AN ABSTRACT OF THE DISSERTATION OF

Karen P. Dierksen for the degree of Doctor of Philosophy in Microbiology presented on June 12, 1996.

Title: Regulation of Exopolysaccharide Synthesis

Abstract approved: [redacted]
Janine E. Trempy

Lactococcus lactis subsp. *cremoris* Ropy 352 and *L. lactis* subsp. *cremoris* Hollandicus produce an exopolysaccharide (EPS) that imparts commercially desirable textural and rheological properties to fermented milk products. This ropy phenotype is expressed under specific environmental conditions. A mucoid EPS phenotype, also expressed under specific environmental conditions, but not involved in the fermentation of ropy milk was identified. The two EPS phenotypes can be expressed individually or concurrently.

Genetic regulators involved in expression of the EPS phenotypes were sought. DNA probes and polyclonal antiserum specific to two regulators of EPS in *Escherichia coli*, Lon protease and RcsA protein, were used to probe ropy and non-ropy strains of *L. lactis*. The two ropy strains of *L. lactis* subsp. *cremoris*, Ropy 352 and Hollandicus, expressed significantly less of the Lon protein than non-ropy strains.

Southern and Western blot analysis was extended to a number of Gram negative and Gram positive bacteria. All of the Gram negative bacteria probed contained DNA sequences that hybridized to the *lon* and *rcsA* gene probes, and all of these bacteria has at least one protein that reacted with antiserum to *E. coli* Lon and RcsA proteins. Two of the Gram positive bacteria contained DNA sequences that hybridized to the *E. coli rcsA* probe. None of the other Gram positive organisms contained DNA sequences that hybridized to the *rcsA* or the *lon* probes. However, all the Gram positive bacteria contained one high molecular weight protein that

reacted with Lon antiserum. In addition, *Streptococcus salivarius* expressed a protein that reacted with RcsA antiserum.

In the course of this study, a second RcsA protein was identified in *E. coli*. The two RcsA proteins are expressed from one *rcsA* gene. One RcsA protein is not the proteolytic product of the other RcsA protein. Limited peptide digest profiles of each RcsA protein reveals almost identical peptides indicating the two proteins share a high degree of homology but are not identical. Ferguson plot analysis strongly suggests that the two RcsA proteins differ by size not by charge. Neither RcsA protein can be detected in cells mutant for *lon* and *rcsB*.

Regulation of Exopolysaccharide Synthesis

by

Karen P. Dierksen

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed June 12, 1996
Commencement June 1997

Doctor of Philosophy dissertation of Karen P. Dierksen presented on June 12, 1996.

APPROVED:

Major Professor, representing Microbiology

Chair of Department of Microbiology

Redacted for privacy

Dean of Graduate School

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

Redacted for privacy

Karen P. Dierksen, Author

ACKNOWLEDGMENTS

To list all the people at Oregon State University who have made my stay a memorable experience would be a monumental task. Faculty, staff, and students from every department I've had contact with have been quick to assist me. I thank you all collectively for your support, encouragement, and your friendship.

I wish to acknowledge those people who have contributed work on this project. Jim Marks performed the Southern blotting experiments. Gary Vaughn assisted with scoring colonies for the ropy phenotype, performed the anthrone and cetylpyridinium chloride polysaccharide assays, and assisted with media and reagent preparations on occasions to numerous to count. Dr. Pui Shing Ho provided information on Ferguson plot analyses. Wolfgang Ebel was always willing to share his technical expertise, provide editorial comment, combine experiments, and work cooperatively on any aspect of our individual projects. I wish to thank Dr. Valerie Stout and Dr. Susan Gottesman for the generous gift of RcsA and Lon antiserum.

I particularly want to thank Dr. William Sandine and Dr. John Fryer for providing wise council, taking an active mentoring role, and serving as role models for professional conduct. You have set a high standard for me to follow.

I wish to acknowledge past and present faculty, staff, and students of the Microbiology Department who have assisted me in so many different ways.

The office staff: Linda Whiteman, Joy Asbury, Carlene Pelroy, and Marilyn Wallace

The Sandine lab: Brent Daniels, Marina Jovanovic, Xintian Ming, Alex Teo, Herb Wyckoff and Debbie Norbratten

The Trempy lab: Don Chen, Jim Marks, T. J. White, Wolfgang Ebel, Mary Jane Nather, Rockie Yarwood, Gary Vaughn, and all the other undergraduate and graduate students who have rotated through the lab during my stay.

The teaching faculty and staff: Dr. Mary Burke who guided me during my Teaching Assistantship and assisted me with dissertation

figures. Cindy Fisher who provided me with bacterial cultures from the OSU Microbiology collection.

My committee members: Dr. Mark Daeschel, Dr. Larry Moore, Dr. Joanne Leong, and Dr. William Sandine. I thank you for the time commitment you made to me when you agreed to serve as members of my committee. I appreciate the suggestions and comments you have made regarding my research project.

For providing financial support during my five year stay at OSU, my appreciation goes to the Eckelman Foundation, the Tartar Foundation and the Microbiology Teaching Assistantship fund.

Dr. Janine Trempy, my Ph.D. advisor, mentor, and friend, your dedication to students and your commitment to providing a quality educational experience will always be an inspiration to me. Thank you for the innumerable hours you spent at the lab bench providing the technical training that has given me the confidence to tackle new problems. Last, but not least, thank-you for sharing your enthusiasm for science. It's been such fun working with you.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF TABLES

DEDICATION

This work is dedicated to my husband, Alan, and our son, David, without whose love, support, and encouragement it would not have been possible.

REGULATION OF EXOPOLYSACCHARIDE SYNTHESIS

INTRODUCTION AND LITERATURE REVIEW

Research topic

Lactic acid bacteria represent a heterogeneous group of microorganisms used in the fermentation of milk, meat, and vegetable materials. Subspecies of *Lactococcus lactis* are used in the production of numerous fermented milk products including cheeses, buttermilk, and sour cream. Many physiological, biochemical, and genetic characteristics of the bacteria have been modified to enhance the organism's commercial usefulness. A detailed understanding of the organism at the genetic level was essential to achieve this end. Strains of *L. lactis* produce an exopolysaccharide that imparts desirable characteristics to fermented milk. This phenotype is unstable and easily lost. Scant information exists about the genetic regulation of this trait. Identification of regulatory genes is a necessary first step in utilizing this characteristic in commercial applications.

Definition of Lactic acid bacteria

Lactic acid bacteria (LAB) represent a heterogeneous group of Gram positive, non-sporeforming microorganisms used in the fermentation of milk, meat, and vegetable materials. They are fastidious organisms generally associated with nutrient-rich habitats such as food products and green plants, but some LAB are normal inhabitants of the mouth, intestines, and mucosal membranes of mammals. Lactic acid bacteria are not able to synthesize porphyrin groups (e.g., heme), do not possess an electron transport chain, and rely on fermentation for energy production. Their major end-product of carbohydrate fermentation is lactic acid (6). As a

result of their lactose-fermenting ability and concomitant acid-producing capacity, LAB perform important functions in the fermentation of specific food products. For example, in the manufacture of cheddar cheese LAB concentrate and stabilize the curd by coagulating protein and expelling moisture. They inhibit or prevent the growth of spoilage or pathogenic microorganisms by increasing the acidity of the environment. Some strains produce specific inhibitory substances as well. Lactic acid bacteria also contribute directly or indirectly to cheese texture and to the formation of flavor compounds (107).

Recent taxonomic revisions list the following organisms in the LAB group: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, and *Vagococcus* (6). The LAB group clusters with the low G + C content of DNA group (55% or less), whereas *Bifidobacterium* and *Propionibacterium* cluster with the high G + C subdivision of the Gram positive bacteria (144).

Lactic acid bacteria used in the dairy industry can be divided into mesophilic and thermophilic classes. The thermophiles include *Lactobacillus delbruekii* subsp. *bulgaricus*, *Lb. helveticus*, and *Streptococcus thermophilus*. These LAB are used to manufacture yogurt, acidophilus milk, and cheeses such as Emmental, Gruyere, and Italian types. The mesophilic LAB that produce most of the fermented milk products include *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris*, and *Leuconostoc mesenteroides* and *dextranicum*. These organisms are used to manufacture cheeses such as Cheddar, Gouda, Camembert, and Cottage, and in cultured buttermilk and sour cream (22).

Lactococcus lactis subsp. *cremoris* is preferred over *L. lactis* subsp. *lactis* in the manufacture of many fermented milk products including cheddar cheese. *Lactococcus lactis* subsp. *lactis* produces higher levels of carbonyl compounds such as acetaldehyde, diacetyl, pyruvic acid, and formaldehyde. These compounds have been implicated in fruity off-flavor defects and harsh or "green" flavor defects in cheese (136, 137). The following physiological characteristics have been used to distinguish *L. lactis* subsp. *lactis*

from *L. lactis* subsp. *cremoris* : (i) growth at 40°C, (ii) growth in 4% NaCl, (iii) growth at pH 9.2, (iv) the ability to hydrolyze arginine, and (v) resistance to lithium chloride (107).

Attempts to isolate new subspecies *cremoris* strains from the environment using the above criteria in conjunction with dilution plating have been largely unsuccessful, whereas isolation of subspecies *lactis* strains from green plant materials is easily accomplished. Two theories have been advanced to account for the difficulty of isolating new *cremoris* strains. (i) Subspecies *cremoris* occur in nature in very small numbers, and they are diluted out using current dilution-plating methods. (ii) They may be present only in milk products where they have evolved as a result of dairy-related practices (108). Salama *et al.* have designed a subspecies-specific 16S rRNA probe for *L. lactis* subsp. *cremoris* and a genus-specific probe for lactococci (103). These probes can be used for the isolation of new strains of subspecies *cremoris*, and as a more definitive method for classification of *L. lactis* at the subspecies level than the current use of physiological characteristics. An alternate method for distinguishing the two subspecies is Southern hybridization analysis using cloned chromosomal genes as probes (43). Using this approach, Delorme *et al.* have shown that the level of divergence between the subspecies varies considerably along regions of the chromosome. In conserved regions, divergence is as low as 10-20%, which agrees with 16S rRNA data, but divergence is as high as 35-65% in variable regions (26).

Functional characteristics of lactococci

Many physiological functions of LAB have important ramifications in the fermentation of dairy products. Acid production, proteolytic activity, aroma formation, production of inhibitory components, bacteriophage resistance, and exopolysaccharide formation influence the quality of the final product.

Acid production

Lactose, the major sugar present in milk, is fermented by lactococci to L(+)-lactic acid. Lactose is phosphorylated to lactose phosphate prior to transport across the cell membrane via the phosphoenol pyruvate-phosphotransferase (PEP/PTS) system. Lactose can also be transported by an ATP-requiring permease system. Once inside the cell, lactose is hydrolyzed to glucose and galactose-6-phosphate by phospho-β-galactosidase. The phosphate or free sugars of glucose, galactose, or lactose can be further metabolized by three different pathways. Glucose is catabolized through glycolysis, galactose-6-phosphate through the D-tagatose-6-phosphate pathway, and galactose through the Leloir pathway (83). The pH drop associated with lactose utilization is important in the clotting or coagulation of milk and in the inhibition of spoilage or pathogenic organisms. Fast acid-producing strains which are required in milk fermentations can be distinguished from slow acid-producing strains by plating on milk-based fast-slow differential agar called FSDA I (56a) or FSDA II (143a). Both media are described by Sandine (107).

Proteolytic activity

Lactococci are nutritionally fastidious organisms. For optimal growth, they require the activity of proteinases and peptidases to supply peptides and free amino acids. The proteinases which have been identified to date are all high molecular weight, membrane-associated, plasmid-encoded proteins (68). Peptidases which have been identified to date are soluble, chromosomally-encoded proteins (83). Proteinases break down large molecules into peptides which are further degraded by peptidases to free amino acids and di- or tripeptides. To give new flavor characteristics to dairy products, and to allow cheeses to be ripened in a more controlled fashion and at an accelerated rate, strains have been engineered with altered or enhanced proteinase or peptidase function (22).

Aroma formation

Lactococci contribute flavor and aroma compounds to fermented milk products and cheeses. Lactic acid, acetic acid, and metabolites from citrate utilization such as acetaldehyde, diacetyl, and acetoin provide aroma in milk products. Lactococci also play an indirect role in the production of flavor compounds in cheese by generating di- or tripeptides and free amino acids which are metabolized to volatile compounds (83). The association of genetic determinants of citrate utilization with plasmid DNA has facilitated their identification and analysis (22).

Production of inhibitory components

The production of organic acids such as lactic and acetic acids and concomitant pH reduction are the primary means of inhibitory action by lactococci against other microorganisms. However, lactococci also produce other inhibitory substances. These include hydrogen peroxide, diacetyl, and bacteriocins. Hydrogen peroxide is inhibitory by itself or in conjunction with the lactoperoxidase system present in milk. In the presence of lactoperoxidase, hydrogen peroxide and thiocyanate (naturally found in milk) react to form an inhibitory substance, hypothiocyanate (83). Diacetyl, an aroma compound, has also been recognized as an inhibitory substance. However, a relatively large amount of diacetyl is required for inhibitory action. This is problematic when diacetyl is used in foods because of its intense aroma at higher concentrations (60). Bacteriocins form a heterogeneous group of antimicrobial substances produced by lactococci. They are differentiated on the bases of antibacterial spectrum, mode of action, and chemical properties. In general, they are defined as proteinaceous compounds bactericidal to susceptible bacteria (24, 65). Bacteriocin production and immunity determinants are often plasmid-linked. Nisin, a bacteriocin produced by *L. lactis*, and the first bacteriocin used in commercial applications, has been granted GRAS (generally regarded

as safe) status in the US for use in process cheeses (65). Strains are being developed that produce increased amounts of inhibitory substances, or that produce a number of different inhibitory substances each with specificity against particular organisms (22).

