AN ABSTRACT OF THE THESIS OF

Eric P. Knoshaug for the degree of Master of Science in Microbiology presented on June 5, 1998. Title: Exopolysaccharide Biosynthesis by a Natural Lactococcal Ropy Isolate

Abstract approved: Redacted for Privacy

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The genes coding for the expression of a ropy exopolysaccharide responsible for commercially desirable textural and rhealogical traits in fermented milk products by a natural lactococcal ropy isolate were sought. Using a transposon mutagenesis vector, pGh9:IS*1*, three mutants lacking expression of the ropy exopolysaccharide were isolated. One of the mutants was chosen for further characterization. Using a Southern hybridization analysis, the interrupted gene was localized to the chromosome. The non-ropy mutant was further characterized and shown to be unable to produce ropy exopolysaccharide in fermented milk.

A 2006 bp fragment of the interrupted gene was sequenced. The DNA sequence over a short region showed homology to sugar transfer enzymes found in exopolysaccharide biosynthesis pathways. The DNA sequence was translated into its predicted amino acid sequences and two partial open reading frames of 236 and 338 amino acid residues in length were identified. These open reading frames were found to exhibit identity to glycosyltransferases present in exopolysaccharide biosynthesis pathways in other bacteria.

Exopolysaccharide Biosynthesis by a Natural Lactococcal Ropy Isolate

by

Eric P. Knoshaug

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed June 5, 1998
Commencement June 1999

Master of Science thesis of Eric P. Knoshaug presented on June 5, 1998.

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Eric P. Knoshaug, Author

ACKNOWLEDGMENTS

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

First, I would like to acknowledge my advisor, mentor, and friend, Dr. Janine Trempy. It was your enthusiasm for science and this project, having a quarters worth of work all ready to go when I first walked into the lab on my first rotation as a graduate student, that furthered my interest in science and dedication to this project. Thank you for the privilege of working in your lab.

I would like to thank my committee members, Dr. Geller and Dr. Bakalinsky, for their time, support, and suggestions and comments regarding my research project. I would also like to thank Dr. Leong for her time.

I would also like to thank members of the Trempy lab: Monica Skinner, Drew Sellers, Gary Vaughn, Howard Peters, Wolfgang Ebel, Karen Dierksen, and all the undergraduate students who have rotated through the lab for their companionship, their help with my project, and their humorous antics that made the lab a fun place to work.

Thanks also to Mary Burke and Susan Williams for entrusting me with the responsibilities of teaching undergraduate students and for showing me how to enjoy teaching as much as they do.

I would also like to thank the office and building staff, Cindy Fisher, Camille Partridge, Marilyn Wallace, Carlene Pelroy, Gayle Jolliffe, and Lynn Wyatt, for their tireless efforts in keeping the labs and department running smoothly.

And finally, I would like to thank all the graduate students and faculty for making the department an enjoyable and inspiring community.

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DEDICATION

This work is dedicated to my wife, Jessica, for always being supportive and allowing me to drag her out to Oregon. This work is also dedicated to my parents for their support and love throughout my struggles and successes.

EXOPOLYSACCHARIDE BIOSYNTHESIS BY A NATURAL LACTOCOCCAL ROPY ISOLATE

INTRODUCTION AND LITERATURE REVIEW

Research Topic

Lactic acid bacteria have historically been used in foods such as cheeses, fermented milks, vegetables, breads, and continue to be an integral part of modern food production and processing. Many functional characteristics possessed by lactic acid bacteria are responsible for their abundant commercial use and have been the subject of many attempts to improve their behavior. Consumers are also becoming more interested in healthy, natural foods and recognize dairy foods produced with lactic acid bacteria as an important part of the diet. Some strains of *Lactococcus* have been used for fermented milk production in Scandinavian countries due to their expression of a thick, viscous exopolysaccharide. Interest in these exopolysaccharides as food additives has increased but before large scale utilization of these exopolysaccharides becomes possible, the gene operons coding for eps biosynthesis must be isolated and characterized.

Definition of Lactic Acid Bacteria

Lactic acid bacteria (LAB) are generally defined as non-spore forming, aero- and acid- tolerant, fastidious, Gram positive cells, that lack an electron transport chain and thus rely upon fermentation for energy production with lactic acid as the major end-product (1). As fastidious organisms, LAB are generally found in nutrient rich habitats such as food products, vegetables, and intestines and mucosal membranes of mammals. Although fastidious, most genes encoding enzymes important for amino acid biosynthesis are present but may be silent (1). Genome sizes of LAB range in size from 1.1 to 2.6 megabases (5). Lactic acid bacteria are a very heterogeneous group and have been historically classified into different genera based on phenotypic characterization but

continue to go through taxonomic revision as better methods of differentiating the genera evolve. Current methods rely upon ribosomal RNA comparisons. Recent taxonomic revision includes the following genera in the LAB group: *Aerococcus, Alliococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella,Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Oenococcus. Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (1). LAB cluster with the low, less than 55%, G + C group. The genera *Bifidobacterium* and *Propionibacterium,* often considered LAB, have been shown to have a high G + C content and are thus not included in the LAB taxonomic group (1).

Commercially Important Characteristics of Lactic Acid Bacteria

Several genera of LAB possess important physiological functions of interest to the dairy industry including production of lactic acid, protein hydrolysis, aroma synthesis, inhibitory substance formation, bacteriophage resistance, IS element activity, and exopolysaccharide biosynthesis. All of these functions are critical to the quality of the finished product and have been studied extensively. LAB used in the dairy industry can be broken down into two groups, mesophilic and thermophilic. The mesophiles have an optimum growth temperature of 30°C and are made up of species in the *Lactococcus* and *Leuconostoc* genera. Thermophiles exhibit optimum growth at 40-50°C and include *Lactobacillus* species and *Streptococcus thermophilus* (34). Regardless of the temperature group, LAB used in dairy processes exhibit similar functions.

Acid Production

Lactic acid production arising from lactose fermentation lowers the pH causing proteins to coagulate and is also the major factor inhibiting adventitious growth of spoilage organisms. The major sugar present in milk is lactose and is utilized by LAB for energy and lactic acid formation. In order to be utilized, lactose must first be transported across the cell wall. This is accomplished by means of the phosphoenol pyruvate

-phosphotransferase system. Lactose is phosphorylated to lactose phosphate and transported across the cell wall. Lactose can also be transported by ATP-dependent permeases (34). Once inside the cell, lactose is hydrolyzed into its glucose and galactose-6-phosphate constituents by phospho-β-galactosidase and enters into another pathway determined by the individual sugar moiety. Glucose is catabolized by the glycolysis (Embden-Meyerhof-Parnas) pathway and galactose-6-phosphate is metabolized by the D-tagatose-6-phosphate pathway. Galactose is metabolized by the Leloir pathway (34) leading to an end product of lactic acid (1).

Proteolysis

Due to the fastidious nature of LAB, a large amount of peptides and amino acids must be present in the media for satisfactory growth. In general, lactococci have been shown to be auxotrophic for isoleucine, valine, leucine, histidine, methionine, arginine, and proline (61). Milk is an excellent growth medium for lactococci containing large amounts of casein and smaller amounts of other proteins and amino acids. Through the combined action of proteinases and peptidases, LAB are able to break down casein into peptides and amino acids that are then transported across the cell wall via specific transport systems. Proteinases have been shown to be attached to the cell wall via a C-terminus anchor and evidence suggests that plasmid DNA may code for the production of certain proteinases (67). The peptidases are intracellular enzymes that hydrolyze short peptide chains into amino acids freeing them for use by the cell. Protein hydrolysis has also been linked to texture and taste in cheese production (34).

Aroma Synthesis

Several flavor and aroma compounds have been shown to be produced in cheese manufacture by members of the *Lactococcus* genera. Lactic acid, acetic acid, and the citrate hydrolysis products, diacetyl, acetaldehyde, 2-3 butylene-glycol, and acetoin, all contribute to aroma. Pyruvate, derived from citrate by citratelyase, is toxic to the cell in

high concentrations and it has been suggested that these aroma compounds are generated as a way of avoiding the accumulation of high levels of pyruvate within the cell (34). Studies suggest that citrate utilization is plasmid borne (67). Additional flavor and aroma compounds may be generated by lactococci via the indirect formation of di- and tri-peptides and free amino acids that are further metabolized into volatile compounds (34).

Inhibitory Compounds

The primary inhibitory compounds produced by lactococci are the organic acids arising from sugar fermentation and the resulting drop in pH. Additional small molecular weight, thermostable molecules with a narrow range of action called bacteriocins are produced by lactococci and have been intensely studied. Nisin has been shown to be active against spoilage organisms such as *Clostridium botulinum* and *Listeria monocytogenes*. Conversely, diplococcin is only active against *Lactococcus lactis* species (34).

Bacteriophage Resistance Mechanisms

Phage that infect *Lactococcus lactis* strains are ubiquitous in cheese plants and are a constant potential for destructive financial losses. Phage resistance is a desired trait in cheese starter cultures (27, 31). Several mechanisms of phage resistance have been elucidated and some of the most prevalent are the restriction-modification systems. These systems cleave unmodified foreign DNA rendering it useless to the cell and have been shown to be plasmid borne (39, 51). Other phage resistance mechanisms include inhibition of adsorption, blocking of DNA penetration, and abortive infection mechanisms. All of these mechanisms have been linked to plasmids (27). Another possible phage resistance mechanism may be linked to exopolysaccharide biosynthesis. One research group noted a correlation with exopolysaccharide production and phage resistance. When a plasmid conferring exopolysaccharide biosynthesis was transferred to a phage sensitive, non-exopolysaccharide producing host, the host became resistant to that particular phage (64). Conversely, another research group showed that an exopolysaccharide producing

strain was a host for many different phage and non-exopolysaccharide producing mutants of that strain were resistant to phage attack (50).

Lactococcal IS Elements

IS elements have been defined as DNA sequences that contain no detectable genes other than those required for transposition and are able to transpose from a donor location to a recipient location. IS sequences are typically 750-1500 base pairs in size and represent the simplest class of insertional elements (52). Four insertional sequence elements have been identified and characterized in *Lactococcus*, IS*S1*, IS*904*, IS*946* (47), and IS*981* (48). These insertion sequences are usually associated with important traits useful for growth in a milk media such as lactose and sucrose metabolism, proteinase activity, nisin production, bacteriophage resistance, and conjugal transfer abilities and may play a role in the phenotypically unstable expression of these genes (48). In lactococci, IS elements have been found in multiple copies on the chromosome and on plasmids. One research group found IS*S1* on the chromosome of 47 of 49 strains tested and on plasmids in 17 of 17 strains tested (44). IS elements have also been found to be part of larger transposons, which often flank a gene that is not able to transpose by itself. IS elements have been shown to have the ability to form cointegrates with other plasmids or integrate plasmids into the chromosome creating novel genetic combinations (48). One group demonstrated that the insertional element IS*946* could integrate plasmids successfully into the chromosome (47). As a virtue of being present in multiple copies on plasmids as well as the chromosome, novel DNA combinations can result from transposition or homologous recombination. In one case, a plasmid free strain was shown to conjugally transfer the lactose fermenting (Lac+) phenotype to a rec$^-$ recipient strain suggesting a chromosomally inserted location for the Lac+ plasmid DNA (48). The abundance of IS elements present on the chromosome as well as plasmids leads to the prediction that due to the sheer number of IS elements present in lactococci, they have a significant effect on commercially important genotypic and phenotypic trait stability (44).

Exopolysaccharide Biosynthesis

Historically, exopolysaccharide producing strains have been used in Scandinavian countries to produce a thick, viscous fermented milk called "villi", "langfil", and "filmjolk" (34, 68). Recently, exopolysaccharide producing strains are finding increased usage in yogurt, sour cream, and whipped toppings to improve rhealogical properties, prevent syneresis, and replace stabilizers. As a result of consumer demands for natural foods, exopolysaccharides have recently begun to be studied more intensely for their potential use as natural stabilizers in other food products such as bakery fillings, canned foods, dry mixes, frozen foods, pourable dressings, sauces, gravies, processed cheeses, and juice drinks (12, 53). Chemical stabilizers can have a negative effect on yogurt taste, aroma, and mouthfeel obviating the potential use for exopolysaccharide producing LAB (7).

