL of 10% sterile lactose was added; 15 g/L agar was added to prepare M17 agar plates.

Table 1. Bacterial Strains used in this study.

Bacterial Strain	**Source**
L. lactis subsp. cremoris Ropy 352	OSU, strain collection
EK 240 (L. lactis subsp. *cremoris* Ropy 352-mutant)	Reference (42)
L. Lactis subso. *lactis* E	OSU, strain collection
L. lactis subsp. *cremoris* 4R-7	OSU, strain collection
L. lactis subsp. *cremoris* ATCC 9596	ATCC
L. lactis subsp. *lactis* biovar *diacetylactis* DRC-1	OSU, strain collection
L. lactis subsp. *lactis* biovar *diacetylactis* DRC-3	OSU, strain collection
L. lactis subsp. *lactis* biovar *diacetylactis ATCC 11007*	OSU, strain collection
L. lactis subsp. *cremoris* R-1	OSU, strain collection
L. lactis subsp. *cremoris* 26-1	OSU, strain collection
L. lactis subsp. *cremoris* Da-1	OSU, strain collection
L. lactis subsp. *cremoris* 18-1	OSU, strain collection
L. lactis subsp. *cremoris* C-13	OSU, strain collection
L. lactis subsp. cremoris ATCC 11454 (nisin +)	ATCC
Leuconostoc mesenteroides	OSU, strain collection
Streptococcus thermophilus R-39	OSU, strain collection
Streptococcus thermophilus MC	OSU, strain collection
Leuconostoc oneos E26	OSU, strain collection
Bacillus cereus ATCC 14579	ATCC
Bacillus cereus ATCC 49064	ATCC
Bacillus subtilis 1A1	*Bacillus* Stock Center

Plasmid DNA Isolation

Small-scale lactococcal plasmid isolation was followed as described in the protocol provided in the MoBio UltraCean® Microbial DNA Isolation Kit. Because the *eps* biosynthesis genes are located on large plasmid (> 20kb) this chromosomal DNA kit was used. The DNA isolated was used for PCR experiments in detecting genes of interest.

Lactococcal plasmid isolation used for plasmid profiling and electrophoresis was carried out as follows with slight modification (65). Ten milliliters of cells were grown to saturation under appropriate conditions, centrifuged to pellet the cells, and the supernatant was decanted. The pellet was resuspended in 200 µL of 25% sucrose with 30mg/ml of lysozyme, 0.1mg/ml RNase and incubated for 15 min. at 37°C. Four hundred µL of alkaline 3% SDS solution/0.2N NaOH was added and mixed immediately, then left to incubate for 7 minutes at room temperature. Three hundred µL of ice-cold 3M sodium acetate was mixed immediately, and incubated on ice for 10 minutes. At this point cellular debris was removed by centrifugation for 15 minutes and the supernatant was transferred carefully to a new microcentrifuge tube. Six hundred fifty µL of room temperature isopropanol was added and mixed well before centrifuging for 15 minutes. The fluid was decanted making sure the pellet was undisturbed. The pellet was resuspended completely in 320 µL of molecular grade water, before adding 200 µL of 7.5 M ammonium acetate with 0.5 mg/ml ethidium bromide, and 350 µL phenol/chloroform (1:1). This was mixed well and centrifuged for 5 minutes. The upper phase was transferred to a new tube and 1 ml ice-cold 100% ethanol was added, mixed well and centrifuged for 15 minutes. The ethanol was carefully decanted and pellet washed with 400 µL of 70% ethanol. The ethanol was removed and the pellet air dried for 10-15 min. The pellet was resuspended in 40uL of Elution Buffer (EB; 10mM Tris-Cl pH8.15).

Screening for unique "Ropy" EPS producing strains on whey agar

To check for the "ropy" phenotype, cultures were streaked onto whey agar plates and incubated for 24 hours at 30°C until defined single colonies were observed. Using a sterile toothpick, colonies were assessed for the production of "ropy" phenotype by the presence of a string of polysaccharide that would appear as the end of the toothpick was pulled away from the colony. A colony producing a polysaccharide "rope" longer than 5mm was considered "ropy."

Screening for EPS producing strains in dairy products for "thickening"

The ability of LAB strains to thicken milk or lactose free milk was assessed by adding 50µL of a overnight culture (24 hours at 30°C for *Lactococcus* spp. and 12 hours at 37°C for other LAB) into 5 mL of either sterile 1% milk or lactose free milk (Organic Valley and Lactaid). Inoculated milk was then incubated for 24 hours at 30°C for *Lactoccocus* spp. and 37°C for other LAB. Presence of EPS in dairy product was measured by the viscosity or thickness of the milk. Thickening of milk is a different and a distinct phenomenon than coagulation. Visually, coagulation can be determined by the separation of solid particles and the liquid whey. Thickening of milk is described as a smooth viscous solution where little to no liquid whey separation is visible. The thickness was scored after comparison to the thickening phenotype of the dairy product produced by *L. lactis* Ropy 352. Thickening was graded as: - no change, + little thickening, ++ some thickening, and +++ very viscous. Each experiment was done a minimum of three times.

Polymerase Chain Reaction

epsM and *epsN*

Polymerase chain reaction (PCR) was used to determine whether the unique *epsM* and *epsN* genes were present in any of the EPS producing strains. Primers were designed based on the nucleotide sequence of *epsM* and *epsN* from *Lactococcus lactis* subsp. *cremoris* Ropy 352 and ordered through Integrated DNA technologies (IDT). PCR reactions were run using a Perkin Elmer Gene Amp PCR system (model 2400) or Eppendorf Mastercycler® gradient machine. PCR reaction mix contained 50-100 ng of both plasmid and chromosomal DNA quantified using NanoDrop ND-1000 UV-Vis Spectrophotometer. Additionally, 1-2μL (20μM) of each primer (Table 2) and Platinum® PCR Super Mix HiFi (Invitrogen Cat. No. 11306016) were added a final volume of 50μL in a 0.5 mL PCR tube. A positive control with *L.lactis* Ropy 352 DNA and a negative no template DNA control were run to ensure purity and functionality of reaction solutions. Touchdown PCR was performed with the following settings: the PCR reaction was subjected to 95°C for 5 minutes followed by 30 cycles of denaturation for (95°C for 30 seconds). Annealing temperatures were set at 60°C, 58°C, 55°C, and 50°C with 5, 5,10, and 15 cycles respectively (30 seconds). The extension temperature was set to 72°C (1 minute). A final extension step was added for 10 minutes to allow complete product extension by the Taq polymerase. PCR products were then analyzed by gel electrophoresis to observe any amplicon. Three separate DNA extractions were performed for each strain and analyzed the same way. 1% agarose was prepared with 1x TAE buffer (40 mM Tris-acetate and 1mM EDTA). DNA bands were visualized by ethidium bromide staining.

NisA

PCR was employed to determine if nisin production was the cause of the inhibitory activity described in the following section. Primers for the structural nisin gene precursor *nisA* were desgined and ordered from IDT (Table 2). A positive control from a known nisin producing strain, *L. lactis ATCC 11454* was used. A modified touch down PCR program was used to optimize *nisA* primers. Touchdown PCR conditions were as described for *epsM* and *epsN* except annealing temperatures were changed to 60°C, 58°C, 50°C, (30 seconds) with 5, 10, and 15 cycles respectively. PCR product was visualized and analyzed as previously described.

epsM and *epsN* DNA Sequence Analysis

For sequence analysis, PCR products were then purified using either QIAquick® PCR Purification Kit (Qiagen, Cat. No. 28104) or MoBio UltraCean® Microbial DNA Isolation Kit. PCR product was purified using NucleoSpin® Gel and PCR Clean-up from Macherey-Nagel. Purified genomic DNA was quantified using NanoDrop ND-1000 UV-Vis Spectrophotometer and was sent to Center for Genome Research and Biocomputing at Oregon State University.

Sequence data was filtered for unambiguous reads and was analyzed by multiple alignment programs. Using the GeneBank data by using BLAST® for sequence homology, DNA sequence was translated into predicted amino acids sequences.

Table 2: Primers used in this study.

Primer	Primer Sequence
epsMFwd2	5'- TAATGAGTCCAGTGATGGCTC-3'
epsMRev2	5'-GTATCATACACGATCCTACTAAGGGA-3'
epsNFwd1	5'-GGCAGGCAGTAGACAAGAATTTTCG-3'
epsNRev1	5'ACCAACTACTGCTCCCAATTCCCG-3'
nisAFwd1	5'-AACTTGGATTTGGTATCTGTTTCG-3'
nisARev1	5'-GCTTACGTGAATACTACAATGACAAG-3'
nisAFwd2	5'-CTGTTTCGAAGAAAGATTCAGGTG-3'
nisARev2	5'- CCTTTGATTTGGTTATTTGCTTACG-3'

Screening for Inhibitory Compounds by Zone of Inhibition Analysis

Lactoccocal cell cultures were grown to saturation in 5mL of M17 broth (24 hours). Fifty μL of the culture was inoculated into 5mL of 1% utra high temperature (UHT) sterile milk and incubated at 30°C overnight. A sterile disc (5mm in diameter) was dipped into the inoculated milk and placed on a BHI (Bacto™ Brain Heart Infusion, 37g/L) agar plate, preseeded with pathogenic or non-pathogenic Gram-positive bacterial strains, *Bacillus cereus* ATCC 14579, *Bacillus cereus* ATCC #1, and *Bacillus subtilis* 1A1-1. To seed the plates, overnight cultures of *Bacillus* spp were incubated at 37°C, aerated and two dilutions were plated (10^{-5} and 10^{-4}). Disc-impregnated plates were incubated overnight at 30°C and static (no aeration). Zones of inhibition were measured using a ruler in millimeters. *Bacillus* strains are Biosafety Level 2 (BSL-2) bacteria. Thus, all inhibition assays involving pathogenic *Bacillus* were conducted in a BSL-2 facility, approved by the Oregon State University's Institutional Biosafety Committee. *Lactococcus lactis ATCC 11454*, a nisin producing strain, was included as a positive control.

pH Sensitivity

Lactococcus spp were grown in M17 broth at 30°C for 24 hours. A 100µL aliquot of the bacterial culture was added to 10 mL of sterile phosphate-buffered saline (PBS, 9g/L of NaCl, Na_2HPO_4 9g/L, and 1.5g/L of KH_2PO_4) that had been prepared at the following pH values [2.0, 2.5, 3.0, 6.2 (control), 8.0, and 9.0] using NaOH or HCl to change the pH. After pH adjustment, the solution was filter sterilized using VWR Vacuum Filtration System, Nylon 0.2 µm. Samples were incubated at 30°C for 2 hours. A 10 µL aliquot was removed from the control pH 6.2 control flask before incubation and serially diluted to determine the initial cell count. After incubation cells were serially diluted, plated on M17 agar plates, and incubated at 30°C for 36 hours prior to enumeration of viable cells. Each diluted sample was plated in triplicate to get average colony forming units per milliliter (CFU/mL) and then log values were calculated to enumerate survival rate. The detection limit was set to 100 cells/ mL (33).