Bacteriophage resistance mechanisms

The infection of dairy cultures by bacteriophages results in large economic losses yearly. Means of inhibiting or stopping phage infection are needed. At least three independent bacteriophage resistance mechanisms have been identified in lactococci: (i) adsorption inhibition, (ii) restriction and modification (R/M), and (iii) abortive infection (abi). Adsorption inhibition occurs when phage are unable to attach to the cell surface in a manner that produces a successful infection. Phage-resistant strains have mutated cell-wall structures that are no longer recognized as receptors by the phage. By complementing spontaneous phage-resistant mutants of *L. lactis* subsp. *lactis* C2 with a genomic library from wildtype C2, Geller *et al.* have identified a membrane protein, designated *pip* (phage infection protein), that is required for phage c2 infection. One strategy for constructing bacteriophage-resistant commercial strains would be to introduce the mutant *pip* gene into the strains (39). Restriction and modification protects host DNA through modification, generally by methylation, while phage DNA is degraded by restriction endonucleases. Abortive infection refers to defenses which act intracellularly but are not due to restriction and modification. One such system, involving a protein designated Hsp, appears to act by interfering with phage DNA replication. Other systems have been identified but the specific steps they inhibit have not been determined. In all cases, phage burst size is low, few phage are released into the medium, and little or no inhibition of bacterial growth rate and acidification is observed (55). Strategies that have been successfully employed to reduce economic losses due to phage infection include: (i) use of single defined strains and selection of phage resistant mutants in

conjunction with strain rotation (106), (ii) introduction of plasmid-encoded R/M and abi defense mechanisms into commercial strains (105), (iii) rotation of several R/M and abi mechanisms within a single-strain starter system (66), and (iv) the use of antisense mRNA technology (64).

In the past, LAB including lactococci have not been as easily amenable to DNA manipulation as *Escherichia coli* or other well-characterized organisms. Recent advances in DNA technologies for the study of lactococci have been instrumental in (i) elucidating important biochemical pathways, (ii) identifying gene transfer and delivery systems, (iii) identifying gene expression and secretion signals, and (iv) isolating and examining, at the DNA sequence level, genes important to the dairy industry (14). A greater understanding of lactococci at the genetic level has allowed researchers to enhance commercial strains by genetic manipulation of functional characteristics such as those described in the previous sections. A functional characteristic of lactococci that has not been well characterized is the synthesis of exopolysaccharide.

Exopolysaccharides contribute to the rheological and textural properties of fermented milk products by increasing viscosity and decreasing syneresis (liquid exuded at the surface). Interest in exploiting this functional characteristic has increased in recent years, in part, due to the demand by consumers for more natural products.

Exopolysaccharide formation

Expression of exopolysaccharide by lactococci is the focus of this research project. The background material has been divided into six sections: (i) definition of exopolysaccharide, (ii) natural habitat of lactococci and environmental roles of exopolysaccharides, (iii) general categories of exopolysaccharides, (iv) industrial

applications and uses, (v) structural analyses and rheological properties, and (vi) polysaccharide phenotype instability.

Definition of exopolysaccharide

Bacterial cells synthesize a number of polysaccharides. Some of these polysaccharides are located in the cytoplasm and are used as carbon and energy sources. Others are components of the bacterial cell wall. A third group of polysaccharides are extracellular. These polysaccharides may be present as capsular material directly attached to the cell surface or may be secreted into the environment. Both capsular and unattached polysaccharides may be produced by the same organism; distinguishing between them can be difficult. Bacteria producing these polysaccharides have been described as capsule-producing, mucoidy, slime-producing, or ropy, dependent on their characteristics on solid media, in liquid culture, or upon microscopic examination. Sutherland proposed that the general term exopolysaccharide (EPS) be used for all polysaccharides found outside the bacterial cell wall (118). The term EPS as defined by Sutherland is used in this work.

Natural habitat of lactococci and environmental roles of exopolysaccharides

Isolates of *L. lactis* subsp. *cremoris* are found in association with dairy products. Attempts to isolate them from the natural environment have been unsuccessful. In contrast, isolates of other subspecies of *L. lactis* are readily detected in association with green plant materials. Although the roles of EPS in the survival of lactococci in the natural environment remain unknown, the association of lactococci with plants suggests the EPS may function to protect the bacteria from desiccation, may function as an adhesive agent, and may be involved in plant-bacterial interactions. In other bacteria, EPS has been shown to decrease susceptibility to

phagocytosis and phage attack, provide higher oxygen tension, and participate in the uptake of metal ions [reviewed by (16, 74, 132)]. Regardless of the functions provided by EPS in the natural environment, EPS is not required for cell viability in the rich milk environment. EPS can be removed without adverse effects on growth, and mutants unable to produce EPS are easily isolated (16).

General categories of exopolysaccharides

Exopolysaccharides can be broadly subdivided into homo- and heteropolysaccharides; polymers composed of a single monomer unit, or those formed from several types of monomer units, respectively (118). A specific substrate, usually sucrose, is required for synthesis of homopolysaccharides such as levans, mutans, and dextrans. Other homopolysaccharides such as bacterial cellulose are produced from any of several carbon sources. Specific substrates are not involved in heteropolysaccharide synthesis, and there is no apparent correlation between the composition of the EPS produced and the ability of the organism to utilize or not utilize any particular substrate. The complexity of EPS varies greatly. However, most are formed from relatively simple disaccharides to octasaccharides, and may include one or more acyl group. Organisms synthesizing heteropolysaccharides are widely distributed among prokaryotic and eukaryotic genera (119).

Industrial applications and uses of exopolysaccharides

Properties of EPS can be exploited as functional components of fermentation by bacteria, or the EPS itself can be isolated for use as a biopolymer in other applications. In addition to applications in the food and dairy industries, EPS have application in the cosmetic, chemical, medical, waste treatment, and oil industries. They are often used as thickeners, viscosifiers, or drag-reducing agents. Exopolysaccharides provide a matrix for immobilization of enzymes,

cells, or biomedical materials. They function as chelating agents in metal recoveries, waste treatment, and in water purifications [Reviewed in (111)]. The desirable properties of EPS are attributable to the capability of EPS to modify aqueous environments. These qualities include among others, high viscosity at low concentration, unique gelling properties, compatibility with high salt concentrations, adhesive and film-forming properties, and binding capacity [Reviewed in (75)].

Dextrans were the first microbial polysaccharides of industrial importance to be isolated. Dextrans synthesized by *Lactobacillus, Leuconostoc,* and other genera have a number of applications including use as plasma extenders and as chromatography components (DEAE dextran) (111). Xanthan, which is synthesized by strains of *Xanthomonas campestris*, was the second microbial EPS isolated, and is the most profitable EPS to date. Xanthan gum was given FDA (U. S. Food and Drug Administration) approval for use in foods in 1969 (23). It is used in bakery fillings, frozen foods, salad dressings, processed cheeses and in numerous other applications (111). The most recent microbial polysaccharide to gain FDA approval for use in foods (1990) is gellan gum, a polysaccharide synthesized by *Aureomonas elodea* ATCC 31461 (23). Isolation of other microbial polysaccharides with novel characteristics continues to generate commercial interest.

Interest in EPS produced by LAB has arisen in recent years not only for their rheological properties in fermented milk products but as safe alternatives as food additives in other applications as well. From an industrial standpoint, low yield and genotypic instability are serious problems to be overcome. However, because LAB are currently used in food and dairy products, the perception of health-conscious consumers is that EPS produced by LAB are from more natural sources than EPS from strains such as *Xanthomonas* or *Pseudomonas*. (23).

Structural analyses and rheological properties

It has been well-established that the thickening trait exhibited by strains of LAB in fermented milk products is associated with EPS expression (16, 17, 83, 107). However, attempts to correlate EPS structure to rheological properties has largely been unsuccessful. The structures of EPS from several ropy lactococci (85, 86) and ropy lactobacilli (67, 145) have been determined. All are structurally distinct and have different monosaccharide compositions. The presence of many structurally distinct EPS in strains exhibiting the ropy phenotype suggests that it is not one specific EPS structure that confers the ropy property to fermented milk products. Toba *et al.* suggested that rheological properties of ropy fermented milk might be the result of interactions between the EPS and milk proteins (124). This interaction had previously been observed by Schellhaass and Morris in scanning electron micrographs (SEM) of ropy and non-ropy strains of *Streptococcus thermophilus, L. cremoris,* and *Lactobacillus bulgaricus* (109a). SEM micrographs by both groups support the hypothesis of milk protein-EPS interactions. SEM micrographs by Toba *et al.* from a ropy strain (*L. cremoris* SBT 0495) showed that the EPS formed a network that attached long chains of bacterial cells to a milk protein matrix. Casein micelle clusters were attached to each other by the EPS, making large conglomerates. Micrographs of a non-ropy variant showed bacterial cells embedded in small casein micelle chains and clusters; no conglomerates were observed. Toba *et al.* proposed that the conglomerates present in the ropy culture increased the adhesiveness of the milk giving it the characteristic ropy consistency (125).

Exopolysaccharide phenotype instability

Although interest in utilizing the viscosity-enhancing characteristics of the ropy EPS-producing strains has increased in the last decade, the instability of the ropy phenotype at the genetic

level remains an impediment to wide-spread use of these strains. Growth at high temperature and/or repeated serial transfer of a culture frequently leads to loss of the phenotype (16). This is thought to be due to the loss of plasmid DNA (140). In a number of strains, loss of a single plasmid has been associated with loss of the phenotype (89, 135, 138)

Limitations on the industrial exploitation of EPS arise from the genetic instability of the producing strains and/or low production of the EPS. An understanding of regulation at the genetic level is paramount in addressing these problems. The observation of plasmid-linkage to many essential functions of lactococci was the beginning of serious interest in genetic research in these organisms. Gene transfer techniques were developed and recombinant DNA techniques made cloning and genetic analyses of genes encoding functions conferring traits important to industry possible (141).

Although significant advances have been made in assigning specific genes to phenotypic expression of many functional characteristics, genetic information regarding regulators of EPS expression in lactococci is lacking. On the other hand, regulators of EPS expression have been identified and well characterized in *Escherichia coli*. Evidence suggesting that many structural and regulatory genes are essentially the same in Archaea, Bacteria, and Eukarya continues to accumulate [reviewed in (1)]. For example, eight of the 12 open reading frames (ORF) from the *his* operon in *L. lactis* are homologous to *his* genes in *E. coli*, and the order of the genes is similar in both organisms (25). More applicable to EPS regulation is the recent identification of an *eps* (exopolysaccharide) gene cluster from *Streptococcus thermophilus* Sfi6. The amino acid sequences deduced from the thirteen genes in the *eps* cluster share homology with capsule or lipopolysaccharide synthesis gene products from both Gram positive and Gram negative microorganisms. This *eps* gene cluster, when expressed from a multicopy plasmid, is sufficient for expression of EPS in the non-EPS-producing heterologous host, *Lactococcus lactis* MG1363 (114).

The evidence for conservation of many genes and proteins among distantly-related organisms was used in the present work as

rationale for probing lactococci with DNA and antibody probes specific for polysaccharide regulators from *E. coli*. A description of the regulators and the pathway for polysaccharide synthesis in *E. coli* is outlined below and illustrated in Figure 1.

Exopolysaccharide regulation in *Escherichia coli*

A complex network of regulatory proteins is involved in the expression of colanic acid capsular polysaccharide in *E. coli*. Colanic acid, also known as M-antigen, is a type I EPS as defined by Jann and Jann (59). Type I EPS (slime layer) are loosely attached to the bacterial cell, whereas type II EPS (capsular) are firmly attached to the outer membrane (59). Colanic acid, a hexasaccharide of D-glucose, D-galactose, L-fucose, and D-glucuronic acid in ratios of 1:2:2:1 (77), is synthesized at the cell membrane utilizing nucleotide-sugar intermediates and glycosyl lipid carriers. Glycosyl lipid carriers are also required in LPS (lipopolysaccharide) biosynthesis. Sequestration of glycosyl lipid carriers in the LPS pathway is postulated as an explanation for EPS production predominantly at low temperature or on nutrient-poor media. Presumably, under these conditions, cells grow more slowly and less glycosyl lipid carriers are needed for LPS biosynthesis and therefore, are available for EPS biosynthesis (118). Colanic acid is synthesized by numerous enteric bacteria including strains from the genera *Salmonella* and *Aerobacter* (50).

A number of biological roles for type I EPS from microorganisms other than *E. coli* have been demonstrated. For example, they serve as virulence factors in strains of *Pseudomonas, Erwinia,* and *Xanthomonas* (74), and are involved in symbiotic processes such as infection of root hairs, nodule invasion, and nitrogen fixation in *Rhizobium* sp. (76). In *E. coli,* the one biological role for colanic acid that has been demonstrated is protection from desiccation (94), and the only environmental signal that has been shown to induce capsule synthesis thus far is osmotic shock (112).

Ten regulatory regions involved in expression of colanic acid capsular polysaccharide in *E. coli* have been identified; *lon* (56) [also known as *deg* (15), or *capR* (77)], *rcsA* (49), *rcsB* (49), *rcsC* (49), *rcsF* (40), *ops* (147), *capS* (77), *capT* (77), *hns* (112), and *dsrA* (112).

Lon protease

Lon, the first ATP-dependent serine protease to be identified and purified, is a protein of 784-787 amino acids. It has a subunit M_r of 88,000, migrates on SDS-PAGE with an apparent molecular weight of 94,000 Da., and functions as a tetramer or octamer in its native state (45). Lon was named La protease by Swamy and Goldberg in 1981 when they purified it as one of eight soluble endoproteases from *E. coli*. The six cytosolic serine proteases they purified were named Do, Re, Mi, Fa, So, and La they said, "In the hope that, when we actually work out the pathway for protein degradation, we shall also discover a pleasant melody." (120). Although most researchers today refer to the protease as Lon, Goldberg and co-workers continue to use the name La.