Bacterial Polysaccharides

LAB are capable of producing several different polysaccharides. These polysaccharides can be found in the cytoplasm as carbon and energy sources, components of the bacterial cell wall, or external to the cell (9). Exopolysaccharides (eps) are defined as the polysaccharides that are found external to the cell, either attached or excreted as free polysaccharides (58). Many different types of microorganisms from Gram positive and Gram negative bacteria to algae produce eps (9). Ambiguity concerning nomenclature describing these eps exists as they have been described as mucoidy, slime producing, and ropy. One strain of *Lactococcus lactis* ssp. *cremoris* has been shown to produce at least two distinct forms of eps, ropy and mucoidy, driven by environmental conditions (13). Ropy exopolysaccharide (ropy eps) is distinguished by viscous ropes greater than five mm in length originating from the colony when the colony is touched. Conversely mucoidy eps imparts a slimy appearance to the colony but does not produce viscous ropes. Another research group suggests that environmental signals may trigger two modes of eps production resulting in the biosynthesis of mixed polymers (17). The roles of substrate, temperature, and oxygen requirements in the regulation of eps biosynthesis is varied and

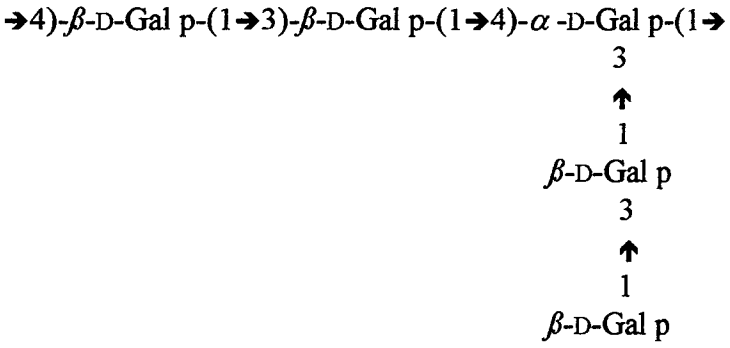
does not provide a definitive answer to regulation of eps expression. One recent study showed the presence of a Lon-like protein in several lactococcal strains (14). Lon protease is a regulatory protease responsible for degrading short-lived regulatory proteins, such as RcsA, which activates colanic acid polysaccharide biosynthesis in *Escherichia coli* (57). The study showed less of the lon-like protein in ropy eps strains than in non-ropy eps strains of *Lactococcus* suggesting a regulatory role in lactococcal ropy eps biosynthesis for this highly conserved enzyme (14).

Roles of Exopolysaccharides

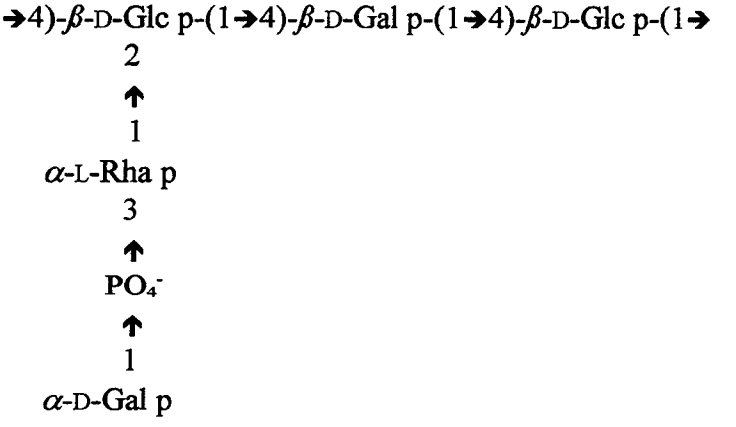
Several functions of eps have been suggested such as protection from desiccation and phagocytosis, adhesion, increasing oxygen tension, and facilitation of nutrient uptake (9). Protection from bacteriophage has been hypothesized as another function of eps but in the case of mesophilic *Lactococcus* strains, that hypothesis remains unsolved (50, 64). It is apparent that eps does not act as an energy source in lactococci. One research group observed that a species of *Streptococcus salivarius* ssp. *thermophilus* possess enzymes capable of degrading the eps but no such data exists for lactococci (17). In any case, eps is not necessary for survival and eps biosynthetic mutants are easily isolated (9).

Exopolysaccharide Composition and Structure

Exopolysaccharides can be broken down into two types, homo- and hetero-polymers. Homopolymers are composed of a single type of sugar monomer. Specific substrates are required for the production of some homopolymers. Sucrose is the required sugar source for the production of dextrans, levans, and mutans (9, 53). Although homopolymers are made of similar sugar moieties, different repeating unit sizes, structures, and molecular weights are common (9, 24, 53). Compositional and structural data of an eps isolated from a *Lactococcus lactis* ssp. *cremoris* strain revealed a homopolymer composed of galactose with the structure (24):



Heteropolymers consist of many different monomers such as glucose, galactose, arabinose, mannose, and rhamnose, and no correlation between carbon source and final eps composition has been made (9). Of all the differing lactococcal heteropolymers examined, no similarities in composition or structure has been observed yet all give a thick, ropy consistency to fermented milk (6, 9, 10, 56). Exopolysaccharide isolated from a *Lactococcus lactis* ssp. *cremoris* strain was reported to be composed of rhamnose, glucose, and galactose in molar concentrations of 1:1.45:1.75 respectively, had a molecular weight of $1.7x10^6$ and the following structure (36, 63):



Other compositions of eps isolated from *Lactococcus lactis* ssp. *cremoris* strains were found to be galactose and glucose in molar ratio of 2:1 (24) and predominantly galactose and glucose, but also including small amounts of rhamnose, arabinose, and mannose (10).

Commercial Uses of Exopolysaccharides

The commercial usefulness of eps arise from their wide range of functions and is readily exploitable due to their hydrophilic nature and solubility in water. Important functions include high viscosity at low concentrations, gelling, antifreeze behavior, stability in high shear, pH, and temperature changes, surface-active dispersing and flocculating capacity, adhesive and film-forming properties, binding capacity for metal ions, proteins and lipids, and biodegradability (30). Exopolysaccharides have applications in a wide range of industries such as the dairy, food, cosmetic, chemical, medical, waste treatment, and oil. Their useful functions include thickeners, viscosifiers, drag reducing agents, and matrix for flavor compounds, enzymes, cells, and biomedical material immobilization (53). One author notes that due to the specific ordering of many differing sugar residues, they are capable of forming ordered chain conformations often in dilute solutions allowing gel formation and stabilization (9). One study showed a drastic increase in moisture retention within a low-fat cheese matrix made with an eps producing strain (43). Several studies show ropy eps strands interacting with the bacterial cells and protein matrix of fermented milk and casein in yogurt (62). This interaction provided an increase in viscosity even after stirring and shearing the eps from the bacterial cell (58). One group suggests that strains maintaining their eps as capsules rather than excreting the eps, provides better characteristics to low-fat cheeses (43). Currently the only microbial eps used to any appreciable extent in industry are dextran, produced by *Leuconostoc mesenteroides*, xanthan gum, produced by *Xanthomonas campestris*, and recently, gellan gum, produced by *Aureomonas elodea* ATCC31461 (12). Xanthan gum was approved by the U. S. Food and Drug Administration (FDA) for use in foods in 1969. Today it is used in many foods such as bakery fillings, canned foods, dry mixes, frozen foods, pourable dressings, sauces, gravies, processed cheeses, and juice drinks but is best known for its use in oil recovery (12,53).

Current health trends are providing a strong stimulus for finding new and more natural food additives, such as lactococcal eps. Consumers demand fresh, minimally processed foods without chemical additives that are low in fats and sugars as well as

providing potential remedies to such ailments as heart disease, osteoporosis, fatigue, and memory loss (70). Two potential concepts relating to these types of healthy foods are probiotics and prebiotics. Probiotics work on the concept that establishing a healthy flora of intestinal microbes through eating foods rich with these microbes causes the host to be more resistant to disease causing organisms. Prebiotics accomplish this same goal using specific nutrients targeted to the desirable microbes already present in the intestinal tract (70). As result, one study tested fermented milks using *Lactobacillus acidophilus*, *Lactobacillus casei* ssp. *casei* and *Bifidobacterium* isolated from human intestine (62). Other studies are underway to examine the benefits of LAB eps in healthy foods. One study utilizing eps producing lactococcal strains showed a drastic increase in moisture retention and meltability in low-fat mozzarella cheese that is typically dry and hard. This low fat mozzarella cheese would be similar in quality to normal mozzarella cheese without the fat content and without unnatural fat replacers (43). Other researchers have noted that eps produced by ropy lactococci can play a role in reducing serum cholesterol levels (37), and an eps produced by a *Lactobacillus* strain exhibits anti-tumor activity (62). Another group studied eps producing strains in yogurt production and found that yogurts made with eps producing strains retained an increase in viscosity after stirring (7) and in yogurts produced with less total milk solids of 12% (68). Normal yogurt is made with 17% total milk solids. Additional sensory studies revealed that yogurt made from a ropy eps strain had a smoother mouthfeel than yogurt made with a non-ropy eps strain. An encouraging result was that the sensory panel preferred the yogurt made with the ropy strain in less total milk solids (12%) rather than the normally used total milk solids (17%). This is significant because quality yogurt can be made with less total milk solids in countries such as Mexico where shortages of milk supply occur (68). Ropy eps strains could thus be financially lucrative in developing countries for dairy food production as consumption of these foods is steadily rising. Mexico experienced a 153% rise in yogurt consumption from 1980-1990 (68). A study of Scandinavian fermented milks showed that a mixture of 60-70% eps producing to 30-40% non-eps producing cells was ideal for making a desirable consumer product but is difficult to maintain due to the instability of eps production (32). Increased eps production is not always a beneficial trait. Yogurts made

with ropy eps strains in 14-17% total milk solids were found to be unacceptable due to the unpleasant mouthfeel of excessive ropiness (68). Another study showed that fermented milk made with *Lactobacillus kefiranofaciens* K1 had excessive ropiness and was also unacceptable (62).

Commercial uses of lactococcal eps have been limited due to the fact that eps production has been shown to be unstable and lost at higher temperatures or with repeated transfers (64, 10). This instability is typical of plasmid encoded traits. Several research groups have linked eps biosynthesis with plasmids of various sizes: 4.5-megadaltons (mDa) (65), 17 mDa (38), 18.5 (64), 26.5 mDa (63), and 30 mDa (38, 66). One group noted that the instability of eps production in a plasmid free strain may be due to reversible DNA rearrangements (17, 48). Another problem associated with lactococcal eps use is low yields typically less than 500 mg/L (12). Yields of 150 mg/L (36), 200-240 mg/L (7), 220-600 mg/L (10), have been observed and one research group noted that when a ropy eps strain is inoculated in conjunction with a non-ropy eps strain, eps yield can reach 800 mg/L suggesting a stimulatory effect by the non-ropy eps strain (9). Due to the heterogeneity of lactococcal eps, the requirements for maximal production are probably strain specific. Unbuffered media (18), lower temperatures (10) and aerobic environments may help to produce greater quantities (9).

Studies examining the specific enzymes needed for eps biosynthesis are limited but are beginning to show that very specific enzymes are utilized to assemble, polymerize, and export the eps heteropolymer. These enzymes include sugar transfer enzymes such as gluco- and galacto- transferases and polymerases (9). In two recent studies, operons encoding eps biosynthesis in LAB have been isolated and both operons contained genes having homology to eps genes in other bacterial species (56, 63). With increased knowledge on how eps biopolymers are synthesized, polymerized, and exported, advanced techniques of molecular biotechnology have the potential to develop novel eps specifically suited to individual parameters (12).

Experimental Approach

The goal of this research project was to isolate the genes responsible for ropy exopolysaccharide biosynthesis in a natural lactococcal ropy isolate which produces a viscous, ropy exopolysaccharide in fermented milk. IS*S1*, carried on the temperature sensitive replicon pGh9:IS*S1*, was used to create insertional mutations in the ropy exopolysaccharide genes of a natural lactococcal ropy isolate. Using this insertional mutagenesis approach, mutants failing to express the ropy exopolysaccharide have been selected and characterized. The interrupted gene was sequenced using unique attributes of the pGh9:IS*S1* vector.