Determination of Growth Curves

Streptococcus thermophilus R-39 and *Lactococcus lactis* subsp. *cremoris* Ropy 352 were grown to saturation in M17 broth overnight. One hundred µl and 300 µl of each respective bacterial culture was back-diluted into fresh 5 mL M17 broth. Using Spectronic 20D+ spectrophotometer, optical density (OD:600 nm) was recorded every 1-2 hours from time of inoculation. Optical density was plotted over time.

For transformation of *S. thermophilus* R-39, bacterial cells were also grown in a modified M17 broth with varying percent glycine incorporated (1%, 2%, and 3%). Glycine is incoporated to bacterial membrane causing leakiness. Same instrument and method was followed to determine growth curve.

Co-culturing

Five milliliters of overnight cultures of *Streptococcus thermophilus* R-39 and *L. lactis* subsp. *cremoris* Ropy 352 were combined and incubated at 42°C for 4 hours to promote cell death of *Lactococcus* and release of DNA for uptake by *S. thermophilus* R-39 bacteria over 4 hours. Serial dilutions were plated on M17 agar plates and incubated at 42°C overnight. Isolated single colonies were then phenotypically checked for expression of the ropy phenotype with the use of a sterile toothpick as described previously (17).

Bacterial Transformation (Electroporation)

Transformation of *S. thermophilus* R-39 cells was accomplished following an electro-transformation protocol designed for *Lactococcus* (103). Briefly, competent *S. thermophilus* R-39 were prepared by growing 50 mL cultures in M17 containing 0.5% lactose broth overnight at 37°C. Fifty mL fresh M17 broth containing 6% glycine was added to the 50ml cultures to obtain a final glycine concentration of 3%. Cultures were incubated at 37°C until $O.D._{600}$ reading was 1.0-1.5. Cells were pelleted by centrifugation at 5-7 *kg* for 20 minutes at 4°C. After the cells were pelleted, an ice cold 100 mL washing solution (0.5M sucrose + 10% glycerol) was

used to resuspend the pellet into solution. The cells were pelleted a second time, and washed 2x with 10 mL ice-cold poration/storage buffer (0.5M sucrose +10% glycerol). Two mL the cell culture was aliquoted into ice-cold eppendorf tubes for storage of competent cells in a -80°C freezer. The BioRad Gene Pulser™ apparatus was set to 25μF capacitor, 1.5 Kvolts, 200 ohms. Transformation of competent *S. thermophilus* R-39 cells was as follows; 5μL of plasmid DNA (approximately 1-5μg) from strain *L. lactis Ropy 352* was added to microfuge tube with 45μL competent *S. thermophilus*. Transferred the pDNA and cells into a cold 0.2 cm electroporation cuvette and given one pulse. Ice-cold recovery medium (LM17 + 0.5M sucrose) was added and left on ice to incubate for 10 minutes. Cells were transferred from the cuvette to a new microfuge tube, then incubated for 2 hours at 37°C in the recovery media. Serial dilutions were made and plated into M17 agar plates and left to grow at 37°C. Colonies were screened for the ropy phenotype with the use of a sterile toothpick. Ten plates of a 10^{-7} dilution of transformed *S. thermophilus* R-39 each containing 150-200 single colonies were screened.

Chapter 4

Results

Exopolysaccharide Production in Milk

The change in viscosity was assessed phenotypically, using the scale described in Figure 4, and compared to the results of Ropy 352. For brevity, *L. lactis* subsp *cremoris* Ropy 352 will be addressed as Ropy 352. Fifty µl of saturated overnight cultures from 80 different LAB strains were added to two dairy media, lactose free milk and 1% low fat milk.

Figure 4. Viscosity scale for phenotypic assessment

Figure 4. The change in viscosity or thickening event was assigned: +, little to no change; ++, moderate thickness some milk particulates start to aggregate; and +++, very thick and viscous solution.

Eighty LAB strains were screened for EPS phenotype; eleven Lactococcal strains showed EPS phenotypic characteristics similar to Ropy 352 in either one or both of the dairy media tested (lactose free or 1% fat milk) (Table 3). Of the eleven EPS producing LAB strains, seven belonged to *cremoris* subspecies and four

belonged to *lactis* subspecies, with three being *diacetylactis* biovar. One strain, *L. lactis* subsp. *lactis* E, induced significant viscosity, similar to Ropy 352, in both dairy media. Interestingly, the other LAB strains thickened more effectively when cultured in lactose free milk as compared to 1% milk.

In previous studies by E. Knoshaug *et. al* (42), investigating essential genes for the unique ropy EPS phenotype, a mutant form of Ropy 352 was generated by insertional mutation to *epsN*, one of the essential *eps* biosynthesis genes. The mutant Ropy 352 strain, named *L. lactis* EK 240, failed to produce the unique ropy EPS. In this study, when *L. lactis* EK 240 was inoculated in dairy media, *L. lactis* EK 240 failed to change the viscosity of both dairy media, 1% milk and lactose free milk.

Additionally, other LAB, known to produce EPS, were tested for ability to alter the viscosity of dairy medium as well. Overnight cultures (50µl) of *Leuconostoc spp* and *Streptococcus thermophilus* were added to 5 mL of the dairy media (1% milk or lactose free milk) and viscosity was recorded as indicated previously. *Leuconostoc spp*. exhibited thickening properties only when cultured in lactose free milk; no change in viscosity was observed in regular 1% fat milk (Table 4). While *S. thermophilus* MC induced a slight thickening in both milk media, this was not due to EPS but through coagulation of milk caused by low pH. Culturing *S. thermophilus* R-39 in milk media did not induce coagulation or thickening. Coagulation is evident by the observable yellow-pigmented liquid whey separation and clumping of milk solids.

Table 3. *Lactococcus lactis* exopolysaccharide phenotype in milk media.

Bacterial strain	Lactose free milk	1% Milk
Uninoculated	-	-
L. lactis subsp *cremoris* Ropy 352	+++	+++
EK 240 (Ropy 352 *eps*N mutant)	-	-
L. lactis subsp. *cremoris* Da-1	+++	++
L. lactis subsp. *cremoris* ATCC 9596	+++	+++
L. lactis subsp. *cremoris* R-1	+++	+
L. lactis subsp. *cremoris* C-13	+++	++
L. lactis subsp. *cremoris* 4R-7	+++	++
L. lactis subsp. *cremoris* 26-1	++	+++
L. lactis subsp. *cremoris* 18-1	+++	+
L. lactis subsp. *lactis* biovar *diacetylactis* DRC-1	+++	+
L. lactis subsp. *lactis* biovar *diacetylactis* DRC-3	+++	+
L. lactis subsp. *lactis* biovar *diacetylactis* ATCC 11007	+++	++
L. lactis subsp. *lactis* E	+++	+++

Table 3. Listed are the EPS positive strains out of the 80 total LAB screened. Cultures were grown to saturation in M17 broth at 37°C. Five µL of each cultures was inoculated into 5mL of lactose free milk and 1% fat milk and incubated at 37°C for 24 hours.

Table 4. Lactic Acid Bacteria exopolysaccharide phenotype in milk media.

Bacterial strain	Lactose free milk	1% Milk
Leuconostoc mesenteroides	+++	-
Leuconostoc oneos E26	+++	-
Streptococcus thermophilus MC	+	+
Streptococcus thermophilus R-39	-	-

Table 4. Cultures were grown to saturation in M17 broth at 37°C. Five μL of each cultures was inoculated into 5mL of lactose free milk and 1% fat milk and incubated at 37°C for 24 hours.

Identification of Unique Ropy EPS Phenotype

LAB strains that demonstrated the capacity to change milk viscosity were investigated for the ropy phenotype when cultured on whey agar plates. Certain natural lactococcal ropy isolates have the capacity to produce two distinct exopolysaccharides, phenotypically described as mucoidy and ropy (17). Ropy 352, the model organism for this study produces both the mucoidy and ropy exopolysaccharides. Ropy EPS is characterized as a formation of "ropes" or "strings" when touched (Figure 5). In a single colony of *Lactococcus*, a formation of ropes greater than 5mm in length originating from the colony is considered ropy phenotype (17). Of the eleven Lactococcal strains with thickening properties when cultured in liquid milk media, none demonstrated ropy EPS when cultured on whey agar plates (Table 5). Although *L. lactis* subsp. *lactis* E induced significant viscosity, similar to Ropy 352, in both liquid milk media, this strain also did not display the ropy characteristic, on whey agar plates, that is unique to Ropy 352.

Figure 5. *Lactococcus lactis* subsp. *cremoris* Ropy 352 on Whey Agar

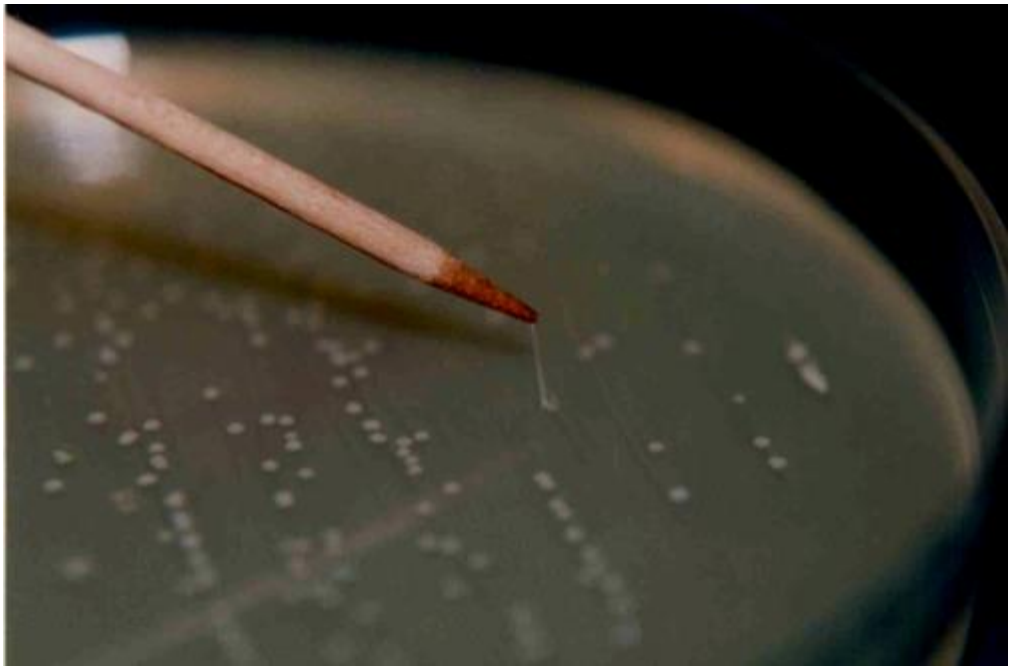

Figure 5. Whey agar plates were incubated at 30°C for 24 hours. Colonies were touched with a sterile toothpick to test for the ropy EPS phenotype.