At the same time that the six serine proteases were purified, two metaloendoproteases were identified: Pi, a periplasmic protease, and Ci, a cytosolic protease. Subsequently, Goldberg *et al.* purified a ninth protease named Ti (57). This protease, which is the only protease in *E. coli* other than Lon shown to be ATP-dependent, is commonly referred to as Clp (48).

Lon protease plays an important role in the degradation of highly abnormal proteins and of certain short-lived regulatory proteins in *E. coli*. Its role in a variety of cellular responses resulted in the initial identification of *lon* as three separate mutant genotypes: *lon*, *deg*, and *cap* (46). The *lon* designation was assigned in 1964 by Howard-Flanders *et al.* to a class of UV-sensitive mutations that resulted in cells forming *long*, non-septate filaments and dying after exposure to UV irradiation (56). At the same time, Markovitz identified three loci involved in overproduction of colanic

acid capsular polysaccharide: *capR, capS,* and *capT* (78). In 1973, *deg* mutants, cells with a reduced capacity to degrade abnormal proteins, were isolated by Bukhari and Zipser (15). The *lon, capR,* and *deg* mutant phenotypes were subsequently shown to map to the *lon* gene (18). *CapS* (*E. coli* K-12 map position 22.5 min)and *capT* (map location unknown) have not been well-characterized and it is not known if these loci are the same as other regulators of colanic acid described in the following sections (46).

Lon protease, the most-studied protease of E coli, has unusual properties that distinguishes it from proteases previously studied. (i) Although Lon, like other serine proteases, is sensitive to diisopropylfluorophosphate, it differs from the classic serine proteases in two important ways. First, Lon does not contain the canonical catalytic triad residues (aspartate, histidine, and serine), and second, Lon requires ATP for catalytic activity. The ATP-dependence for catalytic activity may insure that non-specific or inappropriate degradation of proteins does not occur. (ii) Lon has an ATPase activity that is necessary for protein breakdown; both protein degradation and ATP hydrolysis are Mg^{2+} dependent. (iii) Lon is a DNA-binding protein, and DNA, in particular single-stranded DNA acts to activate Lon's catalytic function. (iv) The *lon* gene contains a heat-shock promoter which is under control of the *rpoH* -encoded sigma factor (Reviewed in (44, 45)).

Early studies by Markovitz demonstrated that some of the structural genes involved in colanic acid biosynthesis were deregulated in *lon* mutants. For example, his studies revealed higher levels of phosphomannose isomerase, an enzyme necessary for synthesis of GDP-fucose, and higher levels of UDP-Gal-4-epimerase, an enzyme necessary for the interconversion of UDP-Gal and UDP-Glu (77). To determine how *lon* regulates colanic acid structural genes, Trisler and Gottesman isolated, mapped, and characterized *lac* operon fusions to a number of these structural genes. Five genes which were expressed at low levels in *lon* + strains and expressed at higher levels in *lon* mutants were mapped to a cluster near 45 minutes on the *E. coli* linkage map. The genes were designated *cpsA, B, C, D,* and *E* (capsular polysaccharide

synthesis) (131). Isolation of fusions to these genes facilitated the subsequent identification of regulatory mutations that affected capsular polysaccharide synthesis. Three regulatory genes were identified and characterized: *rcsA, rcsB,* and *rcsC* (49). Further analysis of the regulatory genes led to the development of a model for *cps* regulation in *E. coli*. In the original model proposed by Stout and Gottesman (115), RcsC receives an environmental signal and transmits that signal through RcsB. RcsB then directly or indirectly activates expression of the *cps* genes. In the absence of Lon or under environmental conditions that have not yet been identified, RcsA accumulates and participates in activation of *cps* (115). Genetic evidence in support of this model, and the characteristics of RcsA, RcsB, and RcsC are presented.

RcsB and RcsC: A two-component sensor pair

RcsB is a 23,600 Da. cytoplasmic protein that shares homology at both the N- and C-terminus with effectors in two-component sensor pairs. Residues at the C-terminus share homology with protein domains involved in DNA binding and activation. N-terminal residues share homology with effector domains involved in activation by phosphorylation (47). Site-directed mutagenesis of a conserved aspartate residue (position 56) that is phosphorylated in other two-component regulators, leads to an inactive protein (47). This suggests that RcsB is activated by phosphorylation, and that Asp-56 is the phosphorylation site in RcsB. RcsC, a 104,000 Da. membrane protein, is the primary candidate as the kinase which donates a phosphate to RcsB. RcsC has a cytoplasmic N-terminus, a 270 amino acid periplasmic domain, and a 600 amino acid cytoplasmic C-terminus domain. The C-terminal domain of RcsC shows homology to the N-terminal effector domain of RcsB and to other members of the FixJ class of two-component sensors. A second domain in the C-terminus of RcsC shows homology to kinases in the FixJ class of sensor pairs. When the appropriate environmental signal is received, RcsC is proposed to phosphorylate

RcsB, and phosphorylated RcsB is proposed to interact with RcsA to turn on expression of the *cps* genes (47, 115). Genetic evidence suggests that RcsC has phosphatase activity as well as kinase activity.

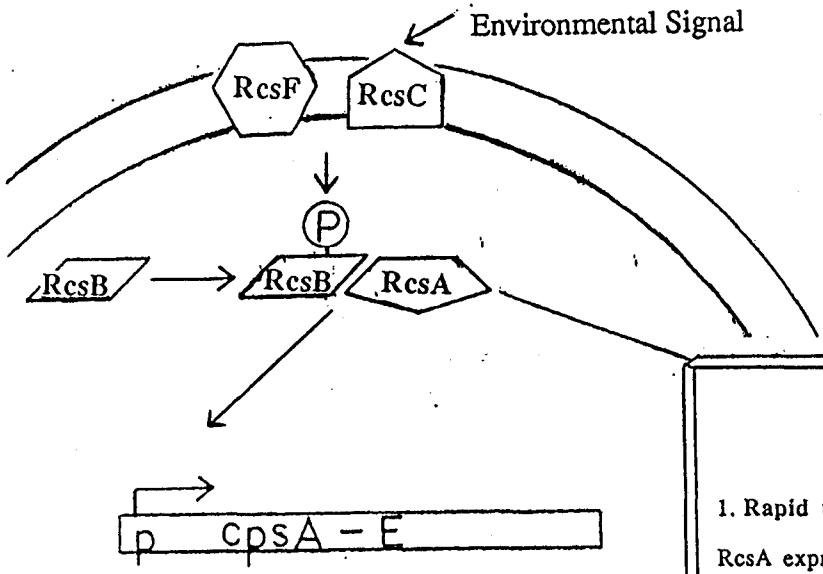
RcsF, a 14.6 kDa. protein that shares no similarity to other proteins in GenBank including protein kinases, may also play a role in phosphorylation of RcsB (40). When overexpressed from a plasmid, *rcsF* confers a mucoid phenotype in *lon*$^+$ *rcsB*$^+$ *rcsA*$^+$ cells, but not in *rcsB* mutant cells. This stimulation of capsule synthesis is RcsA-dependent, and genetic evidence suggests the role of *rcsF* is to promote phosphorylation of RcsB (40).

RcsA, an unstable regulatory protein

The limiting component in this complex of regulatory proteins is RcsA, an unstable protein which is rapidly degraded in *E. coli* wildtype for Lon protease activity (49, 116). RcsA is a highly basic (pI 9.9) 23,500 Da. protein. RcsA migrates on SDS-PAGE with an apparent molecular weight of 27,000 Da. RcsA shares homology with the LuxR family of two-component sensors (116) which includes UhpA and FixJ, sensors that share the most homology with RcsB and RcsC (115).

Unstable proteins, such as RcsA, are frequently control points in the regulation of cellular responses to specific environmental, metabolic, or developmental signals. These unstable regulatory proteins have short half-lives, are normally in low abundance in the cell, and have multiple levels of control over their synthesis and their availability (48). RcsA has been shown to meet these criteria. Stout and coworkers have demonstrated that RcsA expressed from multi-copy plasmids has a half-life of three minutes in *lon*$^+$ cells and approximately 30 minutes in *Δlon* cells. RcsA protein has not been detected in *lon*$^+$ cells when expressed from a single chromosomal *rcsA* gene, although RcsA is detectable from a single copy gene in *Δlon* cells. $RcsA_{62}$ (RcsA*), a dominant allele of RcsA with a single base change that results in a Met -> Val substitution,

Figure 1. Model for Regulation of *cps* in *Escherichia coli*



Levels of Control of RcsA

1. Rapid turnover of RcsA

RcsA expressed from multi-copy plasmids has a half-life of three minutes in *lon*$^+$ cells and approximately 30 minutes in *Δlon* cells. RcsA protein has not been detected in *lon*$^+$ cells when expressed from a single chromosomal *rcsA* gene, although RcsA is detectable in single copy in *Δlon* cells.

2. Control over synthesis of RcsA

Transcription of *rcsA* is silenced by H-NS, a histone-like protein that acts as a silencer of a number of other *E. coli* genes.
DsrA, an 85-nt RNA, acts as an antisilencer of *rcsA* transcription.

3. Control over the activity of RcsA

RcsA requires the presence of RcsB to activate the *cps* genes.
Is there an effect from the two forms of RcsA on *cps* transcription?

can be detected in *lon*$^+$ cells. The Met -> Val substitution is postulated to increase RcsA-RcsB interactions, thereby protecting RcsA from Lon-dependent degradation (116). Sledjeski and Gottesman have demonstrated that transcription of *rcsA* is silenced by H-NS, a histone-like protein that acts as a silencer of a number of other *E. coli* genes. H-NS silencing can be overcome by overproduction of DsrA, an 85-nt RNA (112).

Another locus involved in overproduction of capsular polysaccharide, designated *ops*, was identified and mapped by insertion mutagenesis near 62 minutes on the *E. coli* K-12 linkage map. Unlike the effect from other regulatory mutants, the overexpression of polysaccharide in the *ops* mutant is dependent on composition of the growth media (147). To date, the gene(s) has not been cloned or characterized.

This literature review has focused primarily on the functional characteristics of lactococci that are of commercial interest, including expression of a ropy exopolysaccharide. The types of polysaccharides produced by microorganisms and the uses for polysaccharides have been described. A model for regulation of polysaccharide expression in *E. coli* is presented. Little information currently exists regarding regulators involved in expression of a ropy polysaccharide produced by some strains of *L. lactis*.

Research objectives

Research objectives for this study were two-fold: (i) to develop a means for identifying strains of *L. lactis* that express the commercially desirable ropy phenotype, and (ii) use probes specific for polysaccharide regulatory genes in *E. coli* to determine whether or not similar regulators were present in lactococci.

MATERIALS AND METHODS

Bacterial strains and plasmids.

All bacterial strains and plasmids used in this work and their sources or derivations are listed in Table 1. Large-scale plasmid isolations (1 liter) were by the Birnboim method (9), small-scale isolations (5 to 10 ml) were as described by Sambrook *et al* (104). Briefly, for large-scale isolations this entailed growing a 1 liter overnight culture with appropriate antibiotic, pelleting the cells, and resuspending them in 10 mls TE (Tris-EDTA). Twenty milligrams lysozyme was added, and the cells were incubated 5 minutes at room temperature to weaken the cell wall. Cells were incubated 10 minutes on ice in 20 mls Bernie #2 (400 mg NaOH, 5 ml of 10% SDS, 45 mls dH_2O) for cell lysis and to denature proteins and DNA. Fifteen mls of 5 M KOAc was added to bring the pH to neutral, precipitate SDS-proteins and lipids and to renature the DNA strands. The suspension was incubated on ice 30 minutes with occasional mixing with increasing vigor until the flocculent material was in 1/4" chunks. The lysed cells were centrifuged at 10 k RPM for 15 minutes at room temperature to pellet chromosomal DNA and proteins, and the plasmid-containing supernatant transferred to a fresh centrifuge bottle. Fifteen mls of ice-cold isopropanol was added to precipitate the plasmid DNA, the DNA was centrifuged at 10 k for 15 minutes, and the supernatant decanted. The DNA pellet was dried 2 to 3 minutes before being resuspended in 5 mls of $T_{50}OAc_{100}E_1$ (50 mM Tris, 100 mM Na-acetate, 1 mM EDTA). The DNA was reprecipitated with 15 mls cold 95% ethanol (EtOH), centrifuged, washed in 70% EtOH, and dried. The DNA was resuspended in 4 mls TE, 1.2 gm CsCl was added per ml TE, and 300 ul of 10mg/ml ethidium bromide (EtBr) was added. The suspension was centrifuged at 10 k RPM for 10 minutes at room temperature, and the clear supernatant was transferred to an ultracentrifuge tube. The sample was centrifuged at 40 k RPM at 20°C for 14 to 16 hours. The bottom plasmid-containing band was pulled into salt-

saturated isopropanol and extracted until no EtBr coloring could be seen. The plasmid DNA was precipitated with 0.1 volume 3 M NaOAc and 0.7 volumes isopropanol and dried. It was resuspended in 400 ul $T_{50}OAc_{100}E_1$, transferred to an eppendorf tube, precipitated with 95% EtOH, and washed in 70% EtOH. After drying, the DNA was resuspended in 100 ul TE. DNA purity and concentration were determined by the ratio of O.D. $_{260/280}$ and agarose gel electrophoresis.

For small-scale plasmid isolations, overnight cultures were resuspended in 150 ul of lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8). Two hundred microliters of SDS-NaOH (200 mM NaOH, 1% SDS) was added for cell lysis, protein precipitation, and DNA denaturation. One hundred fifty microliters of 5M KOAc neutralization solution was added, the cell debris and proteins pelleted, and the supernatant transferred to a fresh tube. The DNA-containing supernatant was extracted twice using a 50:50 mix of Tris-buffered phenol and chloroform isoamyl alcohol (24:1), and precipitated with 100 ul of 350 mM NaOAc and 800 ul of 95% EtOH. The pellet was washed with 500 ul 70% EtOH and resuspended in 50 ul TE.