MATERIALS AND METHODS

Bacterial Strains and Growth Media

Bacterial strains and plasmids used in this study are detailed in Table 1. Lactococcal stock glycerols were maintained in 11% reconstituted nonfat dry milk (NFM) containing 20% glycerol at -70°C. For subsequent experimentation, *Lactococcus* cultures were streaked on 7% whey agar (64) or grown in M17 based media (60). In general, lactococcal strains were grown at 30°C and without shaking if grown in broth. Lactococcal strains were grown in M17+lactose (M17L) except MG1363 which was grown in M17+glucose (M17G). Whey media was prepared in two parts as follows: Part I was made by dissolving 70 grams (gms) of sweet whey powder (Tillamook County Creamery, Tillamook OR.) in 500 milliliters (mls) of double distilled water (ddH_2O), mixing for 20 minutes, and centrifuging for one hour at 7000 rpms to remove particulate matter. The supernatant was fortified with 19 gms of β- Na glycerophosphate, 5 gms of yeast extract and brought up to 600 mls in volume. Part II was made by combining 15 gms of agar with 400 mls of ddH_2O and 3 drops of Anti-foam A (Sigma). Parts I and II were autoclaved separately for 10 min. and promptly removed. Part I was mixed into part II after cooling to 55°C and poured into sterile petri plates. M17 media was prepared as follows: five gms of polypeptone peptone, phytone peptone, beef extract and either lactose or glucose, 2.5 gms of yeast extract, 0.5 gms of ascorbic acid, 19 gms of β- Na glycerophosphate, and 1 ml of 1 M $MgSO_4$ was added to 1 liter (L) of ddH_2O and autoclaved for 20 minutes. *Escherichia coli* was grown on LB agar or in LB broth (49). This media was made as described: 10 gms of tryptone and 5 gms of yeast extract and NaCl were added to 1 L of ddH_2O. If plates were needed, 15 gms of agar was added. The media was autoclaved for 20 min. and for plates, cooled and poured into sterile petri plates. Antibiotics were used as follows; Erythromycin (em) concentration for lactococcal strains: 5 µg/ml when pGh9:IS*S1* was in plasmid form or 2 µg/ml when pGh9:IS*S1* was inserted into the chromosome, for *E. coli* TG1: 100 µg/ml (33).

Table 1. Bacterial Strains and Plasmids

Strain/Plasmid	Relevant Phenotype/Characteristic	Reference
Strain		
Lactococcus lactis sp. *cremoris*		
natural ropy isolate	ropy	(13)
MG1363	non-ropy, plasmid free	(19)
EK1002	natural ropy isolate harboring pGh9:IS*S1*, ropy, em^R	This work
EK1038	EK1002 transposon mutant non-ropy, em^R	This work
EK1238	EK1002 transposon mutant non-ropy, em^r	This work
EK1338	EK1002 transposon mutant non-ropy, em^R	This work
EK48X	EK1238 excision mutant, non-ropy	This work
EK217X	EK1238 excision mutant, ropy	This work
Escherichia coli		
TG1	plasmid free	(49)
EK1238H	harboring pEK1238H, em^R	This work
Plasmid		
pGh9:IS*S1*	em^R	(33)
pEK1238H	pGh9:IS*S1* containing a 2006 bp insert from EK1238 chromosomal DNA restricted with *HindIII*, em^R	This work

em^R: erythromycin resistant

Electroporation

Electroporation was carried out as described (26, 15). Cells were grown for 48 hr in M17 media supplemented with 1% sucrose and 0.5% glycine. Cells were pelleted, washed in cold 0.3 M sucrose three times, and resuspended in 200 µl of 0.3 cold M sucrose. DNA was added to the cells and the mixture was transferred to a chilled electroporation cuvette (0.2 cm gap). The cells were shocked (2.5 kV, 200 ohms, 25 µF) and resuspended in 8 mls of growth media supplemented with 1% sucrose and 50 ng/ml em. Cells were allowed to recover for 2 hours before plating on whey agar containing 5 µg/ml em for lactococcal strains and LB agar containing 100 µg/ml em for *E. coli* strains.

Plasmid DNA Isolation

Small scale lactococcal plasmid preparations were carried out as previously described (40). Briefly, a 5-10 ml overnight culture was pelleted, resuspended in 200 µl of 25% sucrose containing 30 mg/ml of lysozyme, and incubated at 37°C for 15 minutes. Four hundred microliters of fresh 3% SDS, 0.2 N NaOH solution was added, mixed immediately, and incubated for 7 min. at room temperature. Three hundred microliters of cold 3 M Na acetate was added and immediately mixed. Cell debris and chromosomal DNA were removed by centrifugation for 15 min. and the supernatant was transferred to a clean microfuge tube. To this tube, 650 µl of isopropanol was added and mixed well. The DNA was pelleted by centrifugation for 15 minutes. After resuspension in 320 µl of sterile ddH_2O, 200 µl of 7 M ammonium acetate containing 0.5 mg/ml of ethidium bromide, 175 µl phenol, and 175 µl of 24:1 chloroform/isoamylalcohol was added and mixed well. After centrifuging for 5 min., the upper phase was transferred to a new tube, ethanol precipitated in 1 ml of 95% ethanol, washed in 0.5 ml of 70% ethanol, dried, and resuspended in 40 µl of 0.01 M Tris, 1 mM EDTA (TE).

Large scale plasmid isolations for lactococcal strains were a combination of two protocols and carried out as follows (4,40). One liter of cells was grown to saturation in appropriate growth media and pelleted. The pellet was resuspended in 20 mls of 25% sucrose containing 30 mg/ml of lysozyme and incubated for 15 min. at 37°C. Forty milliliters of freshly made 3% SDS, 0.2 N NaOH solution was added, mixed immediately, and incubated at room temperature for 7 minutes. Fifteen milliliters of cold 3 M Na acetate was added, mixed immediately, and incubated on ice for 10 minutes. Cellular debris and chromosomal DNA were removed by centrifugation for 15 min. and the supernatant was transferred to a new tube. Thirty milliliters of isopropanol was added, incubated on ice for 10 minutes, and centrifuged. The pellet was dried, resuspended in 10 mls of 50 mM Tris pH 8.0, 100 mM Na acetate, 1 mM EDTA, and agitated at 37°C until resuspended. DNA was precipitated with 20 mls of 95% ethanol, pelleted, washed with 5 mls of 70% ethanol, dried, and resuspended in 4 mls of 1x TE. To this solution, 300 µl of

10 mg/ml ethidium bromide and 5.2 gm of CsCl were added and mixed well. This solution was added to an ultra-centrifuge tube, weighed carefully, sealed, and centrifuged at 58,000 rpms for 16 hours at 20°C. The lower plasmid band was removed with a syringe and the ethidium bromide was removed by repeated extractions with water saturated butanol. The plasmid DNA was pelleted by adding 2 volumes (vol.) of sterile ddH_2O, 1 vol. of 3.5 M Na acetate, and 0.7 vol. of isopropanol followed by centrifugation. The pellet was dried, resuspended in 0.5 ml of 50 mM Tris pH 8.0, 100 mM Na acetate, 1 mM EDTA, precipitated with 95% ethanol, pelleted, washed in 70% ethanol, dried, and resuspended in 200 µl of 1x TE.

Small scale *E. coli* plasmid preparations were carried out as described (49). After overnight growth in 5 mls of LB containing appropriate antibiotics, cells were pelleted and resuspended in 150 µl of lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0). Sixty microliters of fresh lysozyme solution (80 mg lysozyme, 1 ml water) and 200 µl of fresh SDS/NaOH (0.2 N NaOH, 1% SDS) was added and mixed until the cells lysed. One hundred fifty microliters of 5 M K acetate was added and mixed until a white precipitate formed. Cellular debris and chromosomal DNA were removed by centrifugation and 250 µl of phenol and 250 µl chloroform/isoamylalcohol (24:1) were added. After thorough mixing and centrifugation for 3 min., the top phase was removed and added to 80 µl of 0.35 M Na acetate and 1 ml of cold 95% ethanol. After 5 min. at room temperature, the DNA was pelleted, washed with 0.5 ml cold 70% ethanol, dried, and resuspended in 50 µl of 1x TE.

Large scale plasmid isolations for *E. coli* followed the Birnboim method (4). One liter of cells grown in LB containing appropriate antibiotics was pelleted and resuspended in 10 mls of 1x TE containing 20 mg of lysozyme and incubated at room temperature for 5 minutes. Following addition of 20 mls of Bernie #2 solution (400 mg NaOH, 5 mls of 10% SDS, 45 mls sterile ddH2O), cells were incubated 10 min. on ice. Fifteen mls of 5 M K acetate was added and incubated for 30 min. with occasional mixing. Cellular debris and chromosomal DNA were removed by centrifugation for 15 min. and the supernatant was transferred to a new tube followed by the addition of 30 mls of isopropanol, incubation on ice for 10 minutes, and centrifugation. The pellet was dried, resuspended in 10 mls of 50

mM Tris pH 8.0, 100 mM Na acetate, 1 mM EDTA, and agitated at 37°C until resuspended. DNA was precipitated with 20 mls of 95% ethanol, pelleted, washed with 5 mls of 70% ethanol, dried, and resuspended in 4 mls of 1x TE. To this solution, 300 µl of 10 mg/ml ethidium bromide and 5.2 gm of CsCl were added and mixed well. This solution was added to an ultra-centrifuge tube, weighed carefully, sealed, and centrifuged at 58,000 rpms for 16 hours at 20°C. The lower plasmid band was removed with a syringe and the ethidium bromide was removed by repeated extractions with water saturated butanol. Plasmid DNA was pelleted by adding 2 volumes of sterile ddH_2O, 1 vol. of 3.5M Na acetate, and 0.7 vol. of isopropanol followed by centrifugation. The pellet was dried, resuspended in 0.5 ml of 50 mM Tris pH 8.0, 100 mM Na acetate, 1 mM EDTA, precipitated with 95% ethanol, pelleted, washed in 70% ethanol, dried, and resuspended in 200 µl of 1x TE.

Chromosomal DNA Isolation

Chromosomal isolations were performed as described (49). Cultures were grown in 30 mls of the appropriate growth media for 48 hr. and pelleted. Pellets were resuspended in 1 ml of 0.1 M EDTA, 0.1 M Tris pH 8.0 containing 15 mg/ml lysozyme and incubated at 37°C with gentle rocking for 1 hour. Two mls of 0.01 M Tris, 0.1 M NaCl, 1% SDS, pH 8.0 were added and rocked at room temperature for 1 hour. Three mls of phenol were added and rocked for 1.5 hours, after which the aqueous phase was removed to another tube. Two volumes of ice cold 95% ethanol were added, mixed well, and set on ice for 10 minutes. Chromosomal DNA was spooled onto a glass Pasteur pipette, dried, and dissolved in 1 ml of 1x TE.

Lactococcal Insertional Mutations

Insertional mutants were created using the transposon mutagenesis plasmid pGh9:IS*S1* (33). Transformants were grown to saturation in M17L containing 5 µg/ml of em, diluted 100 fold into M17L without em and incubated 150 min. at 28°C. This culture

was shifted to 38°C for another 150 min. and plated on whey agar containing 2 μg/ml of em at 38°C. Mutants were screened for loss of ropy eps expression on whey agar plates. The ropy phenotype is indicated by rope formation greater than 5 mm when the colony is touched with a sterile loop (65).

Mutant Characterization

Lactococcal strains to be characterized were brought out of frozen stocks on whey agar plates, inoculated into 5 mls of M17L, and grown for 48 hours. After commercial 2% milk was sterilized by steaming for 30 min., 3 mls of milk were inoculated with 0.5 ml of the culture. The milk was incubated for 18 hours at 30°C and visually examined for coagulation and ropy eps expression. The milk was examined again after 24, 48, 72 and 96 hours. Positive coagulation was indicated by a solid clump in the tube. Ropiness was indicated by inserting a sterile wire loop and pulling long ropes out of the milk when the loop was withdrawn. Growth curves were prepared as follows. Sixty mls of M17L was inoculated with 1.5 mls of a 48 hour culture and mixed well. An initial OD_{600} reading was taken and readings were subsequently taken every 30 minutes. Results were graphed and the doubling time taken from the graph.

Cloning of the Interrupted Gene

Cloning of interrupted genes was performed as described (33). Chromosomal DNA was isolated and digested with *HindIII*. Fragments were circularized and transformed into *E. coli* TG1 cells and plated on LB containing 100 μg/ml of em at 28°C. Plasmids were isolated and digested with *Hind III* and *EcoRI* to verify the presence of an insert.

E. coli Transformation

Transformation of *E. coli* was achieved as described (49). After growth overnight in LB, cells were diluted 50 fold, grown to OD_{600} = 0.5-0.6, and pelleted. Cells were resuspended in 10 mls of cold 0.1 M $CaCl_2$ and iced for 15 minutes. The cells were pelleted and resuspended in 5 mls of cold 0.1 M $CaCl_2$ and iced for 15 minutes. Cells were pelleted and resuspended in 1 ml of $CaCl_2$ and iced for 2 hours. DNA was added and the cells were iced for 30 min. followed by heat shock at 42°C for 1 min. and icing for 2 minutes. One ml of SOC broth was added and incubated at 37°C for 1 hr and plated on LB agar containing 100 µg/ml em. SOC broth was made by adding 20 gm of tryptone, 5 gm of yeast extract, 0.58 gm of NaCl, 0.19 gm of KCl, 2 gm of $MgCl_2 \cdot 6\ H_2O$, and 2.5 gm of $MgSO_4 \cdot 7\ H_2O$ to 1 L of ddH_2O and autoclaving for 20 minutes. Prior to use, 2 mls of 20% glucose were added for every 100 mls used.