Table 5. LAB "Ropy" Exopolysaccharide Phenotype on Whey Agar.

LAB strains	Ropy measurement (mm)
L. lactis subsp. *cremoris* Ropy 352	10
EK 240 *(*Ropy 352 *eps*N mutant)	0
L. lactis subsp. *lactis* E	0
L. lactis subsp. *cremoris* 4R-7	0
L. lactis subsp. *cremoris* ATCC 9596	0
L. lactis subsp. *lactis* biovar *diacetylactis* DRC-1	0
L. lactis subsp. *lactis* biovar diacetylactis DRC-3	0
L. lactis subsp. *cremoris* R-1	0
L. lactis subsp. *cremoris* 26-1	0
L. lactis subsp. *cremoris* Da-1	0
L. lactis subsp. *cremoris* 18-1	0
L. lactis subsp. *cremoris* C-13	0
L. lactis subsp. *lactis* biovar *diacetylactis* ATCC 11007	0
Leuconostoc mesenteroides	0
Streptococcus thermophilus R-39	0
Streptococcus thermophilus MC	0
Leuconostoc oneos E26	0

Production of Inhibitory Compounds

Other characteristics that are beneficial for the possible food applications of the EPS producing LAB strains is to screen for any inhibitory against among other microorganisms, especially to food pathogens. Although, none of the newly identified EPS producing LAB exhibited ropy phenotype and the exact function of EPS is still largely unknown, the link between EPS and inhibitory activity was investigated. Bacteria are capable of producing inhibitory compounds that are able to retard or suppress growth of other microorganisms. LAB are known to produce proteinaceous toxic compounds called bacteriocins. *L. lactis* subsp. *lactis ATCC 11454*, a known nisin producing strain, was used as a positive control. This positive control strain produced the largest zone of growth inhibition against the *Bacillus* strains used in this study, as measured in millimeters (Table 6). A negative control was performed using only sterile uninoculated milk to verify that zones of growth inhibition were not due to contamination (Table 6). Zones of growth inhibition were successfully formed by several of the *Lactococcus lactis* species cultured in dairy liquid medium against two strains of pathogenic *Bacillus, B.cereus* ATCC 14579, *B. cereus* ATCC 49064, and a non-pathogenic *Bacillus* strain, *B. subtilis* 1A1 (Table 6).

The majority of the newly discovered EPS producing *Lactococcus lactis* strains had some antimicrobial activity, as measured by zones of growth inhibition, against one or more of the *Bacillus* species (Table 6). Both *L. lactis* subspecies, *cremoris* and *lactis,* are capable of producing compounds that can inhibit growth of *Bacillus* species. *L. lactis subsp. lactis* biovar *diacetylactis* ATCC 11007 seemed to inhibit the growth of only the pathogenic strains of *Bacillus cereus* and not the non-

pathogenic *B. subtilis*. Four strains; *L. lactis* subsp. *cremoris* Ropy 352, *L. lactis* subsp. *lactis* E , *L. lactis* subsp. *cremoris* 26-1 and *L. lactis* subsp. *cremoris* 18-1, showed no inhibitory activity toward any of the *Bacillius* species, indicated by the absence of measurable zone of growth inhibition.

Table 6. Inhibitory activity of EPS producing *Lactococcus lactis*

Cell Concentration	*B. cereus* ATCC 14579		*B. cereus* ATCC 49064		*B. subtilus* 1A1	
	10^{-4}	**10^{-5}**	**10^{-4}**	**10^{-5}**	**10^{-4}**	**10^{-5}**
Bacterial Strains	**Inhibition Zones (mm)**					
Uninoculated Dairy	0	0	0	0	0	0
L. lactis subsp. *lactis ATCC 11454*	3	3	4	3	9	8
L. lactis subsp. cremoris Ropy 352	0	0	0	0	0	0
L. lactis subsp. *lactis* E	0	0	0	0	0	0
L. lactis subsp. *lactis* biovar *diacetylactis* ATCC 11007	2	3	0	3	0	0
L. lactis subsp. *lactis* biovar *diacetylactis* DRC-3	4	0	3	3	3	4
L. lactis subsp. *lactis* biovar *diacetylactis* DRC-1	2	0	0	3	3	2
L. lactis subsp. *cremoris* 4R-7	4	3	1	4	3	4
L. lactis subsp. *cremoris* ATCC 9596	5	0	3	3	6	4
L. lactis subsp. *cremoris* R-1	2	4	4	4	3	2
L. lactis subsp. *cremoris* 26-1	0	0	0	4	0	0
L. lactis subsp. *cremoris* Da-1	5	4	10	3	4	4
L. lactis subsp. *cremoris* 18-1	0	3	0	0	0	0
L. lactis subsp. *cremoris* C-13	3	3	3	0	4	3

Table 6. Zone of inhibition was analyzed by saturating a sterile disk with uninoculated 1% fat milk (negative control) or 1% fat milk pre-inoculated with *Lactococcus* strains and placing the disks on BHI agar plates pre-inoculated with appropriate *Bacillus* spp. Bacterial clearing was measured by its diameter in millimeters.

Presence of DNA Sequence for the Bacteriocin, Nisin

Results from the study examining the EPS producing *Lactococcus lactis* strains' ability to inhibit growth of *Bacillus* strains suggested that some of the EPS producing strains had antimicrobial activity, yet this characteristic was not linked to EPS production. Nisin is a bacteriocin that is a natural antimicrobial peptide produced by *Lactoccocus* spp. NisA is the main precursor for the active mature nisin. Using touchdown PCR and primer sets specific to *nisA,* a PCR product was not identified from any of the EPS producing *L. lactis* strains, including those that inhibited the growth of *Bacillus* species (data not shown). To verify that the correct primers were designed, a known nisin producing strain, *L. lactis* subsp. *lactis ATCC 11454,* served as a positive control. In a PCR reaction, the DNA from this strain resulted in a distinct band for both *nisA* primer sets tested indicating primer design and PCR program was successful (data not shown).

pH sensitivity of EPS Producing *Lactococcus lactis* Strains

The ability to survive harsh environments, like extreme pH, is a characteristic microorganisms must possess to be considered as probiotic. Probiotic microorganisms, in food or beverages, must be able to withstand conditions as they travel down the gastrointestinal tract. It is believed that probiotics must be viable to provide benefits to the host. The goal is to check if the presence of EPS can protect the strains from harsh environment. To further characterize the EPS producing Lactococcal strains, sensitivity to various pH levels was investigated. Highly acidic

conditions of 2.0, 2.5, and 3.0, and basic conditions of 8.0 and 9.0 were the chosen parameters (Table 7). None of the EPS producing *L. lactis* strains were able to survive or show any growth following exposure to extreme acidic conditions of pH 2.0, 2.5, and 3.0 (Table 7). Alkaline conditions of pH 8.0 and 9.0 seemed to have no negative effect on the strains, where a high survival percentage was attained, 94% - 100% (Table 7). Preliminary data suggest that a few of the EPS producing *L. lactis* strains examined in this study are able to survive a pH as low as pH 3.5 (data not shown).

Table 7. Lactococcal strains, incubated at pH values ranging from 9.0 to 1.0, and the number of viable cells (log CFU/mL) and survival percentage at each pH values.

	Control	Log CFU/mL	Percent Survival[a]	Log CFU/mL	Percent Survival[a]	Log CFU/mL	Percent Survival[a]	Log CFU/mL	Percent Survival[a]	Log CFU/mL	Percent Survival[a]	Log CFU/mL	Percent Survival[a]
Strain	**pH: 6.2**	**pH: 2.0**		**pH: 2.5**		**pH: 3.0**		**pH: 6.20**		**pH: 8.0**		**pH: 9.0**	
Ropy 352*	6.86	0	0	0	0	0	0	7.07	100	7.31	100	6.45	94.1
C-13	8.53	0	0	0	0	0	0	8.19	96.0	8.12	95.2	8.19	96.0
ATCC 9596*	8.58	0	0	0	0	0	0	8.37	97.6	8.34	97.2	8.32	97.0
18-1	6.45	0	0	0	0	0	0	6.48	100.	6.30	97.7	6.80	100
4R-7	8.13	0	0	0	0	0	0	8.61	100.0	8.23	100	8.22	100
E*	8.55	0	0	0	0	0	0	8.54	99.9	8.69	100	8.61	100
26-1	8.03	0	0	0	0	0	0	7.56	94.2	7.85	97.8	7.87	98.1
R-1	6.82	0	0	0	0	0	0	6.63	97.1	6.87	100	6.58	96.5
Da-1	8.10	0	0	0	0	0	0	8.12	100	8.24	100	7.99	98.7
ATCC 11007	8.50	0	0	0	0	0	0	8.67	100	8.55	100	8.47	99.6
DRC-3*	9.31	0	0	0	0	0	0	9.18	98.6	9.30	99.9	9.28	99.7
DRC-1*	9.23	0	0	0	0	0	0	9.12	98.8	9.10	98.6	9.18	99.5

[a]Percent Survival = [final log (CFU/mL)/ control log (CFU/mL)] x 100. Numbers are the average of two trials except (*) strains, which were repeated in triplicate.

Presence of *epsM* and *epsN* genes in Exopolysaccharide Producing LAB

Touch-down PCR was used, to detect if any of the EPS producing LAB characterized in this study possessed the unique ropy *eps* genes, *epsM* and *epsN*. Based on previous research, a prediction was made that a non-ropy EPS producing LAB strain will not harbor *epsM* and *epsN*. Of the eleven *L. lactis* strains initially exhibiting thickening properties in liquid dairy media, one strain showed the presence of *epsM*. As illustrated in Figure 6, lane 11, PCR revealed a fragment of DNA (using primers specific to *epsM*) from *L. lactis* subsp. *cremoris* 18-1 that exhibited similar banding pattern as a fragment of DNA from *L. lactis* subsp. *cremoris* Ropy 352 (Figure 6, Lane 3). Additionally, PCR revealed a fragment of DNA (using primers specific to *epsN*) from *L. lactis* subsp. *cremoris* 18-1 (Figure 7, Lane 12) that exhibited similar banding pattern as a fragment of DNA from Ropy 352 (Figure 7, Lane 3). Interestingly, *epsM* and *epsN* appear to have been detected, based on PCR, in *L. lactis* subsp. *cremoris* 18-1, but this *L. lactis* strain does not express the ropy EPS phenotype when cultured on whey agar.