OSU environmental isolates of *Lactococcus lactis* were identified as subspecies *lactis* or subspecies *cremoris* by their reactions in the following physiological tests: growth at pH 9.2, growth in 4% NaCl, growth at 40° C, and ammonia production from arginine (90, 123). For this identification, pure colonies from each strain were selected from whey agar plates, resuspended in 1.5 ml of 0.1% peptone broth, and 100 ul was used to inoculate triplicate tubes of yeast glucose lemco broth (YGL) (54) and Niven's arginine broth (90). For growth in 4% NaCl, NaCl was added to YGL broth. For growth at pH 9.2, YGL broth was brought to pH 9.2 using 10N NaOH. For ammonia production from arginine, cultures were grown in Niven's arginine broth for 24 hr at 30°C . Nessler's reagent was added 1:1 to cultures, and cultures were scored for production of an orange precipitate. Niven's and Nessler's reagents without bacteria were used as a negative control. Growth at 30°C in YGL broth was used as a positive control for culture viability.

TABLE 1. Bacterial Strains and Plasmids.

Strain	Relevant Genotype or characteristics	Reference or derivation
L. lactis subsp. *lactis*		
Strain B	non-ropy variant	OSU[c]
MG1363	*lac*- plasmid-free	
L. lactis subsp. *cremoris*		
Hollandicus	ropy variant	OSU[c]
LAPT 3001	non-ropy variant	OSU[c]
KTR II	non-ropy variant	OSU[c]
Ropy 352	ropy variant	OSU[c]
Gram positive organisms		
B. subtilis		Bacillus Genetic Stock Cntr
B. stearothermophilus		ATCC 12980
S. aureus		OSU[c]
S. salivarius		OSU[c]
Leuconostoc		OSU[c]
E. coli		
MC4100 background[a]		
JT4000	Δ*lon*-510	SG1030 + P1(SG4144)
KD302	Δ*lon*-510 *rcsA*72::Tn10	JT4000 + P1(SG21081)
KD303[b]	Δ*lon-510 rcsA*72::Tn10	SG20780 + P1(SG21081)
KD317	*lon*$^+$ *rcsB62*::Kan	SG20250 + P1(SG23002)
KD318	Δ*lon*-510 *rcsB62*::Kan	JT4000 + P1(SG23002)
SG20250	*lon*$^+$	(49)

TABLE 1 (Continued)

SG20780 [b]	Δ*lon*	(13)
SG20781 [b]	*lon*$^+$	(13)
SG21081	*lon*+*rcsC*$_{137}$ *cps*+ *rcsA*$_{72}$::Tn10	(13)
SG23002	*rcsB62::Kan*	(116)
Other *E. coli* backgrounds		
DH5α	F$^-$*supE*$_{44}$ Δ*lacU*$_{169}$ (f80*lacZ*ΔM15) *hsdR*$_{17}$*recA*$_1$ *endA*$_1$ *gyrA*$_{96}$ *thi*$_1$ *relA*$_1$	Bethesda Research Labs
SG1030	F$^-$ Δ*lac araD proCYA*221 *zaj*-403::*Tn*10	(131)
SG4144	N99 Δ*lon*-510	(82)
Gram negative organisms		
A. radiobacter		OSU [d]
A. viscolactis		OSU [c]
P. vulgaris		OSU [c]
Ps. aeruginosa		OSU [c]
Rhizobium sp.		OSU [e]
V. anginolyticus		OSU [c]
X. campestris		OSU [c]
Plasmids		
pATC400	pBR322 + *rcsA*+	(128)
pBR322	*bla*$^+$	(10, 117)
pJB100	pBR322 + *rcsB*$^+$	(13)
pVS103	pACYC184 + *rcsA*$^+$	V. Stout

a Δ*lacU*$_{169}$ *ara*D *flb*B *rel*
b *cps*B$_{10}$::*lac*-Mu-*imm*-I
c OSU Microbiology Department strain collection
d OSU strain collection of Walt Ream
e OSU strain collection of Peter Bottomly

Culture conditions and media.

Lactococcal stock cultures were maintained at -80°C in 11% reconstituted nonfat dry milk (NFM) containing 20% glycerol. Frozen stock were streaked on whey agar (135), M17 lactose (M17L) (122), or M17 glucose (M17G) plates, incubated 24 hrs at 30°C and pure colonies used in subsequent experimental procedures. M17L or M17G broth cultures were incubated static for 24 hr at 30°C and used for inoculations and dilutions. One liter of 7% whey agar was prepared as follows: 70 gm sweet whey powder was mixed in 500 ml H_2O, centrifuged at 6 K RPM for 1 hr at room temperature. Nineteen and one half gm of β-glycerophosphate and 5 gm yeast extract were added to the supernatant and the volume brought up to 600 mls with H_2O. The mixture was autoclaved for 10 minutes and rapidly cooled in tepid H_2O. In a separate flask, 15 gm agar was added to 400 ml H_2O and autoclaved 20 minutes. After cooling to 55-60°C the supernatant and agar were mixed together and poured into petri dishes. Medium for M-17 agar plates was made in two parts. First, for 1 liter of plates, 5 gm polypeptone, 5 gm phytone peptone, 2.5 gm yeast extract, 5.0 beef extract, 0.5 gm ascorbic acid, 19 gm β-glycerophosphate, 1 ml of 1 M MgSO4, and 15 gm agar were mixed in 900 ml H_2O in a 2 liter flask, and autoclaved 20 minutes. Five gm carbohydrate, for example glucose or lactose, was mixed in 100 ml H_2O in a 1 liter flask and autoclaved 20 minutes. After cooling to 55-60°C the two parts were mixed together and poured into petri dishes. Elliker medium was made from prepared stock purchased from Difco. Elliker contains the following ingredients per 1 liter: 20 gm tryptone, 5 gm each of yeast, dextrose, lactose, saccharose, 2.5 gm gelatin, 4 gm NaCl, 1.5 gm Na acetate, and 0.5 gm ascorbic acid. *Escherichia coli* cultures were grown in LB broth (104) at 32°C for all experiments involving capsule synthesis and at 37°C for all other manipulations. All bacteria except lactococci were grown with aeration unless otherwise noted. *Xanthomonas campestris* and *Agrobacterium radiobacter* were grown in LB at 37°C and 28°C, respectively. *Proteus vulgaris, Pseudomonas aeruginosa, Alcaligenes viscolactis,* and *Staphylococcus aureus* were grown in

Brain Heart Infusion broth (28) at 37°C. The remaining organisms were grown under the conditions listed: *Vibrio anginolyticus* in Tryptic Soy broth with 3% NaCl (Difco) at 37°C, *Rhizobium leguminosarum* bv. *trifolii* strain USDA 2152 in Yeast Mannitol broth (Difco) at 30°C, and *Bacillus subtilis* in Nutrient broth with 0.2% glucose (28) at 37°C. The following organisms were grown without aeration: *B. stearothermophilus* in BST broth (146) at 60°C, *Streptococcus salivarius* in M17 sucrose broth at 37°C, and *Leuconostoc* in MRS broth (27) at 30°C..

When required, media were supplemented with antibiotics at the following concentrations: ampicillin (Amp) at 50 ug/ml, tetracycline (Tet), chloramphenicol (Cm), and kanamycin (Kan) at 25 ug/ml, and spectinomycin at 100 ug/ml.

Preparation of protein extracts.

In general, proteins extracts were prepared as described below. Modifications for specific experiments are noted. Cultures were grown at 32°C to an optical density at 600nm ($O.D._{600}$) of 0.8 to 1.0. One ml aliquots were transferred to eppendorf tubes on ice, centrifuged 2 minutes at 4°C, the supernatant aspirated, and the pellets washed in 0.01 M $MgSO_4$. Pellets were resuspended in SDS-PAGE loading buffer (75mM Tris pH 6.8, 3% SDS, 20% glycerol, 0.2% bromophenol blue dye, and 0.5% β-mercaptoethanol) (104) and boiled 10 minutes. Samples used for protein quantitation were resuspended in SDS-PAGE loading buffer without bromophenol blue and without β-mercaptoethanol. Protein extracts were loaded directly onto SDS-PAGE gels or stored at -80°C.

For in vivo turn-over studies of RcsA, overnight cultures of cells were diluted into LB broth to an $O.D._{600}$ less than 0.25. Two flasks were inoculated with each cell type. Cells were incubated at 32° C with aeration until $O.D._{600}$ was 0.60. One ml aliquots for Western blot analysis and BCA protein assay were drawn, labeled T=0, and processed as described for protein harvests. Spectinomycin (100ug/ml) (41) was added to one flask of each cell type. The

second flask served as a control to demonstrate entry of spectinomycin into the cells. Flasks were incubated 10 minutes at 32^0 C for uptake of spectinomycin. Two and one-half minutes later (T=2.5), aliquots were drawn and processed as above. Aliquots were drawn at T=5, T=10, T=30, and T=60 minutes post spectinomycin addition. A comparison of $O.D._{600}$ was made between control flasks and spectinomycin containing flasks at to verify protein synthesis had been halted.

For heat shock, *E. coli* and *B. subtilis* cultures were grown overnight in LB, *Lactococcus* in M17L, *Leuconostoc* in MRS, and *B. stearothermophilus* in BST. Cultures were back-diluted to $O.D._{600}$ of approximately 0.2 and incubated at 30°C except *B. stearothermophilus* which was incubated at 55°C. At $O.D._{600}$ of greater than 0.8 but less than 1.2, each culture was divided in half and the cells pelleted at room temperature. One pellet from each strain was resuspended in fresh media prewarmed to 30°C; 55°C for *B. stearothermophilus*. The second pellet was resuspended in media prewarmed to 42°C; 70°C for *B. stearothermophilus*, and incubated for 30 minutes. Cells were pelleted, resuspended in TE/Laemmli buffer (73, 143) and lysed by bead-beating.

For bead-beating, cells in broth cultures were resuspended in TE /Laemmli buffer. Colonies on agar plates were loosened using sterile 0.01M $MgSO_4$ and were harvested by scraping with a sterile glass rod. The cells were pelleted, washed twice in 0.01M $MgSO_4$, and resuspended in TE/Laemmli buffer. Protease inhibitors were added at the following concentrations: N-Tosyl-L-Phenylalanine chloromethyl ketone (TPCK 1mM), Na-p-Tosyl-L-Lysine chloromethyl ketone (TLCK 0.1mM), Phenylmethane sulphonylfluoride (PMSF 1mM), and aprotinin (0.03mM) (53) Cells were lysed by bead beating following manufacturers instructions (Biospec Products minibeadbeater) as follows. The liquid suspensions of cells were transferred to Sarstadt tubes and enough 0.1 mm glass beads were added to fill the tubes. Each sample was bead beat for 5 one minute intervals. Samples were held on ice between beating. Samples were centrifuged 10 minutes, the supernatant recovered, and an equal volume of 2X SDS gel loading

buffer was added. Samples were boiled 10 minutes and stored at -80°C.

Protein quantitation (BCA)

Protein concentrations were determined using the bicinchoninic protein assay (BCA, Pierce). Protein samples processed as described in Preparation of Protein Extracts were resuspended in SDS-PAGE loading buffer lacking dye and β-mercaptoethanol. Samples were diluted 1:8 in 0.1 M $NaPO_4$ buffer, pH 7. A set of protein standards from 25 ug/ml to 1200 ug/ml was prepared using bovine serum albumin (BSA) resuspended in 7 parts $NaPO_4$ buffer and one part sample buffer. Two mls working reagent and 100 ul of each BSA standard or experimental sample was placed in a test tube and incubated at 37°C for 30 minutes. The samples were cooled to room temperature and $O.D._{562}$ nm was measured. A standard curve plotting absorbance vs. protein concentration was used to determine the protein concentration for each experimental sample. Forty ug of cellular protein was loaded per well and separated on an 8% SDS-PAGE gel for Lon blots. Thirty ug of cellular protein was loaded per well on a 14% SDS-PAGE gel for RcsA blots.

SDS-PAGE analysis

Laemmli SDS-PAGE

The method of Laemmli (73) was followed for SDS-PAGE separation of proteins for Lon Western blots and for conservation of RcsA Western blots. A 3% stacking gel was prepared using 0.5 M Tris pH 6.8, 0.4% SDS. A resolving gel was prepared using 1.5 M Tris pH 8.4, 0.4% SDS (gel dimensions 16 X 18 X 0.15 cm). The cathode and anode chambers of a Hoefer Scientific slab gel apparatus were filled with Tris-glycine reservoir buffer (110). Samples were thawed, boiled 2 minutes, loaded into each well, and electrophoresed

at 15 mA until the proteins had migrated through the stacking gel (approximately 2 hours). The current was raised to 30 mA and the proteins were electrophoresed until the dye front ran off the gel (approximately 3 hours).

Tricine SDS-PAGE

The method of Schagger and vonJagow (109) was followed for SDS-PAGE separation of RcsA proteins. A 14% running gel was polymerized together with a 4% stacking gel using 3 M Tris pH 8.45, 0.3% SDS. The cathode chamber was filled with upper buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.25) and the anode chamber filled with lower buffer (0.2 M Tris, pH 8.9). Samples were thawed, boiled 2 minutes, vortexed briefly, and loaded into each well. Samples were electrophoresed at 30 V until the samples had completely entered the gel (approximately 1 hr). Voltage was raised to 110 V and samples were electrophoresed overnight (14 to 16 hrs). In the morning the voltage was raised to 180 V for 30 minutes to tighten the protein bands.