DNA Sequencing

Plasmids containing inserts to be sequenced were isolated using a large scale plasmid preparation. After gel quantitation using Gibco BRL Life Technologies DNA mass ladders, plasmid DNA was sequenced at Central Services Laboratory at Oregon State University. Sequencing primers are listed in Table 2. The initial forward primer, IS*S1*for, was located in the forward end of the IS*S1* element and used to sequence the pGh9:IS*S1* vector. From the vector sequence, a primer, pGh9rev, was generated to the pGh9 backbone on the downstream side of the *HindIII* site to use as a reverse sequencing primer. Sequence generated using these primers allowed new primers to be synthesized to the end of the new sequences permitting another round of sequencing. Using IS*S1*for, EK1238Hf1, EK1238Hf2, and EK1238Hf3 consecutively, the forward strand was sequenced. Using pGh9rev, EK1238Hr1, EK1238Hr2, and EK1238Hr3 consecutively, the reverse strand was sequenced. Sequence data was aligned using a multiple sequence alignment program and the two ends of the insert were determined. The insert DNA sequence was analyzed by the GenBank data base using BLAST to search for homologous

sequences. The insert DNA sequence was also translated into its predicted amino acid sequences in all six frames. Amino acid sequences of any considerable length were analyzed by the SwissProt data base using BLAST to search for homologous sequences.

Table 2. Sequencing Primers

Primer	Primer Sequence
IS*S1*for	5'-AAACTTTGCAACAGAACCAG-3'
pGh9rev	5'-GCCGGATTGTTCCCAGTCACGACG-3'
EK1238Hf1	5'-TATACTTCTCAACATTGTTATTGG-3'
EK1238Hf2	5'-ATAAAATTCAGGATCAACTTTCCC-3'
EK1238Hf3	5'-CATAATTTCTCGCATGCGATACCC-3'
EK1238Hr1	5'-TAATGATCTTCTTGAATGTGAAGG-3'
EK1238Hr2	5'-CGTGTATGATACATATGTTATCCG-3'
EK1238Hr3	5'-CTTAATGCCACTGGAGAATATATC-3'

Transposon Excision

Excision of the pGh9:IS*S1* vector was performed as described (33). Cultures were grown overnight in M17L containing 2 μg/ml em at 38°C, diluted 50 fold in M17L without antibiotics, and grown to saturation at 28°C. Serial dilutions were plated on whey agar without em at 38°C. Colonies were re-streaked onto whey agar containing 2 μg/ml em and whey agar without em to screen for excision of the pGh9:IS*S1* vector at 28°C.

Southern Hybridization

Southern hybridization using enhanced chemiluminescense (ECL) was performed as described by ECL Direct Nucleic Acid Labeling and Detection System by Amersham Life Sciences. Briefly, chromosomal DNA was restricted using *EcoRV* and separated on 0.6% agarose gel. The gel was depurinated by gentle shaking in 250 mM HCl for 15 min.,

rinsed with ddH_2O for 1 min., denatured by gentle shaking in 1.5 M NaCl, 0.5 M NaOH for 45 min., rinsed with ddH_2O for 1 min., and neutralized by gentle shaking in 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5 for 30 minutes. The neutralization step was done twice. A transfer stack was assembled. This consisted of a shallow tray filled with 20x SSC (0.3 M Na_3 citrate, 3 M NaCl, pH 7.0) overlaid by a glass sheet. A wick of Whatman 3MM paper was wetted and placed over the glass contacting the buffer under both sides of the glass sheet and all air bubbles were removed. The gel was placed upside down on the 3MM paper, air bubbles were removed, and Petri-film was placed on the 3MM paper wick to cover any exposed areas not covered by the gel. A piece of nitrocellulose paper, pre-wetted in 20x SSC, was placed on top of the gel and all air bubbles were removed. Two pieces of pre-wetted 3MM paper were placed on top of the nitrocellulose and the air bubbles were removed. A stack of paper towels was placed on top of the 3MM paper followed by another glass sheet and weighted. After the DNA was allowed to transfer overnight, the nitrocellulose membrane was removed and washed in 6x SSC for 5 min. and dried at room temperature. The DNA was fixed to the membrane by UV cross linking with 1200 joules of energy. The membrane was placed in a hybridization tube filled with hybridization buffer containing 0.5 M NaCl and 5% (w/v) blocking agent preheated to 42°C and allowed to pre-hybridize for 1 hour. Approximately 100 ng of probe DNA consisting of either the *SpeI-HindIII* fragment of pEK1238H or the *HindIII-EcoRI* fragment of pGh9:IS*51* was labeled in a 10 µl volume as follows. The DNA was boiled for 5 min., cooled on ice for 5 min., and spun briefly to collect the sample on the bottom. Ten microliters of labeling reagent was added and mixed. Ten microliters of gluteraldehyde was added and mixed. The probe was set at 37°C for 10 min. and used immediately. The probe was allowed to hybridize at 42°C overnight. Following hybridization, the membrane was washed twice in 100 mls of pre-heated primary wash buffer (6 M urea, 0.4% SDS, 0.5x SSC). The membrane was washed twice in 2x SSC for 5 minutes. Developing reagents in a 1:1 mixture were added to the membrane for 1 min. and drained. The membrane was covered in plastic wrap and exposed to the film for various amounts of time. The film was developed using Kodak developer and fixer.

RESULTS

Insertional Mutations Impact Ropy Exopolysaccharide Biosynthesis

Two distinct exopolysaccharides, mucoidy and ropy, are produced by a previously described natural lactococcal ropy isolate (13). The exopolysaccharide of interest, ropy exopolysaccharide (ropy eps), is evident by touching the colony and observing a viscous rope greater than 5 mm in length originating from the colony. Mucoid exopolysaccharide lacks this rope formation. Due to active restriction-modification systems and plasmid incompatibility, transformation with foreign DNA by electroporation into the natural lactococcal ropy isolate proved to be inefficient (13). A mutagenesis vector designed to circumvent these obstacles, pGh9:IS*S1* (33), was therefore exploited. One microgram of pGh9:IS*S1* plasmid DNA was used to transform the natural lactococcal ropy isolate and one transformant colony, EK1002, was generated. The erythromycin resistant (em^R) colony was assayed for ropiness; long ropes greater than five mm in length indicative of wild type ropy eps expression were observed as shown in figure 1. The plasmid complement of EK1002 was isolated and an additional plasmid approximately 4.6 kb in size, pGh9:IS*S1*, was observed. These results indicated that pGh9:IS*S1* had transformed the natural lactococcal ropy isolate yet had not disrupted ropy exopolysaccharide gene expression while in an autonomously replicating state. Insertional mutants were generated by growing EK1002 at 28°C and shifting the temperature to 37°C so as to repress autonomous replication of the vector. Only cells in which the vector inserted into the chromosome could survive on the whey agar containing erythromycin (em). This process is depicted in figure 2. The surviving em^R colonies were assayed for ropy eps expression. Of the 120 em^R colonies that grew on the mutagenesis plates, three colonies named EK1038, EK1238, and EK1338, were identified that did not yield any rope formation when touched with a sterile loop. The mutational frequency in genes essential for ropy eps biosynthesis was 2.5% indicating that only a fraction of insertions disrupted ropy eps biosynthesis and gave the desired non-ropy phenotype. One mutant, EK1238, was selected for further characterization.

Figure 1. Ropy Eps Expression on Whey Agar by EK1002

Figure 1. Whey agar plates containing em were incubated at 30°C for 48 hours. Em^R colonies were touched with a sterile toothpick to test for ropy eps expression.

Figure 2. pGh9:IS*S1* Mediated Insertional Mutagenesis

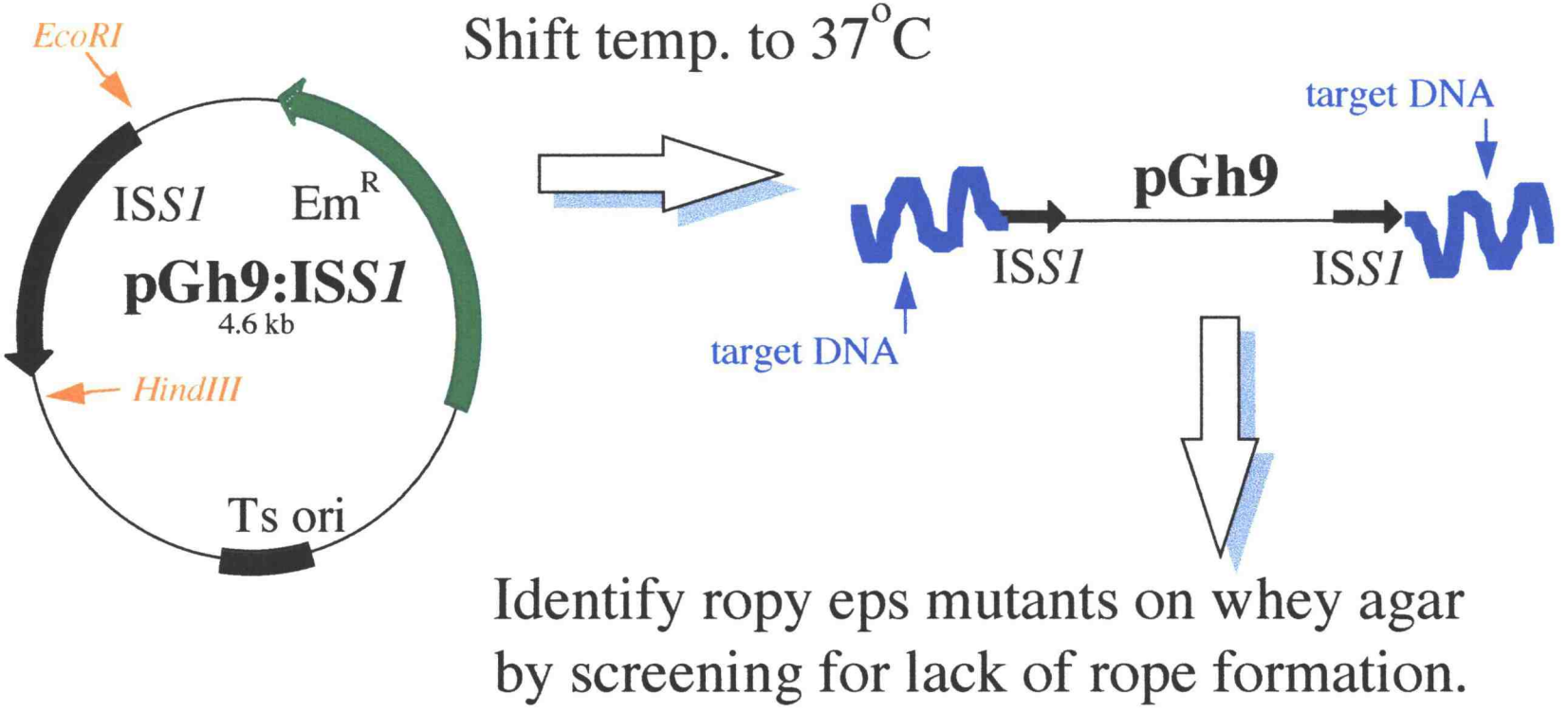


Figure 2. Cells were grown to saturation in M17L containing 5 ug/ml em, diluted 100-fold into M17L without em and incubated 150 minutes at 28°C. The culture was shifted to 38°C for 150 minutes and plated on whey agar containing 2 ug/ml em. Mutants were screened for loss of ropy eps expression by touching with a sterile toothpick and observation of ropes greater than 5 mm in length.