Figure 6. Polymerase chain reaction demonstrating presence of *epsM* gene sequence in EPS producing *Lactococcus lactis* strains.

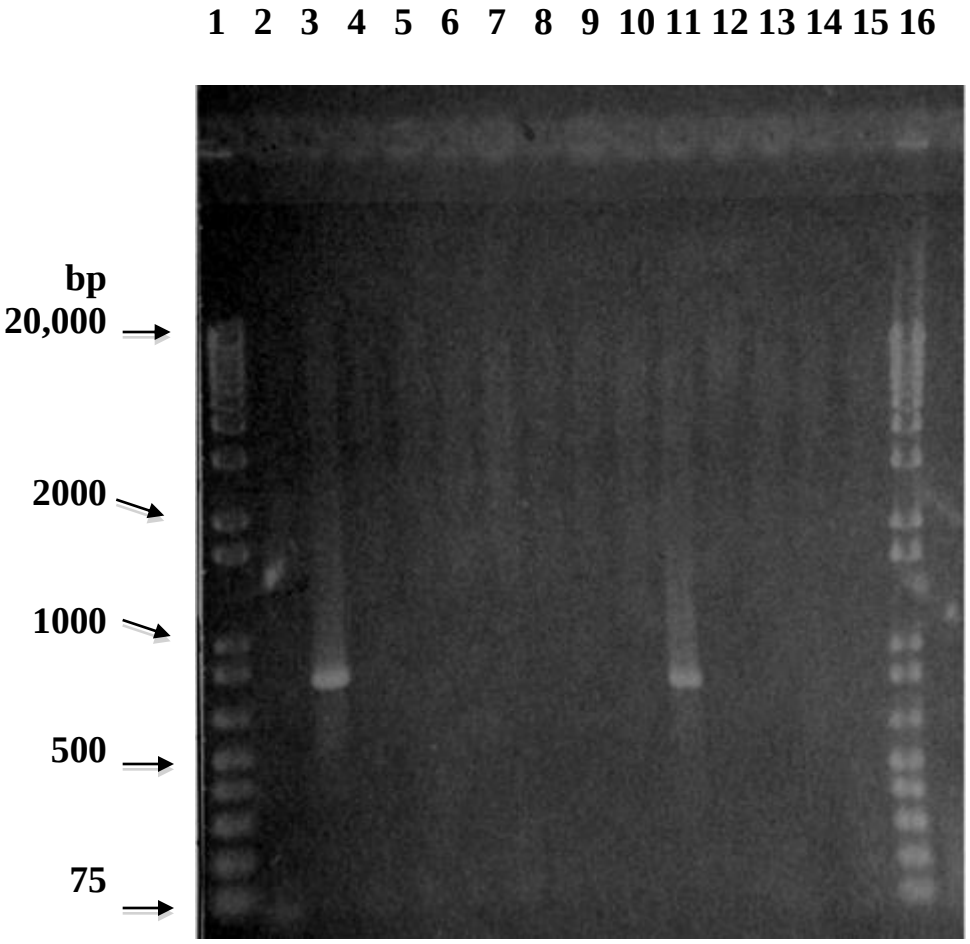

Figure 6. Chromosomal and plasmid DNA was extracted from *Lactococcus* strains. An ethidium bromide stained 1.0 % agarose gel showing DNA fragments produced by PCR amplification of *epsM* gene. Lanes (1) and (16) contains a 1Kb plus DNA ladder (Invitrogen). (2) negative control, sterile ddH_2O, (3); positive control DNA from *L. lactis* Ropy 352. (11) DNA from *L. lactis* 18-1. Lanes (4-10 and 12-15) demonstrate no PCR product amplified from other Lactococcal strains listed in Table 3.

Figure 7. Polymerase chain reaction demonstrating presence of *epsN* gene sequence *in* EPS producing *Lactococcus lactis* strains.

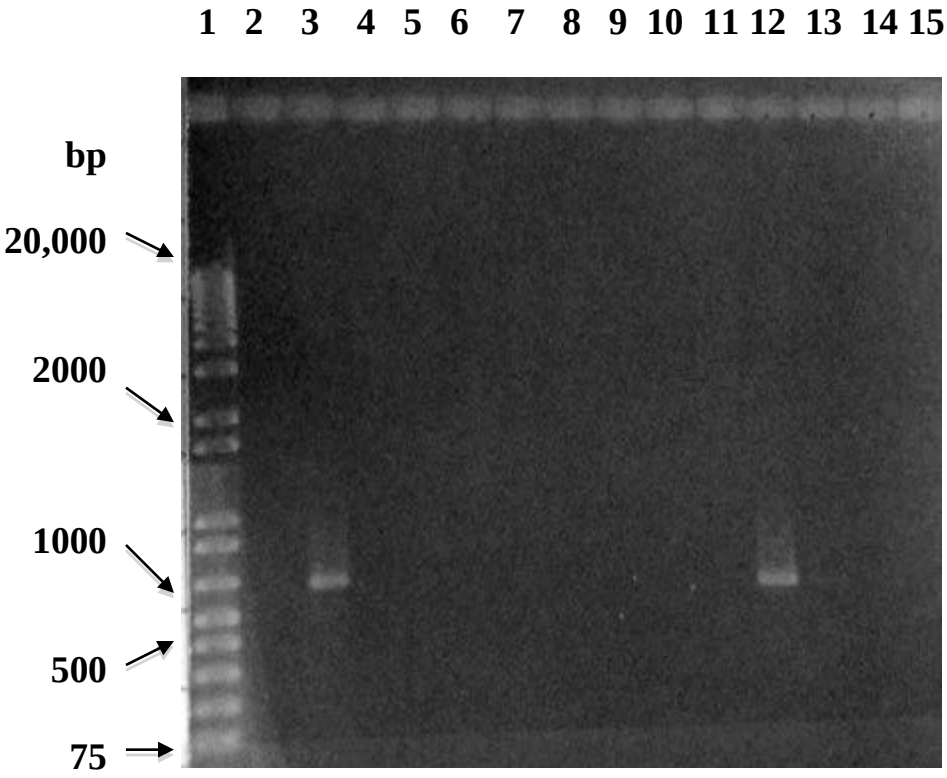

Figure 7. Chromosomal and plasmid DNA was extracted from *Lactococcus* strains. An ethidium bromide stained 1.0 % agarose gel showing DNA fragments produced by PCR amplification of *epsN* gene. Lanes (1) contains a 1Kb plus DNA ladder (Invetrogen). (2) negative control, sterile ddH_2O, (3); positive control DNA from *L. lactis* Ropy 352. (12) DNA from *L. lactis* 18-1. Lanes (4-11) and (13-15) demonstrate no PCR product amplified from other Lactococcal strains listed in Table 3

The other LAB examined in this study (*Leuconostoc* and *S. thermophilus*) were also screened for the presence of the *epsM* and *epsN* gene sequences. Surprisingly, neither *Leuconostoc spp.* exhibited the presence of *epsM* or *epsN* gene sequence (Figure 8, Lanes 4 and 7) despite the thickening properties *Leuconostoc* spp. demonstrated in lactose free milk. *Streptococcus thermophilus* R-39 was the only strain that shown *epsN* sequence and not *epsM*. (Figure 8, Lane 13).

Figure 8. Polymerase chain reaction demonstrating presence of *epsM* and *epsN* gene sequences in *Leuconostoc* spp. and *S. thermophilus.*

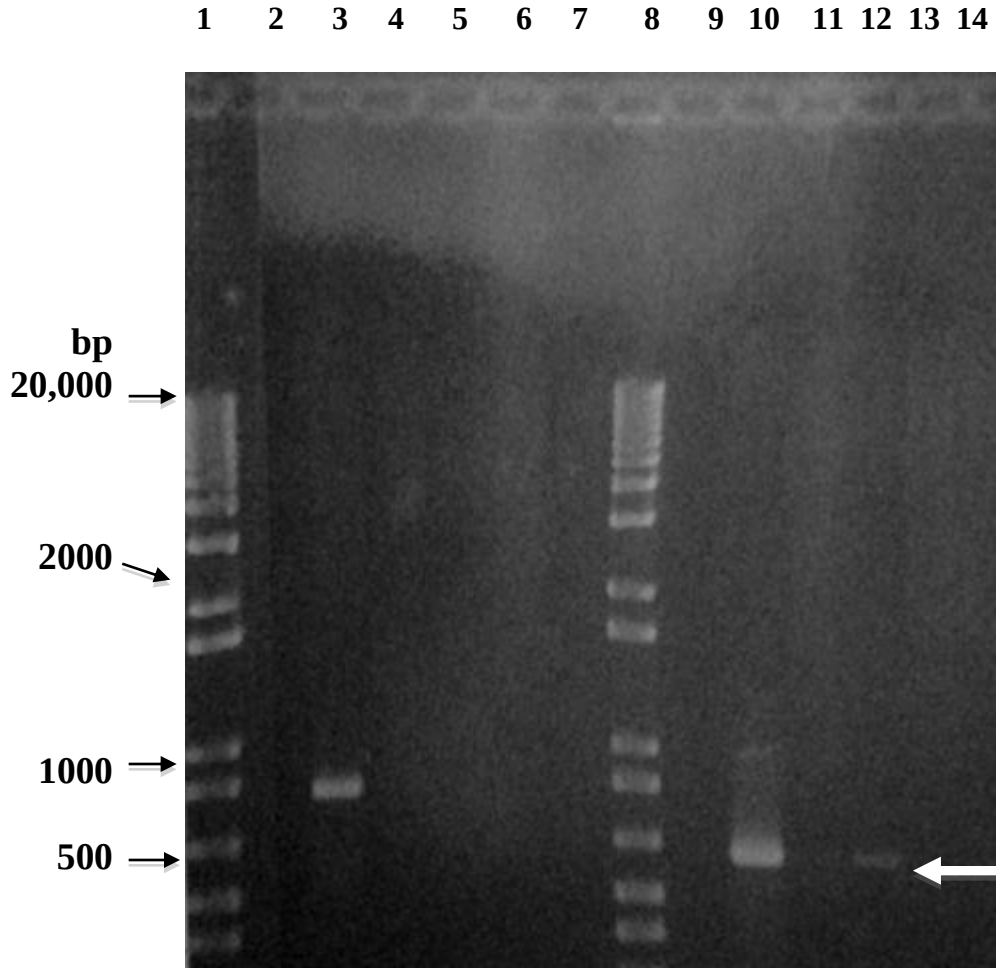

Figure 8. Chromosomal and plasmid DNA was extracted from *Streptococcus thermophilus* strains and *Leuconostoc* spp. An ethidium bromide stained 1.0 % agarose gel showing DNA fragments produced by PCR amplification of *epsN* and *epsM* gene. Lanes (1 and 8) contains a 1Kb plus DNA ladder (Invitrogen). (2 and 9) negative control, sterile ddH_2O, (3 and 10); *epsM/ epsN* positive control DNA from *L. lactis* Ropy 352. (4) *epsM L. mesenteroides* (5) *epsM S. thermophilus* R-39 (6) *epsM S. thermophilus* MC (7) *epsM L. oneos* E26 (11) *epsN L. mesenteroides* (12) *epsN S. thermophilus* R-39 (13) *epsN S. thermophilus* MC (14) *epsN L. oneos* E26. The white arrow indicates *epsN* banding from *S. thermophilus* R-39, similar to *epsN* banding pattern of Ropy 352, lane (10).