Peptide mapping

The peptide mapping technique originally developed by Cleveland *et al.* (21) was used with some modifications. Protein extracts were prepared and separated on a 14% tricine SDS-PAGE gel which had a 2 cm stacking gel and a 11 cm resolving gel. An approximate 2.5 cm gel slice containing the RcsA proteins was cut out, equilibrated against Tris pH 6.8 buffer, and applied at right angles to the original electrophoresis in a second tricine SDS-PAGE gel which had a 5 cm stacking gel and a 23 cm resolving gel. Protease buffer containing 1.5 mg α-chymotrypsin (Sigma) was layered over the gel slices and electrophoresis was performed in the normal manner with the exception that the current was turned off for one hour when the bromophenol blue dye had left the gel slice and

entered the stacking gel to allow protease digestion to occur. Electrophoresis was continued at 30V for 4.5 hours to allow protease digestion of the proteins to continue in the resolving gel. Voltage was raised to 130V and electrophoresis was continued for 16 hours. Peptides were transferred to PVDF membrane and visualized as described in Western blotting conditions.

Construction of Ferguson plots

Ferguson plots (35), graphing the $\log_{10}$ relative mobility (R_f) versus gel concentration, %T (total acrylamide concentration), were generated for each RcsA protein using acrylamide concentrations of 6, 8, 10, 12, and 14 % T in tricine SDS-PAGE gels. Ferguson plots of molecular weight standards at 6, 10, and 12% acrylamide were generated and used to construct a standard curve. The migration distance of each RcsA protein, each molecular weight standard, and a reference marker protein was measured. Relative mobility was calculated by dividing the migration distance of the reference protein band by the migration distance of each RcsA protein and each molecular weight standard. Regression analysis (Microsoft Excel) was used to determine the slope and R_{f0} for each RcsA protein and for the molecular weight standards.

Western blotting conditions

After SDS-PAGE separation, proteins were electroblotted to nitrocellulose membrane in Towbin transfer buffer (20 mM Tris, pH 8.3, 20% MeOH. 150 mM Glycine) (130) (Lon blots) or to PVDF membrane (polyvinylidene difluoride, Dupont) in CAPS [3-(cyclohexylamine)1-propanesulfonic acid; 10 mM CAPS, pH 11.0, 20% MeOH] transfer buffer (RcsA blots) (121).

Towbin transfer buffer

For Lon detection, the gel and nitrocellulose membrane were equilibrated in Towbin buffer for 15 minutes. A "sandwich" was prepared as follows: one Scotch-Brite pad, two pieces of Whatman filter paper, gel, nitrocellulose membrane, two pieces of Whatman filter paper, and the second Scotch-Brite pad. Air bubbles were removed as each layer was added. The sandwich was inserted into a Biorad Transblot cell with the nitrocellulose membrane placed between the gel and the anode. The apparatus was filled with Towbin transfer buffer and proteins were transferred at 280 mA constant current overnight (15 to 16 hours). After transfer, the nitrocellulose membrane was blocked in TBS-T (20mM Tris, 137mM NaCl, 0.1% Tween 20, pH7.6) (129) with 5% nonfat dry milk blocking buffer for two hours at room temperature. The blocking buffer was discarded and the membrane was incubated for 2 hours with polyclonal Lon antiserum (5 ul Ab in 15 ml TBS-T-5% milk; a gift from S. Gottesman). The membrane was washed in TBS-T-milk three times, 15 minutes each. Goat anti-rabbit IgG-Peroxidase labeled antibody (HyClone) (1 ul in 15 mls TBS-T-milk) was added and the membrane incubated 1 hour at room temperature. The membrane was washed in TBS-T as previously described.

CAPS transfer buffer

For RcsA detection, the gel was first equilibrated in CAPS buffer for 15 minutes and the PVDF membrane was pretreated using the NEN-Dupont membrane wetting protocol (1 minute in 95% methanol, 3 minutes in dH_2O, 15 minutes in CAPS). A "sandwich" was prepared as described under Towbin transfer conditions. The sandwich was inserted into a Biorad Transblot cell with the PVDF membrane placed between the gel and the anode. The apparatus was filled with CAPS transfer buffer and proteins were transferred at 280 mA constant current for 7 to 8 hours. After transfer the membrane was air-dried 10 minutes to dehydrate the proteins

binding them tightly to the membrane. The membrane was placed in TBS-T blocking buffer with 1% non-fat dry milk at 4° C overnight. After overnight blocking, the buffer was discarded and the membrane incubated at room temperature for 2 hours with polyclonal RcsA antiserum (1 ul Ab in 15 ml TBS-T-milk; a gift from V. Stout). Washes and incubation with second antibody were as described under Towbin conditions.

Signal detection and film development

Detection of reactive proteins was according to the ECL protocol of Amersham. The membrane was incubated 1 minute with 1.5 ml each of ECL detection reagents A (oxidizing reagent containing H_2O_2) and B (enhanced luminol reagent). Excess reagent was drained off, the membrane wrapped in Saran wrap, and placed protein side up in a film cassette for detection. Film (Amersham-Hyperfilm) was placed on the blot for various exposure times dependent on signal intensity. Films were developed using Kodak developer and fixer.

Southern blotting conditions

Chromosomal DNA was extracted by the method of Pitcher *et al.* using guanidium thiocyanate (96). Lactococcal strains were grown overnight static at 30°C in M17 broth. The cells were harvested, resuspended in TE containing lysozyme at 50 mg per ml and incubated 30 minutes at 37°C. The cells were lysed by incubation for 5 minutes in 5 M guanidium thiocyanate, 0.1 M EDTA, and 0.5% sarkosyl (GES reagent). Lysates were cooled on ice, 7.5 M NH_4OAC was added, and the lysate was incubated an additional 10 minutes on ice. DNA was extracted two or three times with chloroform:isoamyl alcohol (24:1) until the interface was clear. The supernatant was transferred to a clean centrifuge tube and ice-cold isopropanol was added to precipitate the DNA. The DNA was

centrifuged, the supernatant aspirated, and the pellet washed in 70% ethanol. The pellet was dried at 37°C and resuspended in TE. The integrity of the DNA was verified by electrophoresis on a 0.5% agarose gel. The chromosomal DNA's were digested with *HindIII* or *EcoRV* restriction endonucleases, separated on 1% agarose gels, transferred to Zeta-Probe membrane (BioRad) and used as targets for DNA-DNA hybridization by the method of Southern (104, 113). Briefly, this entailed denaturation of the DNA by incubation for 45 minutes with gentle agitation in 1.5 M NaCl, 0.5 M NaOH, a rinse step in distilled water, and neutralization by incubation with gentle agitation for 30 minutes in 1 M Tris (pH 7.4), 1.5 M NaCl. The gel was placed in a glass tray on a solid support overlaid with Whatman 3MM paper that had been prewet with 10X SSC (1.5 M NaCl, M NaCitrate). A sheet of nitrocellulose filter was placed on the gel, air bubbles removed, and two sheets of 3MM paper placed on top. A 5 to 8 cm high stack of paper towels cut to the size of the gel were placed on top of the 3MM paper and weighted down. The glass tray was filled with SSC transfer buffer until the level reached the bottom 3MM paper in order for the liquid to flow by capillary action from the reservoir through the nitrocellulose filter to elute the denatured DNA from the gel onto the membrane. Transfer was allowed to proceed overnight, the nitrocellulose membrane removed, washed in 6X SSC, and dried for 30 minutes at room temperature. The DNA was fixed to the membrane by baking for 30 minutes at 80°C. A 777-bp *XmnI-XmnI* fragment (nucleotides 1870 to 2647) of *lon* was ^{32}P labeled and used as a probe for all *lon* Southern blots. A 556-bp *EcoRV-PstI* fragment (nucleotides 1 to 556) of *rcsA* was ^{32}P labeled and used as a probe for *rcsA* blots. All hybridizations were under conditions of relatively high stringency (1 mM EDTA, 0.25 M Na_2HPO_4, pH 7.2, 7% SDS, 65°C). The membrane was washed, wrapped in plastic wrap and exposed to X-ray film.

Bacterial transformations

Transformation of *E. coli* cells was accomplished using either the $CaCl_2$ method described by Sambrook (104) or by following BioRad specifications for electro-transformation (BioRad). For $CaCl_2$ transformation, cells grown to $O.D._{600}$ of 0.5 to 0.6 were placed on ice for 15 minutes or longer, the cells pelleted by low speed centrifugation, and resuspended in one volume of ice-cold 0.1 M $CaCl_2$. After incubation on ice for at least 15 minutes, the cells were pelleted, resuspended in 0.5 volumes $CaCl_2$ and incubated for 15 minutes or longer on ice. The cells were pelleted a third time, resuspended in 0.1 volume $CaCl_2$ (original volume), and incubated on ice for 2 hours or longer. A glass micropipette was used to transfer 100 ul of competent cells to a chilled eppendorf tube containing approximately 0.1 ug plasmid DNA. Cells and plasmid were incubated on ice for 30 minutes to 2 hours before heat treatment for one minute at 42°C. The heat-treated cells were immediately placed on ice for 2 minutes prior to addition of SOC broth (104). Cells were incubated 1 hour for phenotypic expression prior to plating on selective media. Non-transformed competent cells were plated on selective media as a negative control. Transformant colonies were restreaked to selective media to insure isolation of pure colonies, and plasmid identity was verified by phenotypic observation and/or restriction digest analysis. Competent cells for electroporation were prepared as described for $CaCl_2$ transformation except ice-cold sterile water was used for the first two cell resuspensions and ice-cold sterile 10% glycerol was used as the final resuspension media. The Gene Pulser apparatus was set to 25uF capacitor, 1.5 Kvolts, 200 ohms. Twenty ul cells and 1ul plasmid DNA were placed in a 0.1 cm electroporation cuvette and given one pulse. One ml SOC broth was added to the cuvette and the suspension transferred to an eppendorf tube. Cells were expressed for one hour at 37°C before plating on selective media.

Examination of ropy and mucoid phenotypes

Lactococcal cultures were assayed for expression of ropy and mucoid phenotypes on Elliker (7, 34), M17L, and whey agar plates. Serial dilutions of cultures grown in M17 broth were made using 0.1% (w/v) peptone water and duplicate samples of dilutions yielding 25 to 250 colonies per plate were counted. Anaerobic incubations were carried out in BBL anaerobic jars using a BBL Gas Pak anaerobic system (Baltimore Biological Laboratories). Plates were incubated for six days at 15°C and 20°C, and for three days at 30°C. Colonies were scored for mucoidy on a scale of ++++ (excess extracellular polysaccharide) to (-), (no visible mucoidy). Colonies were scored as ropy if strings of 5 mm or more were detected when the colony was touched once with a wire inoculating loop or sterile toothpick (138).

P1 transductions

P1*vir* lysates were prepared, and P1*vir* transductions were performed as described by Miller (84). For preparation of lysates, an overnight culture of the donor strain was diluted 1:100 in 5 mls of fresh LB broth supplemented with 0.2% glucose, 5 mM $CaCl_2$ and incubated 30 minutes at 37°C. One hundred microliters of a P1*vir* lysate was added and the cells were incubated for 2 or 3 hours until lysis occurred. One hundred microliters of chloroform was added to lyse any intact cells before the cell debris was pelleted. The supernatant was transferred to a sterile screw-capped tube, the lysate spot-titered, and stored at 4°C. For genetic transductions, an overnight culture of the recipient strain was centrifuged, resuspended in 10 mM MgSO4 containing 5 mM CaCl2 and starved for two hours at room temperature. Recipient cells (100 ul) were placed in three small test tubes and P1*vir* from the donor strain was added in the following amounts: tube 1, 10 ul; tube 2, 50 ul; tube 3, 100 ul. The test tubes were incubated 30 minutes without shaking before 100 ul of 1 M sodium citrate was added to chelate the Ca++

and prevent reabsorption of the P1*vir* phage. For phenotypic expression, 1 ml of LB broth was added and cells incubated for one hour prior to plating on selective media. Recipient cells and donor phage lysate were spotted on selective media as negative controls.

RESULTS

Subspecies identification for commercial strains of *Lactococcus lactis*

Two subspecies of *Lactococcus lactis*, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, are important in dairy fermentations. In many applications, *L. lactis* subsp. *cremoris* is the preferred organism because *L. lactis* subsp. *lactis* produces high levels of carbonyl compounds such as aldehyde, diacetyl, and pyruvic acid which have been implicated in fruity off-flavor defects (136, 137). The principal distinguishing characteristics of the two subspecies and the results of physiological tests to identify the two subspecies are shown in Table 2. Of the five commercial strains used in subsequent experimentation, Strain B was the only strain found to exhibit the physiological properties associated with *L. lactis* subsp. *lactis*: (i) growth at 40°C, (ii) growth in 4% NaCl, (iii) growth at pH 9.2, and (iv) the ability to hydrolyze arginine. Strains Hollandicus, Ropy 352, LAPT 3001, and KTR II exhibited characteristics associated with *L. lactis* subsp. *cremoris*. For brevity, *L. lactis* subsp. *cremoris* will be referred to as *L. cremoris*, and *L. lactis* subsp. *lactis* will be referred to as *L. lactis* throughout the remainder of this work.

Expression of Two Distinct Polysaccharide Phenotypes

The descriptive terms, ropy, mucoidy, and slime-producing, have been used interchangeably in reports in the literature describing viscous isolates of LAB (16). However, not all mucoid or slime-producing isolates are ropy. The ropy characteristic has been defined as the ability of colonies to form long strings when touched with a wire inoculating loop (138), and in sensory evaluations ropy cultures have been shown to produce smooth body, higher viscosity fermented milk products that have a decreased susceptibility to

TABLE 2. Physiological properties used to differentiate *Lactococcus lactis* subspecies *lactis* from subspecies *cremoris*.

Strains[a]	Growth at pH 9.2[b]	Growth in 4% NaCl[c]	Growth at 40°C[d]	Ammonia production[e]
Hollandicus	-	-	-	-
Ropy 352	-	-	-	-
LAPT 3001	-	-	-	-
KTR II	-	-	-	-
Strain B	+	+	+	+

[a] *Lactococcus lactis* strain *Hollandicus*; *Lactococcus lactis* strain *Ropy 352*; *Lactococcus lactis* strain *LAPT 3001*; *Lactococcus lactis* strain *KTR II*; *Lactococcus lactis* strain *B*.