Localization of the Insertion

Utilizing the unique characteristics of the pGh9:IS*S1* transposon mutagenesis vector, a fragment of the interrupted gene can be isolated following a simple chromosomal isolation, restriction, and ligation procedure as detailed in figure 3. Chromosomal DNA from the IS*S1* mutant, EK1238, was isolated, restricted with *HindIII*, ligated, and transformed into *Escherichia coli* TG1 cells. Twenty-seven em^R transformants were recovered. The plasmid DNA, pEK1238H, of one transformant, EK1238H, was analyzed by a *HindIII-EcoRI* restriction digest to verify presence of an insert and gauge the size of that insert. Using unique *HindIII* and *EcoRI* restriction sites in pGh9:IS*S1*, two fragments can be resolved: 3.8 kb, corresponding to the pGh9 vector and 0.8 kb, corresponding to the IS*S1* element (figure 2). Transformant plasmid DNA, pEK1238H, was restricted with *HindIII* and *EcoRI* giving fragments of 3.8 kb and 2.7 kb in size. The 3.8 kb fragment co-migrated with the 3.8 kb pGh9 vector fragment and the 2.7 kb fragment was hypothesized to be the 0.8 kb IS*S1* element joined to approximately a 1.9 kb chromosomal insert. The restriction digest results of pEK1238H suggest that a fragment of the IS*S1* interrupted gene (approximate size: 1.9 kb) was isolated from EK1238 chromosomal DNA restricted with *HindIII*. Southern blot analysis was used to localize the IS*S1* interrupted gene and the data is shown in figure 4. A probe consisting of the 1.6 kb *SpeI-HindIII* fragment of pEK1238H insert DNA (figure 3) was constructed and used to probe chromosomal DNA isolated from the natural lactococcal ropy isolate, from EK1238, and from MG1363, a non-ropy, negative control. Chromosomal DNA from these three strains was restricted with *EcoRV* restriction endonuclease before probing. *EcoRV* does not restrict within either the pEK1238H insert or the pGh9:IS*S1* vector. A prediction can be made that this probe will hybridize to a single *EcoRV* DNA fragment (illustrated as a band in a Southern blot) from the natural lactococcal ropy isolate and from EK1238 but will not hybridize to any of the *EcoRV* DNA fragments from the non-ropy MG1363 negative control. As illustrated in figure 4, a single band is detected in the natural lactococcal ropy isolate (lane 1) and in EK1238 (lane 2). In contrast, this probe does not hybridize to any DNA fragments from MG1363. The band in lane 2, EK1238, is

Figure 3. Chromosomal Insert Isolation and Sequencing Using pGh9:IS*1*

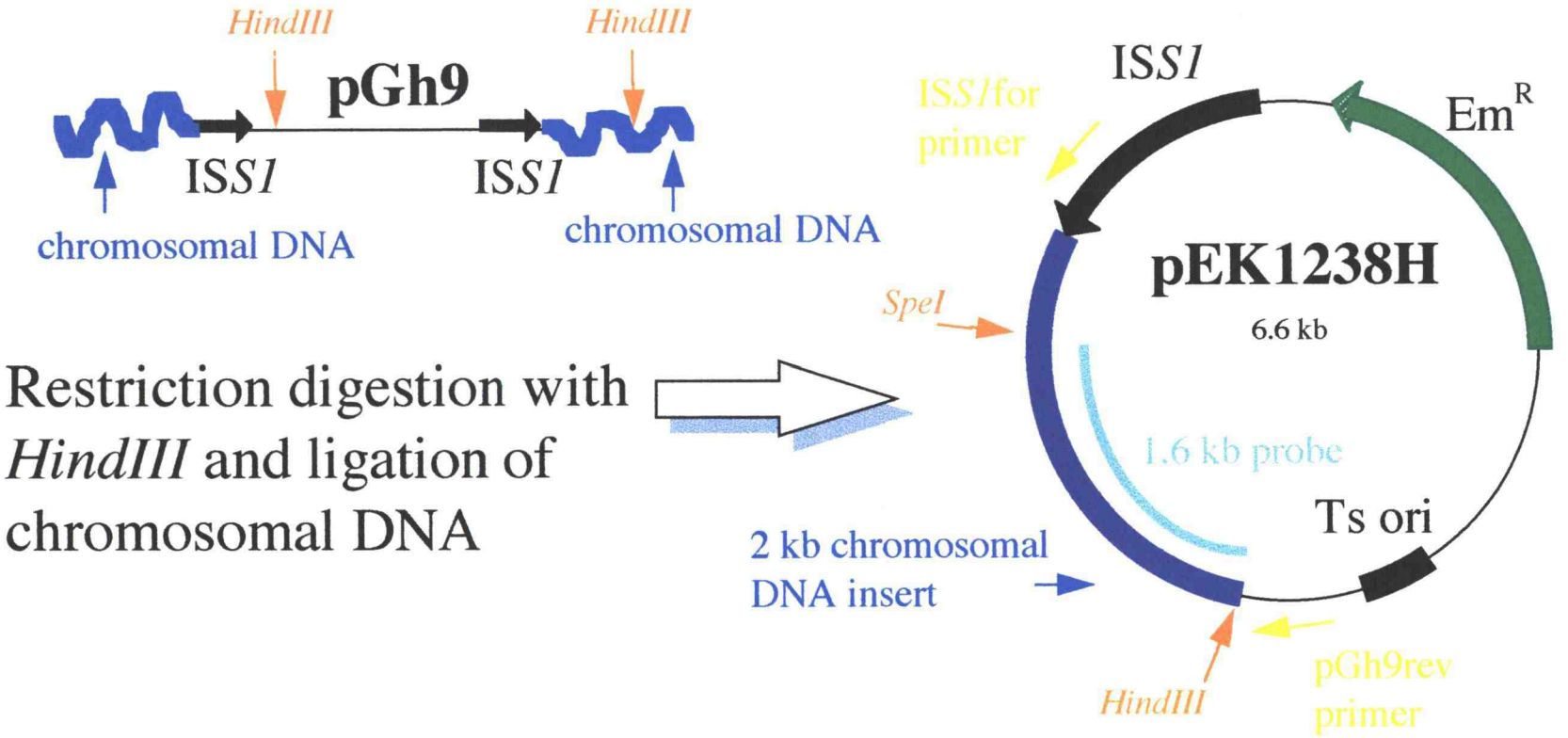


Figure 3. EK1238 chromosomal DNA was isolated and restricted with *HindIII*. Fragments were recircularized by ligation, transformed into *E. coli* TG1, and cells were plated on LB containing 100 ug/ml em at 28°C. Plasmid DNA was isolated and digested with *HindIII* and *EcoRI* to verify presence of an insert.

Figure 4. Southern Blot Analysis Demonstrating Chromosomal Insertion of pGh9:IS*S1*

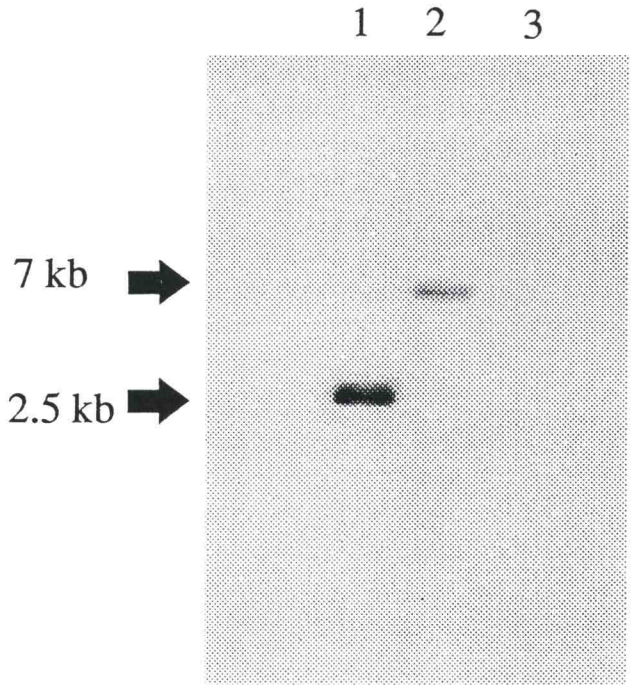


Figure 4. Chromosomal DNA was restricted using *EcoRV* and electrophoresed in 1.0% agarose, transferred to nitrocellulose overnight and UV cross-linked. The blot was pre-hybridized for one hour and probed with the 1.6kb *SpeI-HindIII* fragment of pEK1238H. The film was exposed for 5 minutes. Lane 1: Natural lactococcal ropy isolate; Lane 2: EK1238 (IS*S1* insertional mutant); Lane 3: MG1363 (negative control).

approximately 4.6 kb larger than the band in lane 1, the natural lactococcal ropy isolate, indicative of pGh9:IS*S1* insertion in the chromosome of EK1238. The Southern analysis results suggest a chromosomal location for the interrupted gene of interest as no contaminating plasmid DNA was observed in the chromosomal preparation when unrestricted chromosomal DNA was electrophoresed.

Effects of Insertional Mutagenesis on Ropy Eps Biosynthesis in EK1238

Mutants created using pGh9:IS*S1* must be grown at 38°C or higher in order to maintain the vector as a chromosomal insert. The wild type natural lactococcal ropy isolate grows poorly at 38°C necessitating further characterization of the EK1238 mutant at 28°C so as to use the natural lactococcal ropy isolate as a control. Growth of the IS*S1* generated mutant at 28°C results in renewed autonomous replication of the pGh9:IS*S1* vector and consequently excision from the chromosome generating a heterogeneous population of excisants dependent upon the excision event. After lowering the temperature and allowing the pGh9:IS*S1* vector time to excise, shifting the temperature back to 38°C and plating on antibiotic free media at 38°C allows loss of the vector. After the excision event, 231 colonies of EK1238 were restreaked to whey agar and whey agar containing 5 μg/ml of em at 28°C to screen for em sensitive colonies indicating loss of the vector. After growth for 48 hours, 109 colonies of EK1238 were sensitive to em, giving an excision efficiency of 47%. Of the 109 em sensitive colonies, 64 colonies were restreaked to whey agar for isolation and a ropiness assay. After growth for 48 hours at 28°C, 77% of the excisants yielded long ropes indicating pGh9:IS*S1* excised without taking adjacent chromosomal sequences. In contrast, 23% of the excisants did not yield long ropes indicating pGh9:IS*S1* excised with adjacent chromosomal DNA. One of the non-ropy eps excision mutants, EK48X, and one of the ropy revertants, EK217X, were chosen for a Southern blot analysis and the data is shown in figure 5. In lanes 1 and 2, the natural lactococcal ropy isolate and EK1238 respectively, the bands observed in the previous Southern blot analysis (figure 4) are present. In lanes 3 and 5, the excision mutant EK48X and MG1363 respectively, no bands are evident. In lane 4, the ropy excision revertant

Figure 5. Southern Blot Analysis
Demonstrating Excision of pGh9:IS*S1*

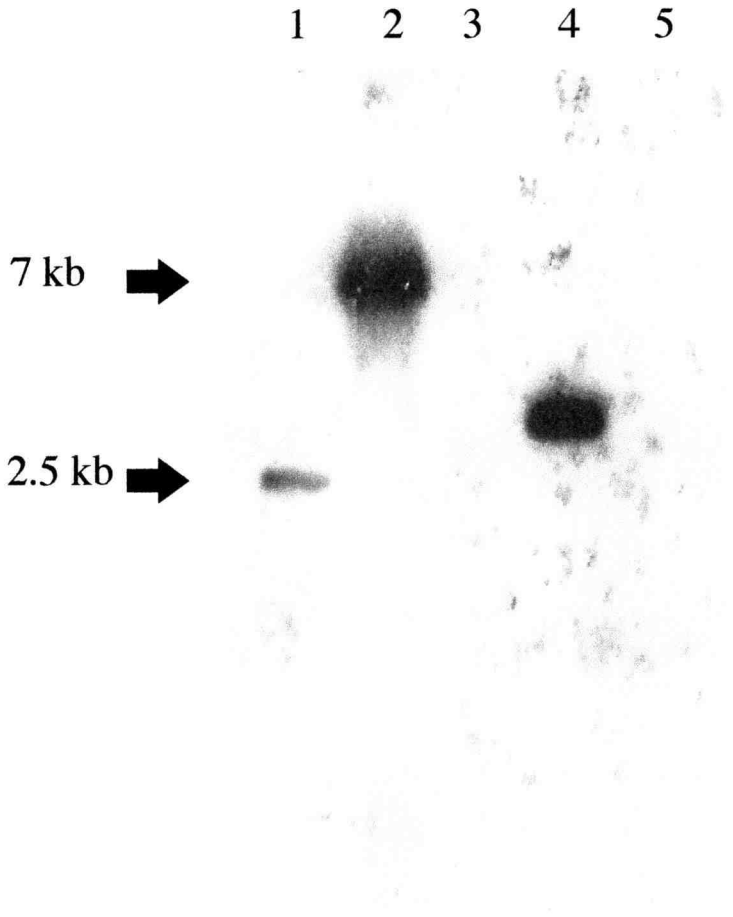


Figure 5. Chromosomal DNA was restricted using *EcoRV* and electrophoresed in 1.0% agarose, transferred to nitrocellulose overnight and UV cross-linked. The blot was pre-hybridized for one hour and probed with the 1.6kb *SpeI-HindIII* fragment of pEK1238H. The film was exposed for 45 minutes. Lane 1: Natural lactococcal ropy isolate; Lane 2: EK1238 (IS*S1* insertional mutant); Lane 3: EK48X (non-ropy excisant); Lane 4: EK217X (ropy excisant); Lane 5: MG1363 (negative control).