Analysis of *epsM* and *epsN* gene sequences from *L. lactis* subsp. *cremoris* 18-1 and *epsN* from *Streptococcus thermophilus* R-39.

PCR product, indicating the presence of *epsM* and *epsN* from DNA extracted from *L. lactis* subsp. *cremoris* 18-1, was sent to Center for Genome Research and Biocomputing (CGRB) for sequencing. Although sequence analysis revealed high homology (99% identity), there were two base pair changes in *L. lactis* subsp. *cremoris* 18-1 *epsM* sequence that resulted in a change in amino acid sequence as compared to the *epsM* amino acid sequence from Ropy 352. The two base pair change in the *L. lactis* subsp. *cremoris* 18-1 *epsM* sequence resulted in a coding change of leucine to isoleucine and asparagine to histidine. (Figure 9).

Figure 9. Image of the alignment of *epsM* from *L. lactis* subsp. *cremoris* 18-1 and *L. lactis* subsp. cremoris Ropy 352.

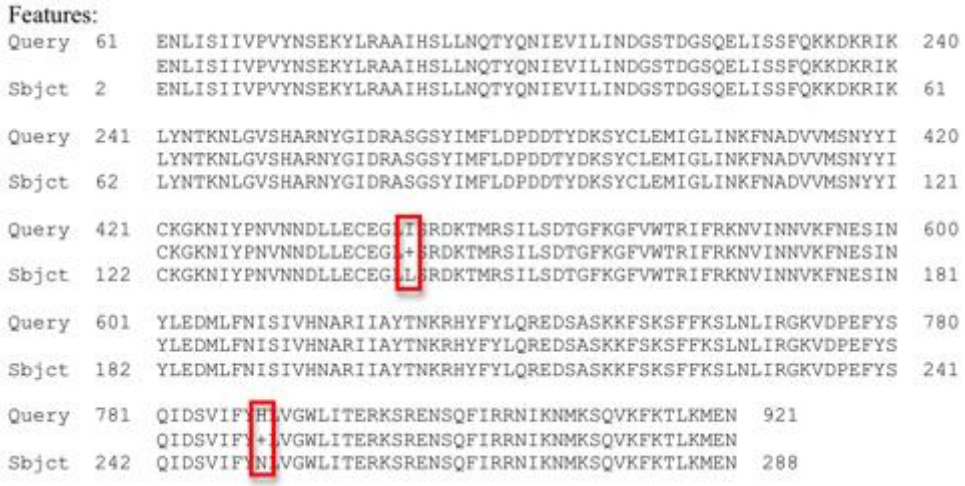

Figure 9. Amino acid sequence result from tBLAST, from sequence data of *L .lactis* subsp. *cremoris* 18-1*epsM*. Query is amino acid sequence from *epsM L .lactis* subsp. *cremoris* 18-1, and subject line is amino acid sequence from *epsM* of *L. lactis* subsp. *cremoris* Ropy 352. Two amino acid differences between the strains is indicated.

Similar to the situation for *epsM*, comparison of the *epsN* sequence from *L. lactis* subsp. *cremoris* 18-1 to that of the *epsN* sequence of Ropy 352 revealed high homology between these two *epsN* sequences (99% identity). However, there were three base pair changes in *L. lactis* subsp. *cremoris* 18-1 *epsN* sequence that resulted in a change in amino acid sequence as compared to the *epsN* amino acid sequence from Ropy 352. The three base pair change in the *L. lactis* subsp. *cremoris* 18-1 *epsN* sequence resulted in a coding change of serine to arginine, serine to alanine, and arginine to histidine. (Figure 10).

Figure 10. Image of the alignment of *epsN* from *L. lactis* subsp. *cremoris* 18-1 and *L. lactis* subsp. *cremoris* Ropy 352.

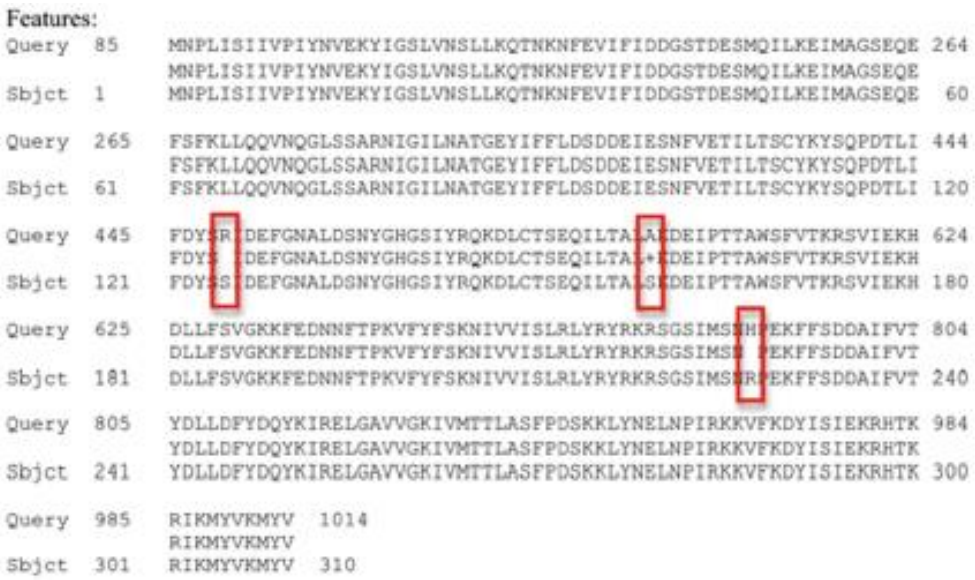

Figure 10. Amino acid sequence result from tBLAST, from sequence data of *L. lactis* subsp. *cremoris* 18-1*epsN*. Query is amino acid sequence from *epsN L. lactis* subsp. *cremoris* 18-1, and subject line is amino acid sequence from *epsN* of *L. lactis* subsp. *cremoris* Ropy 352. Three amino acid differences between the strains is indicated.

Comparison of the *epsN* sequence from *Streptococcus thermophilus* R-39 to that of the *epsN* sequence of Ropy 352 revealed high homology between these two *epsN* sequences (98% identity). However, there were three base pair changes in *S. thermophilus* R-39 *epsN* sequence that resulted in a change in amino acid sequence as compared to the *epsN* amino acid sequence from Ropy 352. The three base pair change, in the *S. thermophilus* R-39 *epsN* sequence resulted in a coding change of serine to arginine, serine to alanine, and arginine to histidine (Figure 11). This was identical to the amino acid changes identified in the *epsN* sequence from *L. lactis* subsp. *cremoris* 18-1.

Figure 11. Image of the alignment of *epsN* from *Streptococcus thermophilus* R-29 and *L. lactis* subsp. *cremoris* Ropy 352.

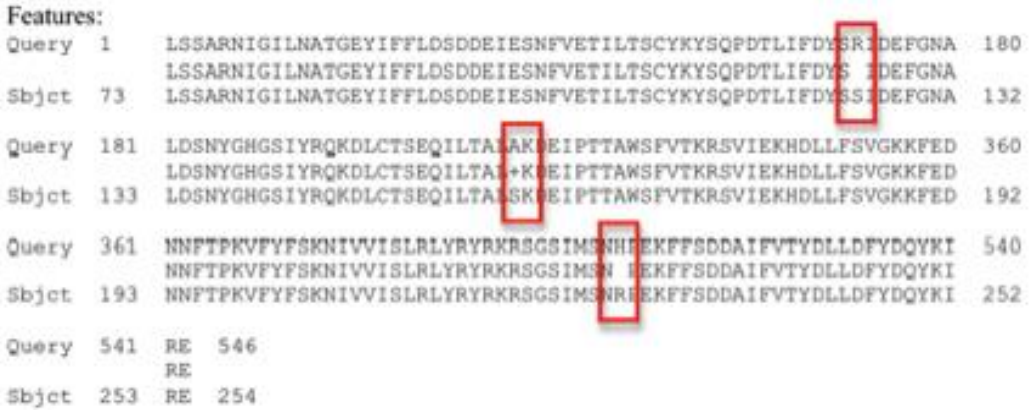

Figure 11. Amino acid sequence result from tBLAST, from sequence data of *epsN S. thermophilus* R-39. Query is amino acid sequence from *epsN S. thermophilus* R-39, and subject line is amino acid sequence from *epsN* of *L. lactis* subsp. *cremoris* Ropy 352. Three amino acid differences between the strains is indicated.

Transfer *epsN* and *epsM* gene from *L. lactis* Ropy 352 to *S. thermophilus* R-39

With the discovery that *S. thermophilus* R-39 did not express a ropy phenotype yet contained the *epsN* sequence and this sequence only differed from the *epsN* sequence of *L. lactis* subsp. *cremoris* Ropy 352 by three base pairs resulting in three amino acid changes, a prediction was made that these three amino acid changes might be crucial to EpsN function and therefore necessary for the ropy phenotype. Additionally, investigation of the importance and role of *epsM* in the biosynthesis of the unique thickening EPS is warranted. One way to test this prediction would be to transfer both the *epsN* and *epsM* plasmid born genes from *L. lactis* Ropy 352 to *S. thermophilus* R-39 and then examine *S. thermophilus* R-39 for the ropy phenotype.