[b] Cultures were grown at 30°C in Yeast glucose lemco broth with pH adjusted to 9.2 using 10 N NaOH. Visible turbidity after 24 hr was recorded as a positive result.

[c] Cultures were grown at 30°C in Yeast glucose lemco broth with 4% NaCl (w/v) added. Visible turbidity after 24 hrs was recorded as a positive result.

[d] Cultures were grown in Yeast glucose lemco broth incubated at 40°C. Visible turbidity after 24 hrs was recorded as a positive result.

[e] Cultures were grown at 30°C in arginine broth for 48 hrs at 30°C. Production of ammonia was determined by formation of an orange precipitate upon addition of Nessler's reagent.

syneresis than those produced by non-ropy strains (16, 69, 142). This study was undertaken to determine culture conditions necessary for expression of the ropy phenotype. Lactococcal strains originally isolated from fermented milk cultures as ropy variants were grown under a set of defined environmental conditions to determine whether environmental conditions affect expression of the ropy phenotype. A surprising result was the observation of two distinct phenotypes, ropy and mucoid (colonies have a glistening, slimy appearance but do not form strings when touched with a loop). Strains were subsequently assayed and scored for both phenotypes as described in Materials and Methods.

Of the five strains initially isolated as ropy variants, three strains, *L. cremoris* LAPT, *L. lactis* Strain B, and *L. cremoris* KTRII did not exhibit the ropy phenotype under any of the conditions selected (data not shown) and were no longer ropy in milk. Two strains, *L. cremoris* Hollandicus and *L. cremoris* Ropy 352, consistently exhibited the ropy phenotype under some of the defined conditions (Table 3), and produced the characteristic ropy milk. One hundred percent of Hollandicus and Ropy 352 colonies exhibited the ropy phenotype at 30°C under aerobic conditions, 83% and 100% respectively were ropy at 20°C aerobic, and 94% and 100% respectively were ropy at 15°C aerobic when plated on whey agar plates. On whey agar plates under anaerobic conditions, the phenotype was exhibited by fewer colonies and only at one temperature for each strain: 6% of Ropy 352 colonies were ropy at 15°C, and 29% of Hollandicus colonies were ropy at 30°C.

In contrast, on Elliker plates a higher percentage of ropy colonies were observed under anaerobic conditions than under aerobic conditions: 100% of colonies of both Hollandicus and Ropy 352 were ropy at 15°C, 23% of Hollandicus were ropy at 20°C, and 73% and 91% respectively were ropy at 30°C.

On M17L under aerobic conditions a small percentage of Hollandicus colonies exhibited the ropy phenotype at 15°C and 20°C (9% and 2% respectively). Ropy 352 did not exhibit the ropy phenotype on M17L. Neither strain was ropy under anaerobic conditions when cultured on M17L.

TABLE 3: Culture medium, temperature and atmosphere influence expression of ropy and mucoid phenotypes in *Lactococcus lactis* subsp. *cremoris.*

Temp. °C Atmosphere	Strains[a]	WHEY		ELLIKER		M-17L	
		% Ropy[b]	Mucoid[c]	% Ropy	Mucoid	% Ropy	Mucoid
15°C /	Holl	94	-	57	-	9	+ +
Aerobic	Ropy	100	-	14	-	0	+ +
20°C /	Holl	83	-	21	-	2	+ +
Aerobic	Ropy	100	+	0	-	0	+ +
30°C /	Holl	100	-	0	+	0	+ +
Aerobic	Ropy	100	++	0	+	0	+ +
15°C /	Holl	0	+	100	-	0	+ +
Anaerobic	Ropy	6	+	100	-	0	+ +
20°C /	Holl	0	+	23	-	0	+ +
Anaerobic	Ropy	0	-	0	-	0	+ +
30°C /	Holl	29	-	73	-	0	+ +
Anaerobic	Ropy	0.7	++	91	-	0	+ +

[a] Full strain designations: *Lactococcus lactis* subsp. *cremoris* strain Hollandicus, *L. lactis* subsp. *cremoris* strain Ropy 352.

[b] Plates at 15°C and 20°C were incubated six days; plates at 30°C were incubated three days. Plates with 25 to 200 colonies were evaluated. Colonies were scored as ropy when strings of 5 mm or more were detected when the colony was touched once with a wire inoculating loop.

[c] Plates at 15°C and 20°C were incubated six days; plates at 30°C were incubated three days. Plates with 25 to 200 colonies were evaluated. Colonies were scored visually for mucoidy: (-) = no observable capsule; (+) = mucoid to (++++) = copious capsular material visible.

In the course of this study, a second EPS phenotype distinct from the ropy phenotype was observed. These colonies presented a glistening, mucoid appearance on agar plates but did not form strings when touched with a wire inoculating loop. This phenotype, referred to as mucoid, was observed under certain environmental conditions in the two ropy strains (*L. cremoris* Hollandicus and Ropy 352) as well as in the three non-ropy strains (*L. cremoris* LAPT 3001, *L. cremoris* KTR II, and *L. lactis* Strain B). When cultures were grown on M17L plates, all five strains exhibited the mucoid phenotype irrespective of temperature and oxygen conditions (Table 3 and Table 4). On whey agar plates, the mucoid phenotype was expressed primarily under anaerobic conditions at 15°C and 20°C. On Elliker plates, the mucoid phenotype was expressed by all five strains at 30°C aerobic, by Strain B and KTRII at 15°C anaerobic and Strain B at 20°C anaerobic (Table 4).

When greater than 10% of Hollandicus colonies were ropy, the mucoid phenotype was not expressed irrespective of media, temperature, or atmospheric conditions. In contrast, the Ropy 352 strain exhibited both phenotypes at 20°C and 30°C when plated on whey under aerobic conditions. Neither phenotype was exhibited by Ropy 352 at 20°C anaerobic on whey and 20°C aerobic or anaerobic on Elliker plates (Table 3).

Structural Evidence for Conservation of Lon Protease in *L. lactis*

A body of evidence exists suggesting that although structurally distinct polysaccharides are produced by different organisms, the biosynthetic genes involved in EPS production are well conserved. The presence of conserved structural genes suggests the presence of conserved regulatory genes as well. My approach in constructing stable ropy LAB strains was to identify regulatory genes which could be modified for constitutive expression of the desired phenotype. A number of regulators of polysaccharide synthesis have been identified in *E. coli*. Previous

TABLE 4: Culture medium, temperature and atmosphere influence expression of the mucoid phenotype in *Lactococcus lactis* subsp. *cremoris* and subsp. *lactis*.

Temp. °C [b] Atmosphere	Strains[a]	WHEY	ELLIKER	M-17L
15°C / Aerobic	LAPT 3001	-	-	+ +
	Strain B	-	-	+ +
	KTR II	-	-	+ +
20°C / Aerobic	LAPT 3001	-	-	+ +
	Strain B	-	-	+ +
	KTR II	-	-	+ +
30°C / Aerobic	LAPT 3001	-	+	+ +
	Strain B	-	+	+ +
	KTR II	-	+	+ +
15°C / Anaerobic	LAPT 3001	+ +	-	+ +
	Strain B	+	+	+ +
	KTR II	+	+	+ +
20°C / Anaerobic	LAPT 3001	+	-	+ +
	Strain B	+	+	+ +
	KTR II	-	-	+ +
30°C / Anaerobic	LAPT 3001	-	-	+ +
	Strain B	-	-	+ +
	KTR II	-	-	+ +

[a] Full strain designations: *Lactococcus lactis* subsp. *cremoris* strain LAPT 3001, *L. lactis* subsp. *lactis* Strain B, and *L. lactis* subsp. *cremoris* strain KTRII.

[b] Plates at 15°C and 20°C were incubated six days; plates at 30°C were incubated three days. Plates with 25 to 200 colonies were evaluated. Colonies were scored visually for mucoidy: (-) = no observable capsule; (+) = mucoid to (++++) = copious capsular material visible.

reports in the literature suggested that two of these regulators, Lon, a negative regulator, and RcsA, an unstable positive regulator were present in organisms other than *E. coli*. Lon protease has been purified from *S. typhimurium* (29). Two genes homologous to the *E. coli lon* gene have been identified in *Myxococcus xanthus* (42, 91, 92) and one gene has been identified in *B. brevis* (58) and *B. subtilis* (100). Under heat shock conditions, a protein similar in size to *E. coli* Lon and immunoreactive to *E. coli* Lon antiserum has been detected in *B. subtilis* (4) and *Caulobacter crescentus* (97). A gene homologous to the *E. coli rcsA* has been identified in *Klebsiella aerogenes* (2) and *Erwinia amylovora* (8). In the present study, both Southern and Western blot approaches were used to probe lactococcal strains for the presence of homologous DNA and protein sequences to Lon and RcsA.

Using DNA sequence information and known biochemical characteristics of Lon protease, a 777 basepair *XmnI* fragment (Figure 2) spanning the proposed serine catalytic active site of Lon protease (3) and excluding DNA coding for the acidic region, basic regions and the nucleotide binding domains and HU-1 coding region (20) was used as a probe for Southern hybridization to *HindIII* digests of chromosomal DNA. A 556-bp *Pst1-EcoRV* fragment (Figure 2) spanning a region at the amino terminus of the *rcsA* gene shown to be conserved in *Erwinia* and *Klebsiella* sp. and postulated to be involved in protein-protein interaction with RcsB was used in Southern hybridization to *HindIII* digests of chromosomal DNA. This probe excluded a region encoding a putative DNA-binding helix turn helix motif (116). No hybridization to lactococcal DNA by the *lon* or *rcsA* probe was observed under the high stringency conditions used.

The lack of homologous DNA sequence did not preclude the presence of homologous amino acid sequence. For some amino acids, for example, asparagine, glutamine, phenylalanine, and tyrosine, *L. lactis* and *E. coli* have opposite codon preferences (133). Although the DNA sequences may differ as a result of codon preference, identical amino acid residues may be encoded by genes present in each organism. Polyclonal antiserum specific to *E. coli* Lon and RcsA proteins was used to probe lactococcal whole cell protein extracts

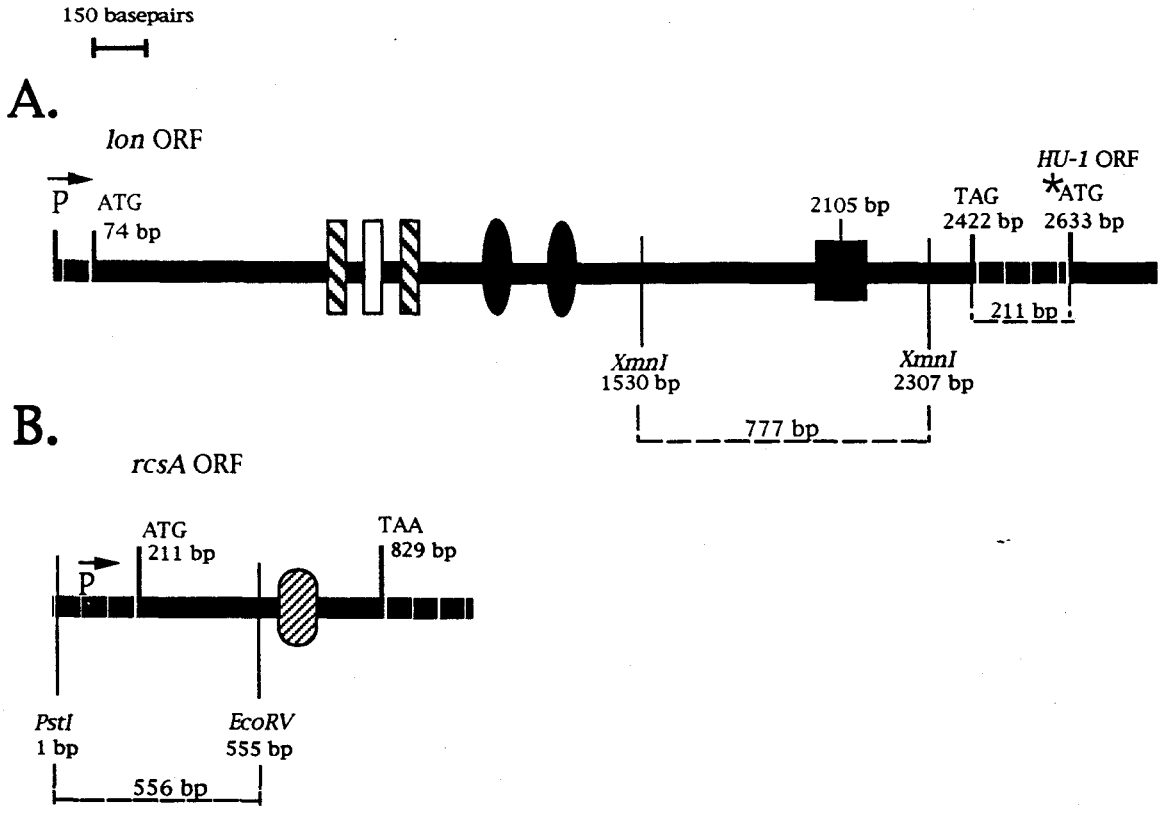


FIG. 2. *lon* and *rcsA* DNA probes used in Southern blot analysis. A. $\overrightarrow{P}$ = *lon* transcriptional start ATG = Lon start codon; ▨ = Region encoding basic amino acids; ▯ = Region encoding acidic amino acids; ● = Region encoding putative ATP binding site; ■ = Serine active site; TAG = Lon termination codon; *ATG = *HU-1* start codon.