EK217X, a band of approximately 3.0 kb is observed. These results suggest that excision of the pGh9:IS*S1* mutational vector can have different effects upon the insertional locus. The literature suggests that an IS*S1* element is maintained in the locus upon excision of the vector, thus a Southern blot analysis was performed to determine the fate of the IS*S1* element. The blot analyzed in figure 5 was re-probed with 0.8 kb *HindIII-EcoRI* fragment of pGh9:IS*S1* corresponding to the IS*S1* element (figure 2) and the data is shown in figure 6. In lane 1, the natural lactococcal ropy isolate, no bands appeared. In lanes 2, 3, and 4, consisting of chromosomal DNA from EK1238, EK48X, and EK217X respectively, several bands common to all lanes are present. These bands represent additional insertion sites of the IS*S1* element. The unique bands of interest are the 7 kb band in lane 2, EK1238, and the 3 kb band in lane 4, EK217X. These bands correspond to the same bands observed in the previous Southern blot (figure 5) using the chromosomal fragment as a probe. In lane 3, the non-ropy EK48X excisant, a band can be seen of approximately 3.2 kb in size. Finally in lane 5, MG1363, no band is observed. This data suggests that an IS*S1* element native to the natural lactococcal ropy isolate or MG1363 is not present and that upon excision of the pGh9:IS*S1* vector, the IS*S1* element is maintained in the target DNA.

A growth curve experiment to compare the generation time of the excision EK48X non-ropy mutant to that of the natural lactococcal ropy isolate was performed. The optical density at 600 nm (OD_{600}) versus time is displayed in figure 7. Generation times were determined to be 1 hr and 20 min. for the natural lactococcal ropy isolate and 1 hr 45 min. for EK48X. This represents an increase in generation time of 24% for EK48X over that of the natural lactococcal ropy isolate. Further characterization of the excision mutant EK48X and the ropy revertant EK217X for ropy eps expression in sterile 2% milk was carried out. The natural lactococcal ropy isolate coagulates and produces long ropes in sterile 2% milk after 16 hours of incubation at 30°C as depicted in figure 8. The non-ropy excision mutant EK48X coagulated the sterile 2% milk after 96 hours but failed to produce ropy eps. The additional time required for EK48X to coagulate the sterile 2% milk is supported by its increased generation time. EK271X, on the other hand, expressed ropy eps in sterile 2% milk after 48 hours of incubation.

Figure 6. Southern Blot Analysis Demonstrating Presence of IS*S1*

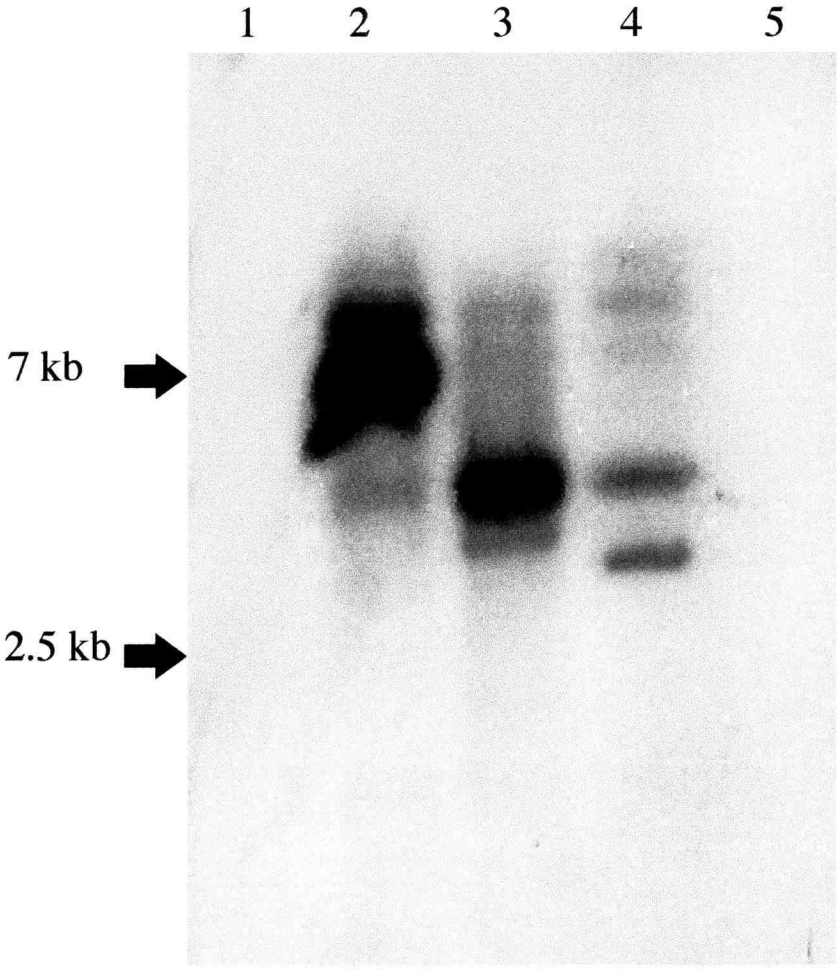


Figure 6. Chromosomal DNA was restricted using *EcoRV* and electrophoresed in 1.0% agarose, transferred to nitrocellulose overnight and UV cross-linked. The blot was pre-hybridized for one hour and probed with the 0.8kb *HindIII-EcoRI* fragment of pGh9:IS*S1*. The film was exposed for 5 minutes. Lane 1: Natural lactococcal ropy isolate; Lane 2: EK1238 (IS*S1* insertional mutant); Lane 3: EK48X (non-ropy excisant); Lane 4: EK217X (ropy excisant); Lane 5: MG1363 (negative control).

Figure 7. Optical Density vs. Time for the Natural Lactococcal Ropy Isolate and the EK48X Excision Mutant

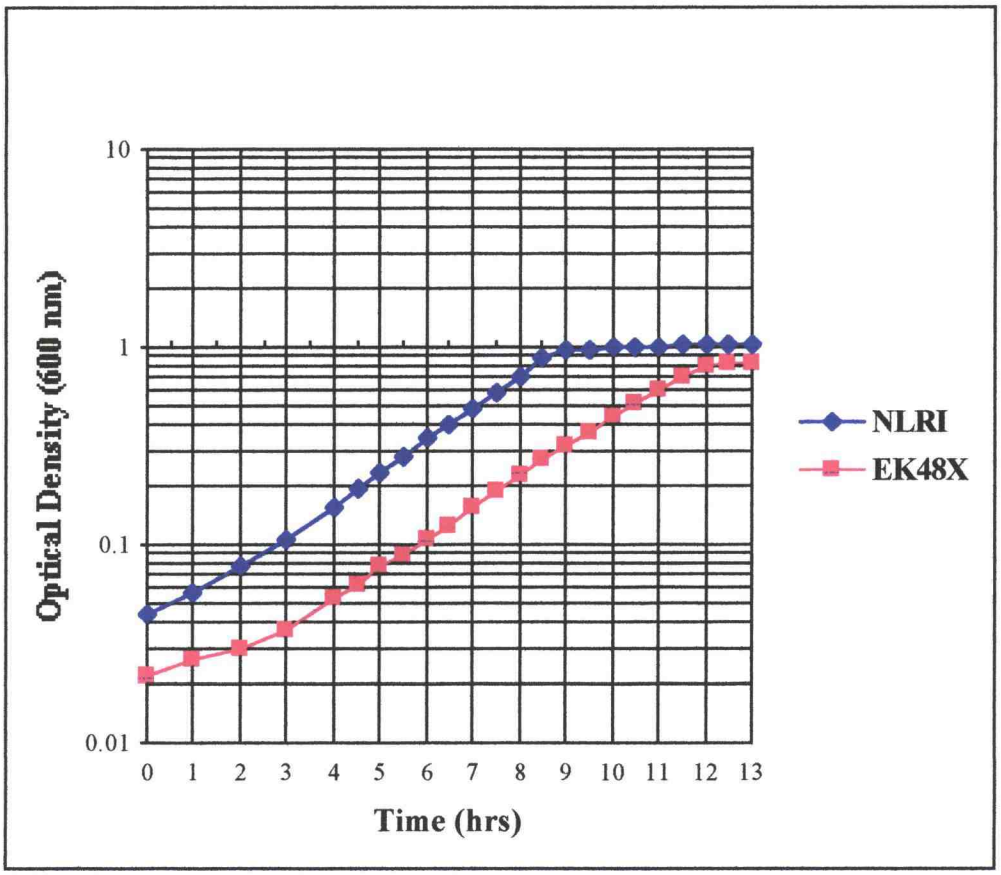


Figure 7. Cultures were grown to saturation in M17L. Sixty mls of M17L were inoculated with 1.5 mls of culture and mixed well. An initial OD_{600} reading was taken and readings were subsequently taken every 30 minutes. NLRI: Natural Lactococcal Ropy Isolate. EK48X: non-ropy excisant.

Figure 8. Ropy Eps Expression in Sterile 2% Milk by the Natural Lactococcal Ropy Isolate

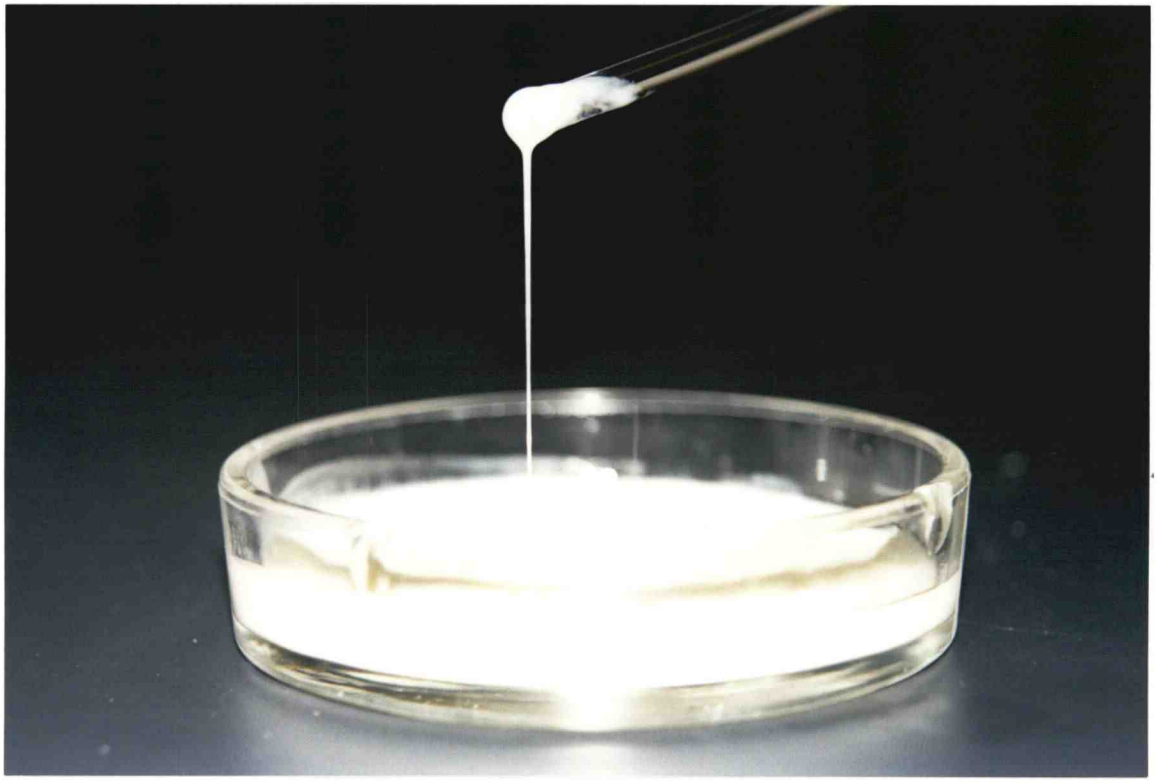


Figure 8. Commercial 2% milk was sterilized by steaming for 30 min. and 10 mls of milk were inoculated with 0.5 ml of an overnight culture. The milk was incubated for 18 hours at 30°C and visually examined for coagulation and ropy eps expression. Ropiness was indicated by inserting a sterile glass rod and pulling long ropes out of the milk.