To verify that *S. thermophilus* R-39 lacked naturally occurring plasmids, a plasmid profile of *S. thermophilus* R-39, *L. lactis* subsp. *cremoris* Ropy 352, and *L. lactis* subsp. *cremoris* 18-1 was created (Figure 12). *S. thermophilus* R-39 lacks naturally occurring plasmids, and if this strain contains *eps* genes, these genes are localized to the chromosome. Similar to *L. lactis* subsp. *cremoris* Ropy 352, *L. lactis* subsp. *cremoris* 18-1 contained several naturally occurring plasmids.

Figure 12. Plasmid profiles of *L. lactis* subsp. *cremoris* Ropy 352, *L. lactis* subsp. *cremoris18*-1 *and S. thermophilus* R-39.

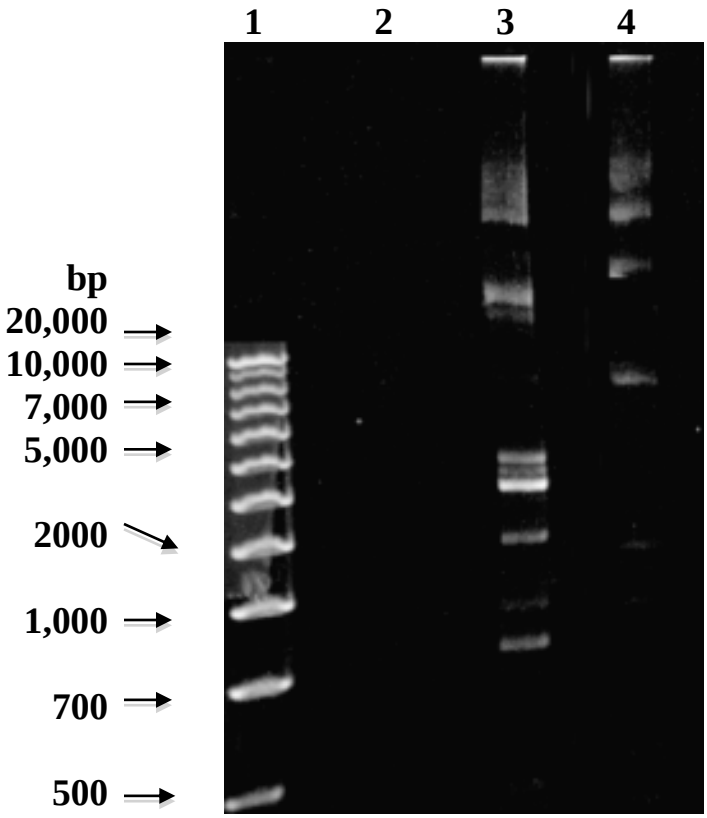

Figure 12. Plasmid DNA was extracted from *Lactococcus* and *S. thermophilus* strains. An ethidium bromide stained 0.5 % agarose gel showing plasmid profiles. Lanes (1) contains a 1Kb plus DNA ladder (Invetrogen). (2) DNA from *S. thermophilus* R-39, (3) DNA from *L. lactis* Ropy 352. (4) DNA from *L. lactis* 18-1.

To maintain GRAS (Generally Regarded as Safe) status, only natural genetic processes (i.e., antibiotic resistant markers can not be used) can be used to transfer the *L. lactis* Ropy 352 *epsN* plasmid born *epsM* and genes into *S. thermophilus* R-39. The difference in growth temperature of the mesophilic of *L. lactis* and thermophilic nature *S. thermophilus* is crucial in designing the experiments. Having a food grade thermophile such as *S. thermophilus* producing the ropy EPS is beneficial in many

dairy food and beverages. Two approaches were designed, one relying on co-culturing to encourage interaction between *L. lactis* Ropy 352 and *S. thermophilus* R-39 and the other using DNA isolated from *L. lactis* Ropy 352 and electroporating this DNA in to *S. thermophilus* R-39. With the first approach, the resulting transconjugant would be selected at a higher temperature (42°C) (to inhibit the growth of the DNA donor, *L. lactis* Ropy 352) and with both approaches, resulting recombinants would be screened for the ropy phenotype. The transfer of *epsM* and *epsN* from the wildtype Ropy 352 was only attempted in *S. thermophilus* R-39 and not with *L. lactis* subsp. cremoris 18-1 that also exhibited presence of *epsM* and *epsN* due to the lack of a selectable trait. Unlike *S. thermophilus spp, L. lactis* subsp. cremoris 18-1 is not able to grow in a high temperature, therefore high temperature can not serve as a selectable trait against Ropy 352.

The first approach, involving co-culturing *S. thermophilus* R-39 with *L. lactis* Ropy 352, resulted in no transconjugants expressing the ropy phenotype. After screening greater than 3,500 potential recombinants for the desired phenotype and not detecting this phenotype, a prediction was made that one of the bacterial strains might be outgrowing the other bacterial strain. Growth rates were determined for both *L. lactis* Ropy 352 and *S. thermophilus* (data not shown). *S. thermophilus* R-39, even grown in mesophilic conditions (30°C), reaches saturation within six hours while *L. lactis* Ropy 352 takes nine hours. The slow growth rate of *L. lactis* Ropy 352 might explain the lack of *S. thermophilus* R-39 ropy transconjugant because the recipient strain, *S. thermophilus* R-39 outgrows the donor strain *L. lactis* Ropy 352 under these growth conditions.

The second approach was to extract DNA from *L. lactis* Ropy 352 and electroporate into competent *S. thermophilus* R-39 cells. Competent *S. thermophilus* R-39 was prepared by supplementing growth media with glycine. Varying concentrations of glycine were tested to see the growth rate of *S. thermophilus* R-39 (data not shown). Three percent glycine was selected to maximize bacterial cell wall leakiness. After screening greater 3,500 electroporated colonies for the ropy phenotype, this approach failed to yield recombinant *S.thermophilus* R-39 with a ropy phenotype.

Chapter 5

Discussion

Phenotypic Characterization of Lactic Acid Bacterial strains in Dairy Milk

Lactococcus lactis, a member of Lactic Acid Bacteria (LAB), are important starter cultures in modern food production of fermented products. LAB produce various compounds as part of this species' natural metabolism and physiology. A unique compound secreted outside the bacterial cell is a polymer structure composed of various sugar linkages called exopolysaccharides (EPS). Exopolysacchrides have been found to enhance desirable characteristics such as mouth-feel, moisture content, and rheology of cheese and yogurt. A natural isolate, *Lactococcus lactis* subsp. *cremoris* Ropy 352 was previously characterized to have the ability to change liquid milk viscosity. The first objective of this study was to find other LAB strains capable of thickening milk. The approach used to address this objective included inoculating lactose free milk and 1% fat milk with LAB strains from Oregon State University's strain collection and observe for changes in viscosity or thickness of the milk based media.

All bacterial taxa are capable of synthesizing polysaccharides that are excreted into the environment (47). Exopolysaccharides are long chained polymers made up of polysaccharides that can either be secreted out into the environment or loosely attached to bacterial cell walls. Composition of EPS structure is influenced by varying ratios of sugar subunits, mainly glucose, mannose and galactose. Eighty *L.*

lactis strains were tested to find if EPS was produced and was capable of thickening milk-based media. Of the eighty strains tested, only eleven *L. lactis* strains demonstrated a milk thickening phenotype similar to Ropy 352 when cultured in milk-based media. EPS phenotype spans all three *Lactococcus* subspecies: *L. lactis* subsp. *cremoris* strains, *L. lactis* subsp. *lactis*. and *L. lactis* subsp *lactis*. biovar *diacetylactis*. *Cremoris* subspecies are commonly preferred in cheese starter cultures because this subspecies imparts desirable flavor and aroma (34). *L. lactis* subsp. *cremoris* ATCC 9596 and *L.lactis* subsp. *lactis* E were able to thicken both the 1% fat milk and lactose free milk similar to Ropy 352. While *L. lactis* subsp. *cremoris* 26-1 showed more thickening capability in 1% fat milk in comparison to the other strains tested, most of the strains appeared to prefer lactose free milk for producing EPS. Lactose free milk contains glucose and galactose, which are simple monosaccharide. The molecular structure of EPS influences thickening properties. Highly branched and high molecular weight EPS will have a greater chance of physically entangling, causing a more viscous solution. The findings suggest that the structural composition of EPS produced by the *L. lactis* strains cultured in 1% fat milk might be different from the EPS produced in lactose free milk.

Streptococcus thermophilus strains and *Leuconostoc* species were cultured in milk-based media as previously described. None of the *S. thermophilus* strains were able to thicken milk; however, they were able to coagulate milk within 24 hours of incubation. Milk coagulation is the event where milk protein casein micelles aggregate to each other due to their hydrophobic nature. Under normal conditions, a surface-active protein, called kappa-casein, prevents casein micelles from sticking to

each other, surrounding casein micelles and resulting in negatively charged casein micelles repelling each other. However, during fermentation, when pH is acidic, this condition neutralizes the negative charged casein micelles, leading to coagulation. Such an event is a crucial step in making cheese. The observation that *S. thermophilus* coagulates rather than thickens milk suggests growth of *S. thermophilus* in milk based media leads to an acidic environment that might inhibit EPS formation.

Both *Leuconostoc mesenteroides* and *Leuconostoc oneos* E26 produced EPS with thickening property only when cultured in lactose free milk. Lactose free milk contains galactose and glucose, achieved by the enzymatic action of lactase. Lactase, also known as lactase-phlorizin hydrolase (LPH), is a member of the β-galactosidase family of enzymes, that cleaves lactose molecules into galactose and glucose.

The same observation was made for the *L. lactis* strains tested, where thicker solution resulted when cultured in lactose free milk compared to 1% fat milk. This observation suggests that biosynthesis of EPS by LAB utilizes the simpler form of sugar or monosaccharide moiety of galactose and glucose more readily than the disaccharide form, lactose.

Additionally, when Ropy 352 is inoculated in coconut and rice milk, thickening is observed (data not shown). Interestingly, Ropy 352, when inoculated in soy and almond milk, did not thicken these non-dairy based milks (data not shown). Growth conditions like carbon/nitrogen source, pH, temperature, and incubation time are known to affect EPS biosynthesis and structure. With the goal to maximize EPS production for industrial purposes, other growth media or carbon sources have been tested. *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 produced three times

more EPS when cultured in glucose than fructose (30). Other EPS producing LAB, *Lactobacillus rhamnosus* C83, *Lactobacillus casie* CG 11 and *Streptococcus salivarius* subsp. *thermophilus*, also showed similar results, in that EPS production is influenced by sugar source. Possiblly, a simple substrate source, such as glucose and galactose, is more favorable for the production of EPS.