B. $\overrightarrow{P}$ = *rcsA* transcriptional start; ATG = RcsA start codon; TAA = RcsA termination codon; ▨ = Region encoding putative helix turn helix motif.

for conservation at the amino acid level. A protein immunoreactive to *E. coli* Lon antiserum and migrating at the reported SDS-PAGE molecular mass of *E. coli* Lon (94 kDa) (Figure 3, lane 1) was observed in the five lactococcal strains probed. The intensity of the signal for the 94 kDa. Lon-like protein was lower in the two ropy strains, *L. cremoris* Ropy 352 (lane 3) and *L. cremoris* Hollandicus (lane 4) than in non-ropy *L. cremoris* KTRII (lane 5), *L. lactis* Strain B (lane 6), and the *lac*$^-$ plasmid-free laboratory strain, *L. lactis* MG1363 (lane 7). The Lon protein band was absent in the Δ*lon E. coli* strain (lane 2). A second immunoreactive protein migrating at a molecular mass of 86 kDa was observed in the *E. coli lon* + strain and in the lactococcal strains. The signal intensity for the 86 kDa. protein was also lower in the two ropy strains than in non-ropy strains and in the laboratory strain. The differences in signal intensity for the 94 kDa. and 86 kDa. proteins in the ropy strains are not due to unequal protein loading; each lane contains 40 ug total cellular extract. In support of equal protein loading, an immunoreactive protein at 76 kDa and several lower molecular mass proteins can be seen that react with equal intensities in all five lactococcal strains.

A similar Western blot approach was used to probe lactococcal strains with polyclonal antiserum specific to *E. coli* RcsA. A protein immunoreactive to *E. coli* antiserum and migrating at the reported SDS-PAGE molecular mass of *E. coli* RcsA (27 kDa) was not observed in the five lactococcal strains probed (data not shown).

Structural evidence for conservation of Lon and RcsA in Gram positive and Gram negative organisms

The results in the previous section provided evidence for structural conservation of Lon protease in *Lactococcus* sp. However, a survey of the conservation of Lon and RcsA in other organisms had not been previously undertaken. In the present study, a number of different Gram negative and Gram positive bacteria were probed with DNA and antibody probes specific to *E. coli lon* and *rcsA* genes

Figure 3. Western blot of *Lactococcus lactis* strains using *Escherichia coli* Lon antiserum.

Equal amounts of protein from whole cell extracts were boiled in sample buffer, fractionated on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and analyzed by Western blotting with preabsorbed polyclonal antiserum specific to *E.coli* Lon protease. Immunoreactive proteins were visualized by enhanced chemiluminescence. 1, *E.coli* SG20250 *lon*+; 2, *E. coli* JT4000 Δ*lon*; 3, *L. cremoris* strain Ropy 352; 4, *L. cremoris* strain Hollandicus; 5, *L. cremoris* strain KTR II; 6, *L. lactis* strain B; 7, *L. lactis* strain MG1363. The position of molecular weight standards are indicated on the left.

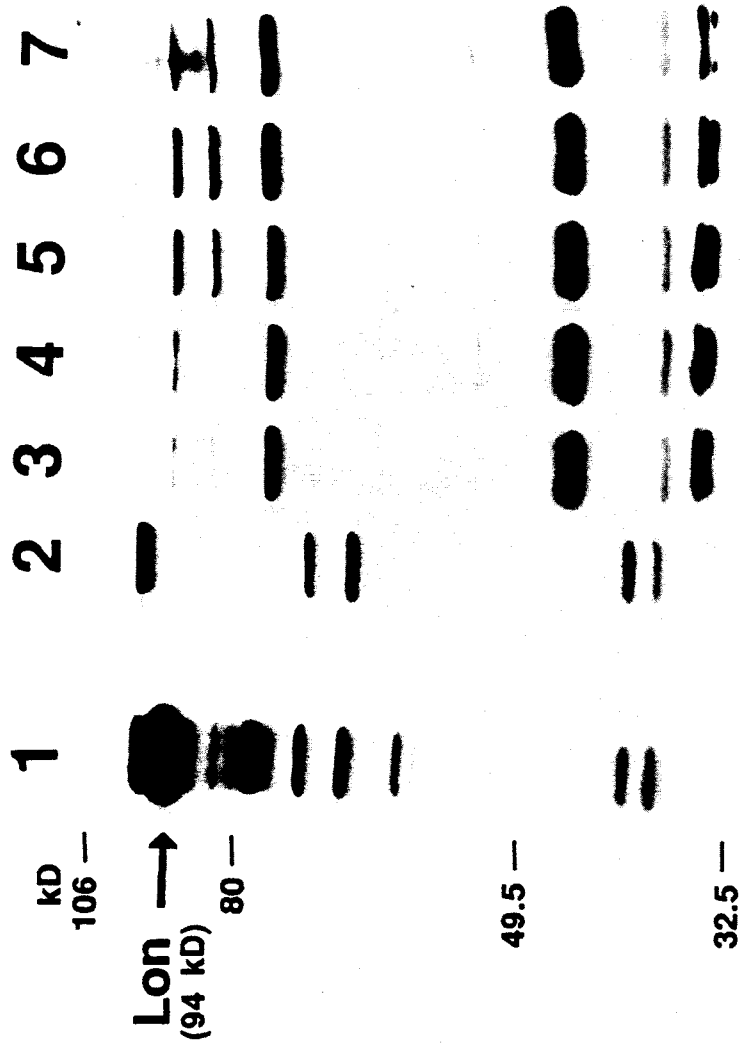


Figure 3.

and to Lon and RcsA proteins. Results for Lon Western blotting are shown in Figure 4. Lon protease was detected in *E. coli lon+* (SG20250) and absent from *E. coli Δlon* (JT4000) (Figure 4, Panel A, lanes 1 and 2). Figure 4, Panel B shows that a protein immunoreactive to Lon antiserum and similar in size to Lon (molecular mass 94 kDa.) was detected in *V. anginolyticus* (lane 2), *X. campestris* (lane 4), *P. vulgaris* (lane 5), *P. aeruginosa* (lane 6), and *B. subtilis* (lane 8). A slightly smaller protein was detected in *B. stearothermophilus* (92 kDa., lane 7), and a higher molecular mass protein was detected in *A. viscolactis* (160 kDa., lane 3). In addition to the immunoreactive protein at 94 kDa., a second immunoreactive protein was detected in *P. aeruginosa* (86 kDa., lane 6) and in *B. subtilis* (35 kDa., lane 8). In a previous study, *B. subtilis* and *C. crescentus* were subjected to heat-shock prior to Western blot analysis with Lon antiserum. In that study, a Lon-like protein (94 kDa.) was identified in both organisms under heat shock conditions (4,97). To examine the impact of heat shock on the expression of the lon-like proteins in *B. stearothermophilus* and *B. subtilis* (Figure 4, Panel C), the cells were heat shocked, lysed by bead-beating, and probed with Lon antiserum. Three immunoreactive proteins were visible in *B. subtilis* (lane 3), the 94 and 35 kDa. proteins seen under physiological conditions and an additional immunoreactive protein at 120 kDa. The immunoreactive protein at 92 kDa. in *B. stearothermophilus* (Lane 4) was more readily detected after heat shock.

For comparison and clarity, the results obtained from Southern and Western blot analyses of all organisms are summarized in Table 5. DNA sequences from γ and β purple bacteria hybridized to the *lon* and *rcsA* DNA probes; the α-purple bacteria were not analyzed by Southern blotting. Proteins immunoreactive with Lon antiserum and RcsA antiserum were observed in all α-, γ-, and β-purple bacteria. An interesting result was my ability to detect a protein immunoreactive with *E. coli* RcsA antiserum in all Gram negative organisms probed other than *E. coli*. In laboratory strains of *E. coli*, RcsA is not detected in *lon+* strains unless *rcsA* is overexpressed from a multicopy plasmid, although, RcsA is readily detected in *lon*

Figure 4. Western blot of Gram negative and Gram positive microorganisms using *Escherichia coli* Lon antiserum.

Equal amounts of protein from whole cell extracts were boiled in sample buffer, fractionated on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and analyzed by Western blotting with preabsorbed polyclonal antiserum specific to *E.coli* Lon protease. Immunoreactive proteins were visualized by enhanced chemiluminescence. (A and B) Extracts of cells grown under physiological growth conditions. (A) Lanes: 1, *E. coli* SG20250 *lon*+; 2, *E. coli* JT4000 Δ*lon*. (B) Lanes: 1, *E. coli* SG20250 *lon*+; 2, *Vibrio anginolyticus*; 3, *Alcaligenes viscolactis*; 4, *Xanthomonas campestris*; 5, *Proteus vulgaris*; 6, *Pseudomonas aeruginosa*; 7, *Bacillus stearothermophilus*; 8, *B. subtilis*. (C) Extracts of cells subjected to heat shock. Lanes: 1, *E. coli* SG20250 *lon*+; 2, *E. coli* JT4000 Δ*lon*; 3, *B. subtilis*; 4, *B. stearothermophilus*. kD, kilodaltons. The position of molecular weight standards are indicated to the right of Panel A.

Figure 4.

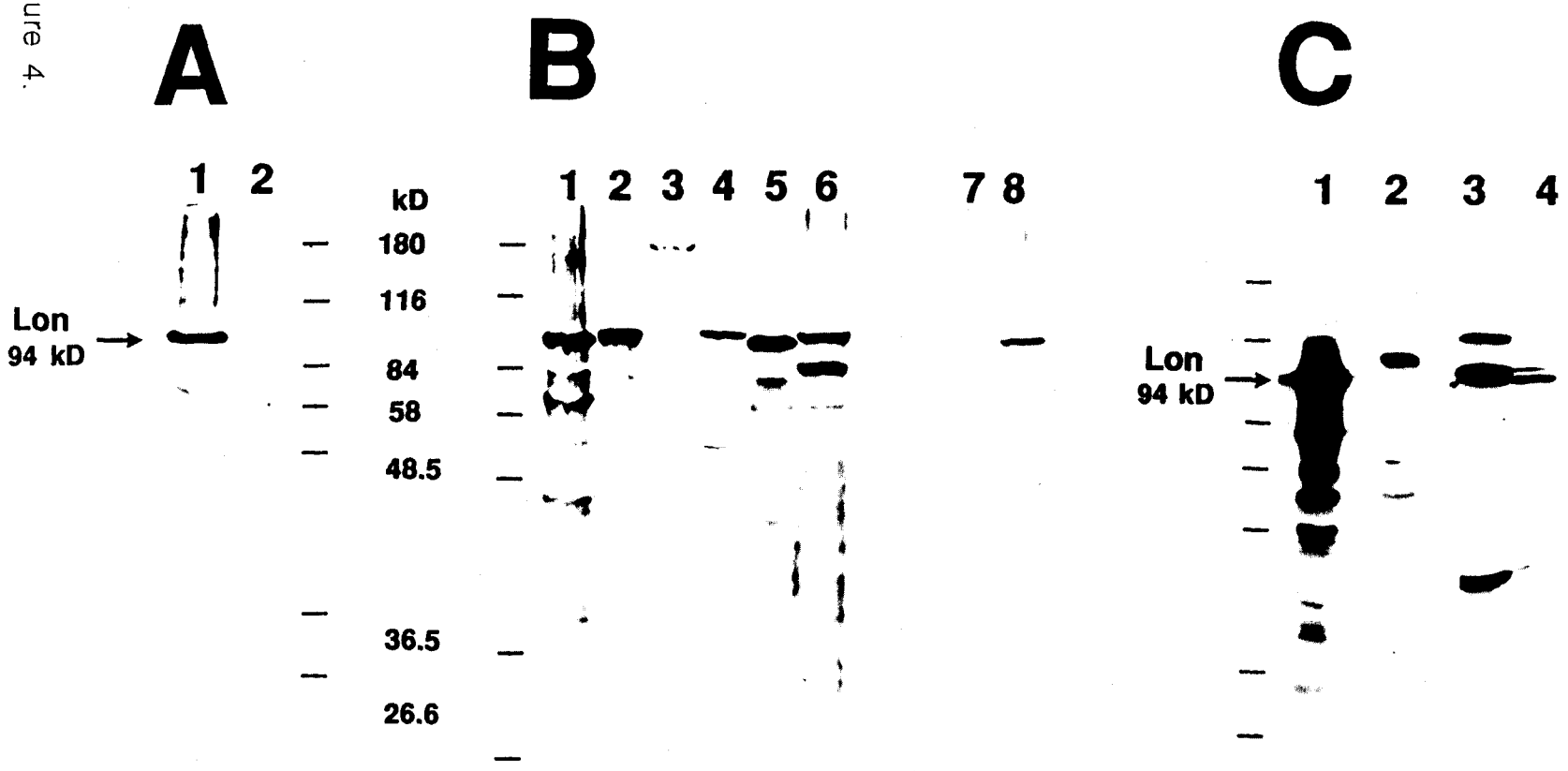

A
B
C
1 2
kD
180
116
84
58
48.5
36.5
26.6
Lon
94 kD
1 2 3 4 5 6 7 8
1 2 3 4
Lon
94 kD

TABLE 5. Evidence for structural conservation of Lon and RcsA.

		RcsA probes		Lon probes	
Strain	Classification[c]	DNA[a]	Protein[b] (MW)	DNA[a]	Protein[b] (MW)
Escherichia coli lon⁺rcsA⁺	γ-purple	+	-	+	+ (94 kDa)
E. coli Δlon rcsA⁺	γ-purple	+	+ (27 kDa)	-	-
Proteus vulgaris	γ-purple	+	+ (27)	+	+ (94)
Pseudomonas aeruginosa	γ-purple	+	+ (26)	+	+ (94, 86)
Vibrio anginolyticus	γ-purple	+	+ (26)	+	+ (94)
Xanthomonas campestris	γ-purple	+	+ (27.5)	+	+ (94)
Alcaligenes viscolactis	β-purple	+	+ (26.5)	+	+ (160)
Rhizobium strain USDA2152	α-purple	ND	+ (33, 34)	ND	+ (100)
Agrobacterium radiobacter	α-purple	ND	+ (31, 35)	ND	+ (100)
Bacillus subtilis 1A1	low G+C G^+	-	-	-	+ (94)
Bacillus stearothermophilus	low G+C G^+	-	-	-	+ (92)
Lactococcus lactis	low G+C G^+	-	-	-	+ (94)
Leuconstoc	low G+C G^+	-	-	-	+ (94)
Streptococcus salivarius	low G+C G^+	+	+ (27)	-	+ (180)
Staphylococcus aureus	low G+C G^+	+	?	-	?