IS*S1* Interrupted Gene Sequence Analysis

Primers to the pGh9:IS*S1* vector (figure 3) were synthesized and the insert of pEK1238H was sequenced as detailed in the materials and methods section. Sequence data for pEK1238H revealed a 2006 bp insert. The DNA insert sequence was scanned against GenBank for homologous DNA sequences. This search revealed potential matches to glycosyltransferase enzymes found in eps biosynthesis pathways in other bacteria. The DNA homology typically spanned regions of 100-274 base pairs with a 60-68% homology suggesting a conserved active domain important for sugar transfer activity. Translation of the insert DNA sequence of pEK1238H into its predicted open reading frames revealed two potential open reading frames, orf1, composed of 236 amino acids (aa), and orf2, composed of 338 amino acids. Both open reading frames are in the same direction but in different translational frames. Both of these amino acid sequences were scanned against SwissProt to find homologous sequences. This search revealed several matches to glycosyltransferase enzymes involved in eps biosynthesis pathways in other bacteria. Four regions of homology were found that contained between 4 and 8 homologous amino acids suggesting conserved active sites. Using the potential matches generated from the SwissProt comparison, multiple sequence alignments were performed to elucidate the percent identity over the entire orf and the results are presented in table 3. The predicted open reading frames show limited identity to putative glycosyltransferases essential for eps biosynthesis in Gram positive and negative organisms. It has been hypothesized that the gene products EpsF and EpsG of *Lactococcus lactis* ssp. *cremoris*, EpsI of *Streptococcus thermophilus*, WcaA of *Escherichia coli*, and AmsE of *Erwinia amylovora* are responsible for the transfer of specific sugar moieties late in the elongation stage of the eps heteropolymer (8, 55, 56, 63). The low overall percent identity suggests that a conserved active domain is what links these homologous sequences. The amino acid sequence identities suggest the open reading frames of the chromosomal insert DNA isolated from IS*S1* insertional mutant EK1238 may encode a glycosyltransferase involved in sugar residue transfer during elongation of the ropy eps polymer. The chromosomal insert DNA sequence of pEK1238H was analyzed for potential transcriptional start sites and

termination sites and none were found. The sequence was also searched for ribosomal binding sites and none were found in a suitable position to be involved with the translation of the predicted orfs.

Table 3. EK1238H Predicted Amino Acid Sequence Identities

Predicted orf	Organism/Gene Product		% Identity	Putative Function	Reference
orf1	*L. lactis* ssp. *cremoris*[a]	EpsG	26	glycosyltransferase	(63)
	S. thermophilus[b]	EpsI	24	glycosyltransferase	(56)
	E. amylovora[c]	AmsE	21	glycosyltransferase	(8)
	E. coli[d]	WcaA	23	glycosyltransferase	(55)
	B. subtilis[e]	GgaB*	26	spore coat biosynthesis	(20)
	S. dysenteriae[f]	RfpA	25	glycosyltransferase	(23)
orf2	*L. lactis* ssp. *cremoris*[a]	EpsF	29	glycosyltransferase	(63)
		EpsG	30	glycosyltransferase	(63)
	S. thermophilus[b]	EpsI	27	glycosyltransferase	(56)
	E. amylovora[c]	AmsE	21	glycosyltransferase	(8)
	E. coli[d]	WcaA	22	glycosyltransferase	(55)
	B. subtilis[e]	GgaB*	25	spore coat biosynthesis	(20)
	S. dysenteriae[f]	RfpA	22	glycosyltransferase	(23)

Full bacterial species names and GenBank accession numbers: a. *Lactococcus lactis* ssp. *cremoris* (U93364) b. *Streptococcus thermophilus* (U40830) c. *Erwinia amylovora* (X77921) d. *Escherichia coli* (U38473) e. *Bacillus subtilis* (U13979) f. *Shigella dysenteriae* (S73325).

* Identity in N-terminus part of protein.

DISCUSSION

The goal of this project was to identify and isolate genes responsible for ropy exopolysaccharide (ropy eps) biosynthesis in a natural lactococcal ropy isolate. Bacterial exopolysaccharides are of interest in industry, particularly the food industry, for their ability to increase viscosity, decrease syneresis, and bestow a smooth texture upon fermented milk products. Increased consumer health awareness associated with LAB also encourages increased use of ropy eps produced by LAB as thickeners and stabilizers in foods and beverages. Many studies have elucidated the structures and compositions as well as localized the genes involved in eps biosynthesis to plasmids, but recently only two studies have focused on characterizing the specific enzymes necessary for eps expression.

Localization of IS*S1* Insertional Interruption

Previous researchers have linked eps biosynthesis to large and small plasmids in mesophilic strains of *Lactococcus* (38, 63, 64, 65, 66) while chromosomally linked eps genes have only been identified in thermophilic organisms lacking plasmids (56). Using an unbiased approach of transposon mutagenesis with pGh9:IS*S1*, three mutants unable to express ropy eps were isolated and one, EK1238, was chosen for further study. A Southern blot analysis potentially revealed a chromosomal location for the pGh9:IS*S1* interrupted locus. A chromosomal position for genes essential for ropy eps biosynthesis in a mesophilic lactococcal organism has not previously been reported. Many strains of *Lactococcus* have been shown to carry a complement of IS elements that are potentially capable of inserting plasmids into the chromosome (44, 48), and the chromosomal location of the interrupted eps locus in this strain may be a manifestation of a chromosomally inserted plasmid.

IS*S1* Interrupted Gene Sequence Analysis

Translation of the DNA sequence generated from the chromosomal fragment isolated from the EK1238 IS*S1* insertional mutant revealed two open reading frames. The amino acid sequences of these open reading frames showed limited identity to sugar transfer enzymes involved in eps biosynthesis from several bacterial species. Typical eps biosynthesis, as shown in figure 9, requires linking the first sugar residue to a lipid-phosphate carrier onto which other sugar residues are transferred resulting in the repeating structure of the eps heteropolymer (36, 22). Elongation of the eps heteropolymer requires specific glycosyltransferases to transfer the appropriate sugar residues to the growing eps chain in the proper sequence to retain the appropriate structure. Glycosyltransferases are enzymes catalyzing the formation of glycosidic bonds between activated nucleotide sugars (donor) and the growing polymer (substrate) (3, 42). The polymerization and export of bacterial eps are still under consideration but the most recent speculation in Gram negative bacteria involves transporting the repeating eps subunits via energy dependent transporters to the periplasmic space where they are polymerized and ultimately exported to the environment (54, 55). Common individual activated sugar precursors such as UDP-D-glucose and UDP-D-galactose are synthesized by enzymes involved in energy production or other pathways and may also used for eps biosynthesis (55). A ropy strain of *Streptococcus thermophilus* has been shown to utilize only the glucose moiety and not the galactose moiety of lactose. The galactose moiety is excreted back into the environment implying that all cellular demands for sugar residues are met by glucose including synthesis of an eps composed of galactose, glucose, and rhamnose (16). Unique activated precursor sugar residues that are needed for eps biosynthesis are probably synthesized by enzymes coded for by the eps gene cluster (55). The structure of most eps heteropolymers contains a backbone of one or two different sugar residues and branches composed of the same or different sugar residues, presumably requiring a different glycosyltransferase for each residue in each position (3, 46, 63). *Lactococcus lactis* ssp. *cremoris* NIZO B40 produces an eps with a backbone of one galactose residue and two glucose residues and a branch containing a rhamnose and a

Figure 9. General Model of Eps Biosynthesis

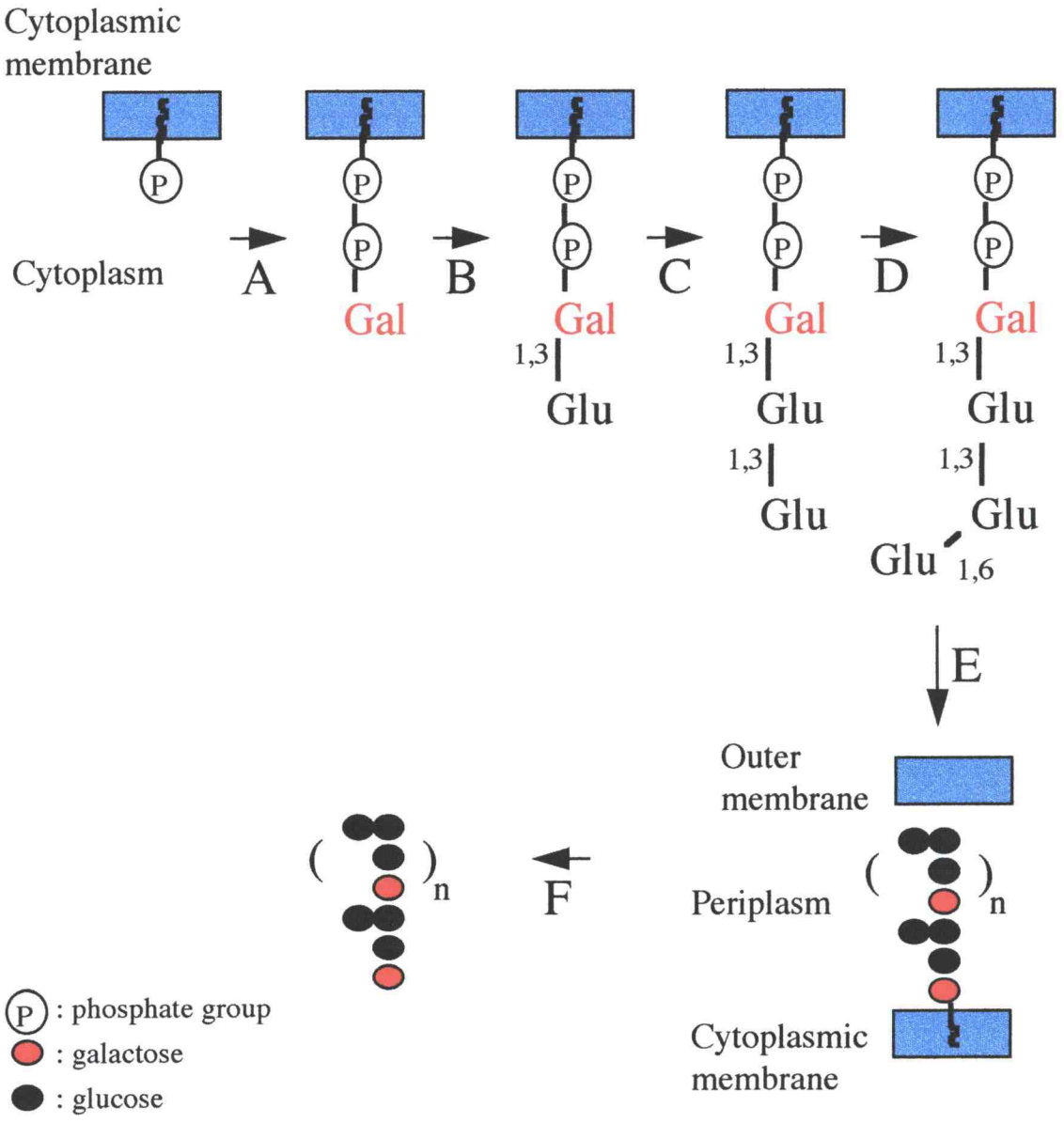


Figure 9. Steps of proposed model for biosynthesis of bacterial eps (21, 39). A: Galactosyltransferase catalyzes the transfer of activated galactose (UDP-Gal) to the lipid carrier. B, C, D: Glucosyltransferases catalyze the specific bond formation of the subsequent transfers of activated glucose residues (UDP-Glu) to the growing eps biopolymer. E: Energy dependent enzymes flip the eps biopolymer to the periplasmic space (47,48). F: Uncharacterized enzymes release the eps biopolymer from the lipid carrier and export the eps chain outside the cell.

galactose residue. Presumably five different glycosyltransferases would be needed to synthesize the eps and five glycosyltransferases were predicted in the eps operon (63). Similarly, *Streptococcus thermophilus* produces an eps with a backbone composed of two galactose residues and one glucose residue with a branch containing a galactose residue. Presumably, at least four glycosyltransferases would be required for eps synthesis and five were predicted in the eps operon (56). Glycosyltransferases must possess specificity to recognize the growing polymer, the appropriate substrate to be added, and be able to catalyze the correct bond formation at the appropriate site. This may explain the presence of five or more glycosyltransferases in several characterized eps operons (8, 55, 56, 63). Each glycosyltransferase catalyzes a different step possibly governed by substrate recognition (the growing eps chain) in order to ensure that the proper sequence of sugar residues and thus structure is constructed (3). This may lead to an inevitable amount of uniqueness in every glycosyltransferase due to the differing substrate recognition sites between enzymes and thus lead to low overall sequence identity among glycosyltransferases involved in eps biosynthesis. Greater localized identity will be present over short regions because independent of substrate recognition, the enzymes must retain similar mechanics to effect sugar residue transfer and formation of a glycosidic linkage. The gene product, ExoA, a glucosyltransferase from *Rhizobium meliloti* (46), was shown to complement an *amsE* mutant of *Erwinia amylovora* yet the two enzymes shared little identity illustrating that enzymes shown to have similar functions differ greatly in their amino acid sequence presumably due to substrate recognition (2, 8, 21). Typical amino acid sequence identities between glycosyltransferases reported in the literature range from a low of less than 20% to greater than 60% with the majority of percent identities ranging between 20-30% (21, 56, 63). The amino acid sequences of the two open reading frames of the chromosomal DNA insert isolated from the IS*51* insertional mutant EK1238 were found to show identity to enzymes important in the transfer of sugar residues to the growing eps chain but the overall identities were low. The low identities may also be due to the incomplete amino acid sequences of the predicted orfs. Limited overall identities between orf1, orf2, and the gene products from two LAB, EpsF and EpsG of *Lactococcus lactis* ssp. *cremoris* and EpsI of *Streptococcus thermophilus,* were observed. The gene

products EpsF and EpsG of *Lactococcus lactis* ssp. *cremoris* are important for the expression of eps by transferring sugar moieties to the growing eps heteropolymer (63). Similarly, the gene product, EpsI, of *Streptococcus thermophilus* is also a glycosyltransferase responsible for adding sugar residues to the growing eps chain (56). Multiple sequence alignments between these glycosyltransferases, orf1, and orf2 were performed and illustrated several conserved regions of 4, 5, 6, and 8 amino acid residues in length. These conserved regions may be important in glycosidic bond formation via the active domain and not in substrate recognition. The IS*S1* interrupted locus of the EK1238 insertional mutant may be responsible for adding sugar residues to the growing eps heteropolymer and the inability to complete the eps heteropolymer leads to the observed non-ropy phenotype of the IS*S1* insertional mutant EK1238.