Leuconostoc species undergoes heterofermentation and is used for its aromatic products to mainly manufacture buttermilk. *Leuconostoc mesenteroides* is capable of producing high amounts of diacetyl, which is synonymous to the "buttery" flavor distinct to butter products metabolized from citrate and pyruvate (100). *Leuconostoc oneos* is used in wine fermentation because of its ability for malolactic fermentation. Malolactic fermentation creates a lower acidity, enhancement of the organoleptic characteristics, and controls the microbiological load of wine (102). Formation of EPS in wine products, described as "ropiness"or "oiliness," is considered to be a quality defect and is deemed unfit for consumers (59). *Leuconostoc* spp. screened in this study might not be beneficial in wine production due to its undesirable ability to produce EPS, however products such as buttermilk can benefit with the production of EPS by *Leuconostoc* spp.

Survival in milk environment requires the bacterium to degrade lactose sugar or metabolize milk protein, casein. The lack of thickening phenotype in regular 1% fat milk can be attributed to the bacterium's inability to produce β-galactosidase to breakdown lactose into glucose and galactose. Alternatively, *L. lactis* strains will breakdown casein as a source of amino acids and substrates for energy production, however this metabolism does not supply the cell with sugar substrates possibly

affecting the strains capability to produce EPS. The ability for lactose fermentation is one of the traits known to be plasmid borne (31). Absence of this crucial enzyme can plausibly explain why some strains did not produce the EPS to thicken 1% fat milk. Future investigation should examine if the LAB strains that thicken lactose free milk will also demonstrate the same phenotypic result in 1% fat milk supplemented with glucose.

Characterization of EPS Producing Lactic Acid Bacteria

Food safety and food quality has been the utmost concern of major food companies. In fermented products that contain live microorganisms, the use of bacterial starter cultures that have evolved sophisticated mechanisms to inhibit growth of other microorganisms is desirable. One objective of this study was to phenotypically describe if a link exists between production of *L. lactis* EPS and microbial inhibitory activity. Some EPS producing *L. lactis* strains produced inhibitory compounds that prevented the growth of pathogenic *B. cereus* and/or non-pathogenic *B. subtilis*, as evident by the presence of measurable zone of growth inhibition. There is no clear correlation between presence of EPS and inhibition of *Bacillus* species growth in that not all EPS producing *L. lactis* strains were able to inhibit the growth of the *Bacillus* species tested. Bacterial strains, such as *L. lactis* subsp. cremoris Ropy 352, *L. lactis* subsp. *lactis* E, *L. lactis* subsp. *cremoris* 26-1 and *L. lactis* subsp. *cremoris* 18-1 induced no measurable zone of growth inhibition in the presence of any of the *Bacillus* species tested, suggesting that EPS is not linked to antimicrobial activity and that other active compounds are involved.

L. lactis strains are known to produce bacteriocins. Bacteriocins are a unique class of compounds excreted by certain organisms capable of inhibiting growth of closely related organisms. Nisin is a well studied and common bacteriocin produced by lactococcal strains. The presence of nisin was investigated with the use of Touch-down PCR targeted to *nisA*. Extracted DNA from EPS producing *L. lactis* strains did not show the presence of the *nisA*, suggesting that the inhibitory compound, exhibited by some of the EPS producing *L. lactis* strains, is not due to the nisin mechanism. There are two forms of nisin; nisin A and nisin Z. Two sets of primers were designed within the region that is identical for both *nisA* or *nisZ*; both sets of primers failed to amplify DNA from the eleven EPS producing *L. lactis* strains. These primer sets did amplify DNA extracted from a known nisin producing strain, *L. lactis* subsp. *cremoris* ATCC 11454. Other types of bacteriocins produced by *L. lactis* have been identified. Three more natural nisin derivatives, Q, U, and F variants, have been identified (45). In this study, only the nisin variants, A and Z, were screened for in the eleven EPS producing *L. lactis* strains. Further studies should include examining the eleven EPS producing *L. lactis* strains for the Q, U, F variants of nisin.

Other lantibiotics, that are completely different in function and structure, include the single peptide, lacticin 481, and the two-component, lacticin 3147 (13). *L. lactis* is also capable of producing non-lantibiotic bacteriocins such as lactococcin, MMFII, G, M, A and 972 (101, 67). Presence of either lantibiotics or non-lantibiotics may account for the results in this study. Alternatively, environmental conditions, such as pH, hydrogen peroxide, and organic acids, created by growth of *L. lactis* could have affected the growth or survivability of the *Bacillus* species. Interestingly,

the eleven EPS producing *L. lactis* strains do not inhibit growth of a non-pathogenic strain of *Escherichia coli* (preliminary data; data not shown), further suggesting that a bacteriocin is indeed at play. Nisin is effective in inhibiting growth of Gram-positive but not Gram-negative bacteria, such as *E.coli*. The architecture of the bacterial outer membrane of Gram-negative bacteria prevents the successful penetration of nisin into the cytoplasmic membrane, and therefore confers protection from bacteriocins produced by Gram-positive bacteria. Incorporation of bacteriocin producing starter culture is appealing for food manufacturers, where this type of culture can potentially be economically advantageous due to the use of fewer additives to achieve longer shelf life. In the United States, purified bacteriocins are classified as a natural food additive, and therefore has a wide range of approved uses. However, in European countries, where they are more hesitant in using food additives, such as bacteriocins, European companies avoid this practice. Interestingly, that the newly characterized EPS producing *L. lactis* strains that did show inhibitory activity do not harbor nisin genes. Furthermore, additional investigation is needed to identify the exact inhibitory compound that caused growth inhibition of *Bacillus cereus* and or *Bacillus subtilis.*

Role of Exopolysaccharide in Harsh Growth Environment (extreme pH)

Exopolysaccharides have been suggested to play a role in bacterial cells' overall survival and fitness. In this study, the role of EPS and its potential protective property was investigated. Additionally, characterizing EPS producing *L. lactis* strains' pH limitations will provide insight to these strains' possible recognition as probiotic. According to the FAO/WHO guidelines, for microorganisms to be

considered probitotic, they must be able to withstand extreme pH as they travel down the gastrointestinal tract (20) The performance of the EPS producing *L. lactis* strains suggest these strains are able to withstand highly alkaline (pH: 8.0 and 9.0) conditions, with a high percentage of viable cells remaining after exposure. However, in extreme acidic conditions no cells survived. Preliminary data suggest that some of the EPS producing *L. lactis* strains are capable of surviving a pH as low as 3.5 (data not shown). In a food system, inoculation of *L. lactis* in milk is capable of reducing the pH to 4.5-5.0 (60). Food systems can potentially provide a protective barrier for the cells to survive the GI tract. In a study comparing ropy and non-EPS producing derivative of *Lactobacillus paraplantarum* BGCG11, all strains survived *in vitro* GI conditions only when included in a food matrix (1% skimmed milk) (63). Additionally, when cultures without a food matrix were exposed to the same conditions, even those strains with a ropy EPS phenotype did not improve survival (63). Although data suggest that the eleven EPS producing *L. lactis* strains in this study are unable to survive acidic conditions below pH 3.5, results might change if these strains are incorporated into a food matrix.

Role for *epsM* and *epsN*

A mutant Ropy 352 strain, *L. lactis* EK 240, was generated when an insertion element disrupted one of the glycotransferase genes, *epsN*. When analyzed phenotypically, this Ropy 352 mutant no longer expressed the ropy EPS phenotype when cultured on whey agar plates (42). In this study, when the Ropy 352 mutant, *L. lactis* EK 240, was inoculated in milk based media, this mutant failed to thicken

either the 1% fat milk or the lactose free milk. The lack of thickening activity further suggested that indeed the disruption in the *epsN* inhibited *L. lactis* Ropy 352 from producing thickening EPS. The majority of the *eps* gene cluster located on pEPS352 is upstream from *epsM* and *epsN* separated by 10,596 base pairs (Figure 13). Despite the location of *epsN* and *epsM* downstream 10,596 base pairs from the main *eps* gene cluster (Figure 13), the mutation in *epsN* was adequate to prevent both the ropy and thickening phenotypes. EpsN is a glycosyltransferase enzyme involved in facilitating sugar linkage formations during EPS biosynthesis.

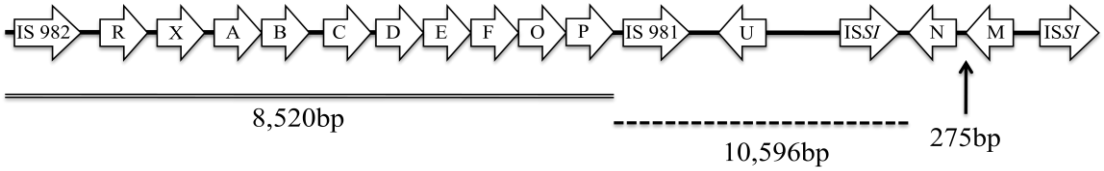

Figure 13. Plasmid pEPS352, *eps* genes cluster from *L. lactis* Ropy 352. Adapted from Knoshaug et al (42).

A study analyzing the phenotype of a *Lactobacillus rhamnosus* GG *welE* (glycosyltransferase) mutant revealed a three-fold decrease in EPS concentrations, and the EPS made by this mutant was structurally different compared to the wildtype EPS (48). Similar observations were made in regards to the role of glycosyltransferases in EPS biosynthesis in *S. thermophilus* (57) and *L. lactis* (104). Possible complications occur when EPS production is synthesized prematurely, meaning that the complete EPS structure was not completed, due to absence of certain enzymes, like glycosyltransferase. The incomplete heteropolysacharides or EPS formation is unable to be recognized by polymerizing and export enzymes.