Previous eps genes have been shown to be transcribed into one large, single mRNA and transcriptional start sites within the eps coding region were lacking (8, 56, 63). This data indicates that the gene clusters coding for eps biosynthesis in these organisms are organized into operons, and the gene products are translated from one species of mRNA. The typical coding structure of the eps biosynthesis operon from the 5' end starts with putative regulatory genes followed by genes involved in preliminary sugar transfer and late sugar transfer, and ends with the genes involved in polymerization and export. The lack of potential transcriptional start sites and termination sites in the chromosomal DNA insert sequence of the IS*S1* insertional mutant, EK1238, can be interpreted. The amino acid sequences show identity to glycosyltransferases typically involved in transfer of sugar residues later in eps heteropolymer elongation and would thus be predicted to be situated towards the middle of the gene cluster. Following the typical structure of eps operons, the transcription of orf1 and orf2 can be predicted to be driven from a promoter upstream of the eps gene cluster and would thus not need any transcription sites. No typical ribosomal binding sites were located in appropriate relation to the orfs in order to be useful in translation. The amino acid sequence of the predicted orfs is incomplete due to the end of the DNA insert sequence.

Effect of IS*S1* Insertional Mutation on Ropy Eps Biosynthesis

Characterization of EK48X, a non-ropy pGh9:IS*S1* excisant, in sterile 2% milk demonstrated that ropy eps was not produced in milk even after 96 hours. As an indication of growth and acid production in the milk, EK48X produced enough acid to coagulate milk only after an extended amount of time. Conversely, EK217X, a ropy excisant, was able to produce ropy eps in sterile 2% milk after 48 hours. Recovery of two types of excisants, non-ropy and ropy, after insertional mutagenesis with pGh9:IS*S1* and subsequent excision suggests that IS*S1* inserted in a gene essential for ropy eps biosynthesis. Southern blot analysis of the non-ropy EK48X excisant shows a total loss of the chromosomal sequence complementary to the probe, indicating excision of the vector and additional sequence flanking the insertion site. On the other hand, the Southern blot analysis of the ropy EK217X excisant shows sequence complementary to the chromosomal probe is still present but the fragment, visualized as a band in the Southern blot, has increased in size. These data suggests that upon excision of the pGh9 vector, different excision events may occur leading to a change in the size of the fragment and retention of the mutation or regeneration of the wild type phenotype. Southern blot analysis data using the IS*S1* sequence as the probe provides some clarification. Presence of a 3.2 kb band in the lane corresponding to EK48X suggests that upon excision, an IS*S1* element was maintained yet due to the loss of the flanking DNA sequence, one or more *EcoRV* restriction sites were lost leading to a larger fragment size. In the case of EK217X, the IS*S1* probe sequence hybridized to the same band as the chromosomal probe, suggesting that an IS*S1* element has been maintained in the insertion site yet is not disrupting ropy eps expression. These Southern blot analyses present further evidence that pGh9:IS*S1* inserted into the genes essential for ropy eps expression by illustrating the retention of the chromosomal fragment sequence and ropy eps expression in the ropy revertant even though an IS*S1* element is still present in the insertion site. Conversely, the chromosomal fragment sequence in the non-ropy excision mutant is absent and with excision of the pGh9 vector the fragment can be hypothesized to be smaller yet appears larger indicating a loss of *EcoRV* restriction sites and subsequently yields larger fragment.

The production of eps is an energy intensive process, potentially using up twice as much energy as cellular growth, and production of eps has been shown to be inversely related to cellular growth rates (29). Thus growth of an organism actively expressing a ropy eps directs a large proportion of available energy to making eps and would thus experience a slower growth rate. A hypothesis may be put forth that a non-ropy eps producing strain, such as a non-ropy laboratory strain or mutant, would then be able to grow faster as the drain on cellular energy is no longer present and cellular ATP can be directed towards growth, DNA replication, and division giving the non-ropy excisant mutant EK48X the potential to grow and divide at an increased rate over the ropy eps expressing natural lactococcal ropy isolate. The growth curve data shows that EK48X grows slower than the natural lactococcal ropy isolate. What may account for this conflicting evidence? The longer generation time for the non-ropy excisant EK48X may arise through a number of different mechanisms. Flanking genes not involved in ropy eps biosynthesis may lie upstream or downstream of the insertion locus and may be impacted by the insertion and subsequent excision. Also the Southern blots showed evidence of insertions occurring at other loci in the mutants. The genes flanking the excised chromosomal fragment and the other loci effected by the insertion and excision events may have detrimental effects to the health of the cell and be responsible for the longer generation time. A longer generation time caused by these other loci impacted by IS*S1* insertion may also be responsible for the extended time required for EK217X, the ropy revertant, to produce ropy eps in 2% milk.

Interestingly, the natural lactococcal ropy isolate grows much slower than that which has been cited for the non-ropy laboratory lactococcal strain MG1363. Exopolysaccharide production provides a large drain of cellular energy levels and as such must provide a selectable advantage over other bacteria in order to justify the large expenditure of energy and the resulting slow down in generation time. The role of eps in lactococci is not clear. Protection from bacteriophage has been suggested but is not a general characteristic. Lactococcal eps provides protection from certain phage attack yet acts as a receptor for others. Lactococci are now primarily grown in man-maintained environments where carbon and nutrient sources are not limited until late in the growth

stage, if the culture is allowed to grow for that long. It seems unlikely that eps expression is a starvation survival strategy as the cultures lactococci are routinely grown in do not provide the starvation stresses and thus the stimulus for eps expression of a natural environment. On the other hand, lactococcal eps may represent a survival strategy in the form of an adhesive. The bacterium receives signals indicating a favorable environment and generates a response in order to maintain its position. In continuous batch cultures grown in chemostats, some lactococci are able to produce eps in order to stay anchored in the growth chamber and thus avoid being removed from the rich environment. In natural environments such as the soil, water, or plants, the functions of other bacterial eps are beginning to be elucidated. Soil organisms utilize eps primarily as an adhesive. The adhesive anchors the bacteria to a matrix such as a soil particle allowing the bacterium to be in close contact with a source of nutrients and the eps may even trap smaller nutrient containing particles (11, 25). Similarly, bacteria present in the water also utilize eps as an adhesive for maintaining their position near a source of nutrients and nutrient trapping (45). In one case, a species of *Pseudomonas*, uses eps as a means of detachment from the surface. By constitively producing one eps the bacteria can attach to surfaces but when starvation occurs another eps is produced that interferes with the adhesive qualities of the constitively produced eps and the bacterium is released. This provides the bacterium with a survival strategy effected by eps expression and allows the bacterium to detach from the now unfavorable surface and find a new surface for colonization (69). Plant-bacterium interactions involving eps are many but some of the best studied involve *Rhizobium*, *Agrobacterium*, and *Erwinia* species. *Rhizobium* infects the roots of leguminous plants forming nodules allowing the bacterium to fix nitrogen that is used by the plant in exchange for carbohydrates produced by the plant. An eps produced by *Rhizobium* has been implicated as necessary for proper infection and establishment of the symbiotic relationship (46). *Agrobacterium* and *Erwinia*, on the other hand are plant pathogens. *Agrobacterium* causes tumor growth while *Erwinia* causes wilting. Both of these interactions require the production of a specific exopolysaccharide (8, 28).

One of the natural habitats of lactococci is postulated to be plants, and eps expression may represent a past survival strategy for lactococci that has been maintained. The eps

expressed by ancestral species of *lactococcus* may have functioned as an adhesive allowing the bacteria to maintain a favorable position in a relatively rich environment associated with the host plant.

The mechanisms by which bacterial eps allow cells to attach to solid surfaces continues to be explored but the mechanisms of gelling are becoming better known. In the best studied case of xanthan gum, strands of the polymer are able to adopt helical structures in solution. These helical structures are stabilized by non-covalent interactions and the short, one residue, side-chains may provide intra-helical stabilization (35, 41). Highly branched polymers such as guar gum, do not form as stable gel solutions due to the lack of stable interactions between strands of the polymer. The branches inhibit non-covalent forces from further stabilizing the gel structure (41). Preliminary compositional data of the ropy eps expressed by the natural lactococcal ropy isolate shows a heteropolymer composed of 54% glucose and 46% galactose with glucose at the branch points and galactose at the end points representing a novel composition for a LAB eps (unpublished data, E. P. Knoshaug, J. A. Ahlgren, J. E. Trempy). Preliminary data is also available for the mucoid eps produced by the natural lactococcal ropy isolate. This polymer is composed of 58% galactose, 29% glucose but also contains 13% mannose eps (unpublished data, E. P. Knoshaug, J. A. Ahlgren, J. E. Trempy). From this data one can postulate that the ropy eps may contain short branches that are able to non-covalently stabilize intra-molecular interactions among the helical strands of the ropy polymer leading to the rhealogical characteristic of long ropes. Conversely the mucoid polymer may have branches such that intra-strand interactions are prevented. Another prediction may be that the two polymers must act in concert to provide the strongly ropy characteristic to fermented milk. Studies have shown that mixtures of xanthan and guar can become even more stable than xanthan or guar alone. This is due to the additional intra-molecular interactions between additional branches of the guar polymer and xanthan. The ropy and mucoid polymers may act similarly when expressed in fermented milk. The branches that inhibited strong stabilization of the mucoid polymer may interact with the ropy polymer backbone and provide further stabilization to the milk giving it the characteristic ropy texture.

Summary

Using insertional mutagenesis, a gene important in ropy eps biosynthesis has been identified in a natural lactococcal ropy isolate. Failure of the insertional mutant to express ropy eps on whey agar and in milk strongly suggests the IS*S1* generated mutation lies within a gene essential for ropy eps expression. Additional support is lent by the excision process where upon excision of pGh9:IS*S1*, two phenotypes were observed, non-ropy and ropy. A fragment of chromosomal DNA was sequenced and this DNA sequence was translated into its predicted amino acid sequence. Two open reading frames demonstrated identity to glycosyltransferase enzymes involved in eps biosynthesis in several different species. The data generated in this project strongly suggests that an insertional mutation was created in a gene essential for ropy eps production and further molecular characterization localized this potential ropy eps gene to the chromosome, which is unique in a mesophilic lactococcal strain. The characterization of the genes responsible for ropy exopolysaccharide in this natural lactococcal isolate will provide another bacterial eps system that may have commercial utility.

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APPENDIX

Biographical Information

Eric Paul Knoshaug was born December 12, 1970 at Fort Belvoir, Virginia to Ronald N. and Judith F. Knoshaug. He was raised in Montana and Colorado. After graduation from Arvada West High school in Arvada, Colorado, he moved to Boulder, Colorado where he earned a B.A. in Biology and Environmental Conservation. Fall term, 1995, he began graduate training in bacterial genetics in the laboratory of Dr. Janine E. Trempy at Oregon State University in Corvallis, Oregon. He was the recipient of a Tartar award for research for the academic years 1995-1996 and was also the department of Microbiology nominee for the Herbert F. Frolander award in 1998.

PUBLISHED ABSTRACTS AND PRESENTATIONS:

Knoshaug, E. P., A. S. Bishop, and J. E. Trempy. 1998. The Search for Genes Defining Exopolysaccharide Expression in *Lactococcus*. 12th annual Biology Graduate Student Symposium, Hatfield Marine Science Center, Newport, OR.

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