The eleven newly characterized EPS producing *L. lactis* strains were screened by Touch-down PCR to determine if the unique *epsM* and *epsN* gene sequences of Ropy 352 were present. Presumably, a non ropy producing strain will lacks *epsM* or *epsN* gene sequences. One strain, *L. lactis* subsp. *cremoris* 18-1 was confirmed to possess both genes. Sequence analysis of *L. lactis* subsp. *cremoris* 18-1 *epsM* and *epsN* genes revealed two and three basepair changes, respectively, giving rise to amino acid changes that might influence the normal function of the *espM* and *espN* gene products. Interestingly, the same three amino acid changes in the *epsN* gene product from *L. lactis* subsp. *cremoris* 18-1 were found in the *epsN* gene product from *S. thermophilus* R-39. Using the full *epsM* translated gene sequence to BLAST search other bacterial genomes for sequence homology to *espM* from Ropy 352, the search resulted only in the identification of a low identity of 35-36% sequence from *Streptococcus pneumoniae* strains. Using the full *epsN* translated gene sequence to BLAST search other bacterial genomes for sequence homology to *espN* from Ropy 352, the search revealed that the top hit came from *S. thermophilus* ND03 with a sequence having 94% identity and the second hit came from *Leuconstoc mesenteroides* subsp. *meseneteroides* ATCC 8293 with a sequence having only 57% identity. *Streptococcus thermophilus* ND03 genome sequence was recently completed and showed high homology with other *S. thermophilus* genomes available, LMG18311 (7), CNRZ1066 (7), and LMD-9 (53). In areas that were unique to *S. thermophilus* ND30, *eps* genes varied greatly with the EPS biosynthesis cluster where six more unique genes, *epsE*, *epsF*, *epsG*, *epsI*, *epsJ*, and *epsP* were found (89).

Sequence data suggest that *epsM* may be more unique because, as to date, only *L. lactis* subsp. *cremoris* Ropy 352 and *L. lactis* subsp. *cremoris* 18-1 are reported to have this gene. Interestingly, no other LAB species whose genome sequence was available in the GeneBank database, show the presence of *epsM* sequence, other than the *L. lactis* subsp. *cremoris* 18-1 newly discovered in this study. Furthermore, even with *L. lactis* subsp. *cremoris* 18-1 *epsM* and *epsN* sequences highly homologous to the *L. lactis* Ropy 352 *epsM* and *epsN* sequences, *L. lactis* subsp. *cremoris* 18-1 failed to express the ropy phenotype on whey agar plates, although this *L. lactis* strain could still thicken milk in a manner similar to Ropy 352. Taken together, these observations suggest that the two amino acid difference in *L. lactis* subsp. *cremoris* 18-1 *epsM* gene product and the three amino acid difference in the *L. lactis* subsp. *cremoris* 18-1 *epsN* gene product may define whether the full ropy phenotype is present or absent. Interesting questions remain about the evolution and gene transfer of *eps* genes among *Lactococcus* and *Streptococcus* species, such as the origin of the unique *epsN* and *epsM* and their role in the biosynthesis of the desirable ropy EPS phenotype.

Transfer of *epsN* and *epsM* from Ropy 352 to *S. thermophilus* R-39

One of the unique genes, *epsN,* that appears to contribute to the ropy phenotype of *L. lactis* subsp. Ropy 352 was discovered to be present in *S. thermophilus* R-39 genome. *epsN,* coding for a glycosyltransferase activity in *S. thermophilus* R-39, contained three base pair changes resulting in three amino acid changes in the *epsN* gene product as compared to the *epsN* gene product of *L. lactis*

Ropy 352. These sequence changes in *epsN* and the absence of detectable *epsM* may account for the lack of the ropy phenotype in *S. thermophilus* R-39. The goal for this experiment was to employ natural methods of gene transfer, so as to retain GRAS (Generally Regarded As Safe) status using either co-culturing or electroporation of DNA isolated from Ropy 352 with the goal to transfer both, *epsN* and *epsM* genes from *L. lactis* Ropy 352 to *S. thermophilus* R-39 and observe thickening phenotype. The distance between *epsM* and *epsN* is only 275 base pairs (Figure 13). Although, *S. thermophilus* R-39 does not harbor sequence similarity to *epsM*, the predication was that due to the high sequence homology between the *epsN* of *S. thermophilus* R-39 and Ropy 352 and small distance between *epsM*, that when homologous recombination event occurs, *S. thermophilus* R-39 will acquire both *epsN* and *epsM* in the process. Mobilizing the *epsM* and *epsN* genes from *L. lactis* Ropy 352 to *S. thermophilus* R-39, a prediction was made that expression of the ropy phenotype would result. Dairy companies, such as yogurt producers, find ropy EPS producing *S. thermophilus* desirable. Yogurt products have a "standard of identity" designation under federal laws, describing that yogurt strickly must "contain the lactic acid-producing bacteria, *Lactobacilius bulgaricus*, and *Streptococcus thermophilus*." Due to manufacturing methods used by yogurt companies, where fermentation is set at high temperature, greater than 40°C, mesophilic strains of EPS producing *Lactococcus* are unable to withstand the thermophilic fermentation conditions and thus benefits afforded by EPS are unavailable. Unfortunately both methods used to transfer the *epsN* and *epsM* genes from *L. lactis* Ropy 352 to *S. thermophilus* failed,

and thus a recombinant *S. thermophilus* R-39 that expresses the "ropy" phenotype has yet to be generated.

Recently it was documented that *S. thermophilus* has the ability to become naturally competent in growth medium lacking peptides or in nutrient starved-environments (23). Natural competence is well studied in *Streptococcus pneumoniae*, where a competence-stimulating peptide or CSP is secreted in the environment and then taken up by the bacterial cell, leading to the expression of competence genes (*ComX*) and later for DNA uptake machinery (36). The same process is thought to occur in *S. thermophilus* in the early stage of competency, when a peptide called ComS is secreted into the environment to lead to the expression of *comX* (24). This event is time sensitive, such that production of ComS occurs in nutrient deficient environment or at saturating phase. A challenge for successful competency in *Streptococci* is that the presence of the alternative sigma factor, ComX is transient and is degraded by a Clp protease. In *S. pneumonia*, ComX is degraded by ClpEP and in *S. thermophilus* a different protease, ClpC degrades ComX (6). To increase frequency of competency in *S. thermophilus* strains, one or the combination of increasing the competency signal of ComS or ComX, and inhibiting the action of the ClpC protease might enhance future efforts to transform *S. thermophilus* R-39 with both the *epsN* and *epsM* genes from *L. lactis* Ropy 352.

The second method that might increase the successful transfer of *L. lactis* Ropy 352 *epsN* and *epsM* genes to *S. thermophilus* R-39 is to create a competent *S. thermophilus* R-39 with incorporation of glycine in the bacterial cell wall, causing leakiness and thus enhancing frequency of DNA uptake through electroporation. *L.*

lactis Ropy 352's *eps* gene cluster is located on a 22kb plasmid called pEPS352. Incompatibility of plasmid is avoided due to the lack of existing plasmids as suggested by the plasmid profile of *S. thermophilus* R-39. Chromosomal and plasmid DNA was extracted from Ropy 352 and was used to electroporate the R-39 strain. Alotough no recombinants resulted, further experiments might use purified pEPS352 to transform, through electroporation, *S. thermophilus* R-39.

Natural methods (selectable marker free) of gene transfer were an integral part of the experimental design in this project due to the possible food application once a successful strain is constructed. Growing concerns about the safety of Genetically Modified Organisms (GMO) is still present and widely debated. Members of the European Union (EU) have more rigid regulations, as it relates to GMO products, in comparison to the United States. Currently, EU products that contain GMO need to be indicated in the label. Although many opponents of GMO are more concerned about GMO plants and how these plants will affect the environment, critics have generalized various types of GMO products, with the goal to ban all GMOs. Despite GMO bacteria mainly being used as "manufacturing factories" of certain food ingredients, flavoring, or additives there is still opposition to their use in the food industry. The consumption of GMO bacteria, such as GMO-Lactic Acid Bacterial strains, falls in a poorly defined area, where some consider them as food when used in fermented products while others regard them as medicine due to probiotic benefits (87). Only time will tell how consumers' perception of GMO products, targeted for human consumption, will evolve.

Chapter 6

Conclusion

This study characterizes the ability of Lactic Acid Bacteria (LAB) to produce exopolysaccharide (EPS) that have unique viscoelastic properties when cultured in dairy media. The goal of this research effort is to discover new EPS producing LAB and to expand understanding of the potential roles and biosynthesis of EPS. Increased understanding will lead to potentially new EPS compounds that can play major roles in developing and enhancing certain food products. Bacterial derived EPS technology is advantageous for the food industry, and also potentially valuable in biotechnology, pharmaceutical, medicine, and petroleum industries.

Eleven *Lactococcus lactis* strains and two *Leuconostoc spp* were described to produce EPS as evident by the ability to thicken milk. A majority of the characterized EPS strains exhibited the thickening phenotype when cultured in lactose free milk. However, none of the *L. lactis* strains exhibited the ropy EPS phenotype when cultured on whey agar plates. Additionally, *L. lactis* strains were examined for production of antimicrobial compounds in which some strains tested positive for the presence of these compounds. Not all EPS positive LAB did produce detectable inhibitory compounds therefore a possible conclusion is that EPS is not linked to the antimicrobial activity. Inhibition of microbial growth was not the result of the nisin bacteriocin. Inhibitory activity might be attributed to other types of bacteriocin or

novel inhibitory compounds. *L. lactis* strains demonstrated high survivability after exposure in highly alkaline (pH 8.0 and 9.0) conditions.

In this study the roles of two unique EPS genes, *epsM* and *epsN* were also investigated. A natural dairy isolate, *Lactococcus lactis* subsp. *cremoris* Ropy 352 had previously been described to contain *epsM* and *epsN* and their functions were linked, by mutational analysis, to the ropy phenotype when cultured in whey agar and in milk. Strain *L. lactis* subsp. *cremoris* 18-1 was identified to contain *epsM* and *epsN* but this *L. lactis* strain does not express ropy phenotype, possibly due to non-functional EpsM and EpsN gene products. Non-ropy LAB, *Streptococcus thermophilus* R-39, was also found to contain the *epsN* gene, with an identical sequence to that of the *epsN* gene from *L. lactis* subsp. *cremoris* 18-1.

This study provides additional insight into the diversity and complexity of bacterial exopolysaccharides. Future directions for this research effort will include chemically characterizing the unique EPS produced in lactose free milk in comparison to the EPS when cultured in regular 1% fat milk. EPS producing LAB strains that did not thicken regular milk should be tested for the ability to degrade lactose. Further investigation in the origin and evolutionary history of the two unique *epsM* and *epsN* is needed to fully understand the role the gene products play. A ropy EPS producing *S. thermophilus* R-39 is still desirable for its potential application in yogurt production. Collectively, answering why and how EPS biosynthesis occurs can lead to advancement in development of “designer” EPS that will contain desirable characteristics. Lastly, other important food industry characteristics such as phage sensitivity, aroma and flavor compounds of EPS producing LAB, especially

Lactococcus strains, need to be a continued area of investigation. The outcome can potentially lead to the development and marketing of novel EPS producing starter cultures for fermented products that provide added health benefits to consumers.

Chapter 7

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