[a] Homologous DNA sequences detected by Southern hybridization. + = positive reaction; - = no reaction; ND = Not done

[b] Protein similar in size to Lon or RcsA and reacting to either Lon antiserum or RcsA antiserum in a Western blot. + = positive reaction; - = no reaction; ? = ambiguous result; MW = molecular weight in kDa.

[c] Classifications based on information from *Bergey's Manual of Systematic Bacteriology* and ref. (93, 144)

mutants. In contrast to *E. coli*, which is non-mucoid under laboratory conditions, the other Gram negative organisms presented a mucoid phenotype under laboratory growth conditions.

DNA sequences from low G+C Gram positive bacteria did not hybridize to the *lon* DNA probe although all organisms contained at least one high molecular weight protein which reacted strongly to *E. coli* Lon antiserum. Hybridizing sequences to the *rcsA* probe were found in two of the six Gram positive bacteria examined (*S. salivarius* and *S. aureus*). *Streptococcus salivarius* which presents a mucoid phenotype on solid media under standard laboratory conditions expressed a protein immunoreactive with RcsA antiserum (27 kDa.). The Western blot results for *S. aureus* were ambiguous; antisera reacted with proteins throughout the entire lane on the SDS-PAGE gel.

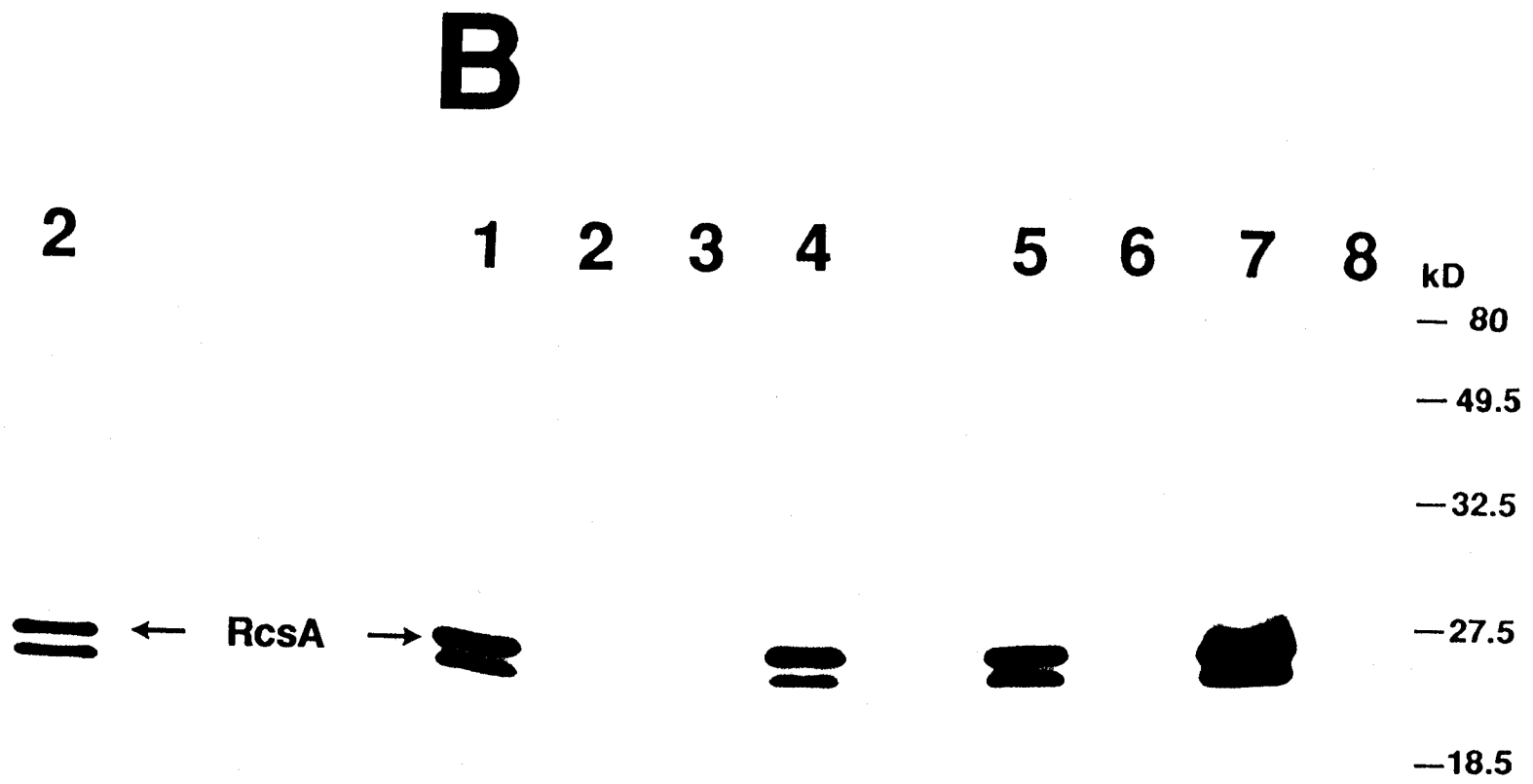
Detection of two *E. coli* proteins immunoreactive with RcsA antiserum.

During the course of the previous study, it appeared that *E. coli* RcsA protein might resolve into two distinct protein bands. To optimize separation of low molecular weight proteins (RcsA reported SDS-PAGE molecular mass 27 kDa.), the glycine cathode buffer of the Laemmli SDS-PAGE system used in the previous study (73) was replaced with tricine as described by Schagger and von Jagow (109). Tricine (pK 8.15) migrates faster than glycine (pK 9.6) in the stacking gel. This shifts the stacking limit to low molecular weight proteins which results in better separation and resolution of proteins in the 5 to 30kDa range. With enhanced separation, RcsA resolves into two distinct protein bands. After fractionation by tricine-SDS-PAGE and Western blot analysis, two proteins immunoreactive with RcsA antiserum and which migrate to the location reported for RcsA (Figure 5, Panel A, lane 2) are detected in Δ*lon E. coli* cells . Neither immunoreactive protein is detected in *E. coli* cells wildtype for *lon* (Figure 5, Panel A, lane 1). Extracts of

Figure 5. Detection of two RcsA proteins in *Escherichia coli* by Western Blot analysis.

Equal amounts of protein from whole-cell extracts were boiled in sample buffer, fractionated on a 14% Tricine SDS polyacrylamide gel, transferred to PVDF membrane, and analyzed by Western blotting with preabsorbed polyclonal antiserum specific to *E. coli* RcsA protein. Immunoreactive proteins were visualized by enhanced chemiluminescence. Panel A: Lanes: 1, SG20250 *lon*+ *rcsA*+; 2, JT4000 Δ*lon rcsA*+. Panel B; Lanes: 1, JT4000 Δ*lon rcsA*+; 2, KD302 (JT4000 $rcsA_{72}$::Tn10); 3, KD302 + pBR322; 4, KD302 + pATC400(*rcsA*+); 5, SG20780 Δ*lon rcsA*+ $cpsB_{10}$::*lacZ*; 6, KD303 (SG20780 $rcsA_{72}$::Tn10); 7, KD303 + pATC400; 8, KD303 + pBR322. The positions of molecular weight standards are indicated on the right.

Figure 5.



lon::Tn*10* *E. coli* and *lon-100* *E. coli* cells were screened with RcsA antiserum. The two RcsA proteins were detected in both *lon* mutants; these proteins were not detected in isogenic *lon*+ counterparts (data not shown).

Stout *et al.* commented that two or three immunoreactive proteins were often detected in their Laemmli SDS-PAGE system when RcsA was overproduced from a multicopy plasmid (116). Using the identical Laemmli SDS-PAGE system as that cited by Stout *et al.* (15% acrylamide concentration, glycine in the cathode buffer, very short running gel), two or three protein bands were detected when overproduced RcsA was overproduced from a multicopy plasmid, but those proteins migrated at a faster rate (12-18 kDa) than RcsA; two protein bands migrating in the gel at the location predicted for RcsA were not detected in the present study using the system cited by Stout (data not shown).

The possibility existed that the replacement of glycine by tricine in the cathode buffer of the Laemmli SDS-PAGE system might contribute to artifactual electrophoretic conditions resulting in the separation of RcsA into two protein bands. To challenge this possibility, the resolving power of different acrylamide percentages and various gel lengths was examined using the traditional Laemmli SDS-PAGE system (glycine cathode buffer). The two RcsA protein bands could only be resolved in a traditional Laemmli SDS-PAGE system using a glycine cathode buffer if a high percent acrylamide gradient (15-20%) was used in a gel runner that spanned at least eight inches (data not shown). This observation suggested that the separation of RcsA into two protein bands using a tricine cathode buffer was not the result of an electrophoretic artifact but rather was the result of increased protein separation and resolution by the tricine system.

Peptide digestion reveals strikingly similar, yet not identical banding patterns.

Peptide mapping by limited proteolysis in SDS-PAGE is a highly reproducible method for generating a pattern of peptide bands characteristic of the protein substrate and the proteolytic enzyme used in the analysis (21). This technique can be used to examine amino acid sequence homology between two proteins. To determine whether the two immunologically related RcsA proteins share common peptides and therefore share similarity in primary amino acid sequence, the two proteins were separated by SDS-PAGE, reelectrophoresed in the second dimension in the presence of the protease, α-chymotrypsin, and subjected to Western blot analysis. Both proteins exhibit strikingly similar, although not identical peptide digestion patterns (Figure 6). The RcsA protein that migrates at a higher molecular weight in SDS-PAGE (designated RcsA1) contains one higher molecular weight peptide fragment not seen in the digestion pattern of the RcsA protein that migrates at the lower molecular weight in SDS-PAGE (designated RcsA2). The digestion pattern for the RcsA2 protein shows one lower molecular weight fragment not seen in the RcsA1 digestion pattern. All other peptide bands are common to both proteins. This result argues strongly against the possibility that one of the proteins is a protein cross-reacting to RcsA antiserum but not related to RcsA. Rather, this result strongly suggests the two proteins share extensive homology in their primary amino acid sequence but are not identical proteins.

Two RcsA proteins differ by size not charge

Two assumptions are made when SDS-PAGE is used to fractionate proteins: (i) all proteins bind the same weight ratio of SDS (1.4 gram SDS per gram protein) which results in identical charge-to-mass ratios (98), and (ii) all SDS-protein complexes assume the same conformation; the size of the complexes vary

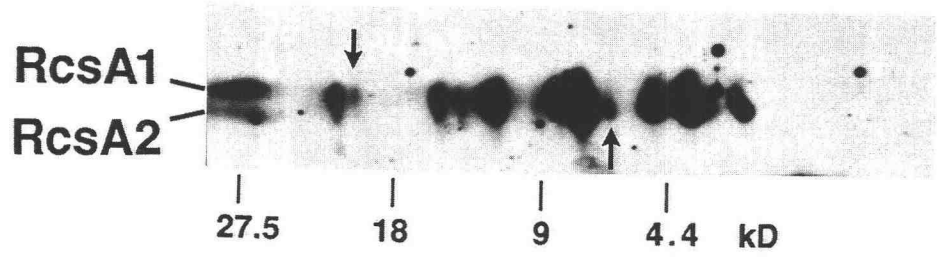


Figure 6. Peptide Map of the two RcsA proteins.

The two RcsA proteins were fractionated on a 14% tricine SDS-PAGE gel, a band containing both proteins was excised, fractionated in the presence of α-chymotrypsin on a second gel at 90° to the first gel, transferred to PVDF membrane, and analyzed by Western blotting with preabsorbed polyclonal antiserum specific to *E. coli* RcsA protein. Immunoreactive proteins were visualized by enhanced chemiluminescence. The top bands are peptide fragments from the higher molecular weight RcsA protein (designated RcsA1). The bottom bands are peptide fragments from the lower molecular weight RcsA protein (designated RcsA2). Arrows indicate the bands unique to each digest. The positions of molecular weight standards are indicated at the bottom.

linearly as a function of molecular weight (99). One or both of these assumptions are violated by proteins that migrate anomalously. Ferguson plots were constructed for both RcsA proteins, and their respective slope K_R (a measure of the retardation of the protein by the gel) and its ordinate intercept Y_o (a measure of the mobility of the protein in free solution) were calculated. Anomalous migration can be detected by comparison of the migration patterns of the two proteins relative to each other and by comparison of their ordinate intercepts to those of the MW protein standards. Rodbard *et al* classified four sets of separation patterns that can be seen by Ferguson plot analysis dependent on whether size and/or charge is the major determinant in the protein's migration in a PAGE gel (Figure 7) (101). Case A, designated "size isomerism", gives rise to the typical migration patterns seen with SDS denatured proteins; all proteins have very similar charge densities due to the binding of SDS, subsequently they have very similar mobility's in free solution, and migrate strictly according to size. In this case, the intersections of their Ferguson plots will be at the point where the %T acrylamide is approximately equal to zero (Figure 7A). In Case B, size and charge fractionation are synergistic; the protein with the higher free mobility has the lower retardation coefficient, and the %T gel concentration at the point of intersection of the proteins is less than zero (Figure 7B). In Case C, size and charge are antagonistic; the protein with the higher free mobility has the higher retardation coefficient, and the %T gel concentration at the point of intersection of the proteins is greater than zero (Figure 7C). Case D, designated "charge isomerism", is seen with proteins that are the same size but which have different free mobility's. In this case, the two proteins have identical retardation coefficients and migration lines from the two Ferguson plots do not intersect (Figure 7D). The results illustrated in Figure 8 support the conclusion that the two RcsA proteins migrate in SDS-PAGE gels as predicted for typical denatured proteins which differ in size (Case A) : as %T acrylamide increases, the separation distance between the two proteins increases as a result of the higher retardation coefficient of the larger protein. Furthermore, the two proteins exhibit very

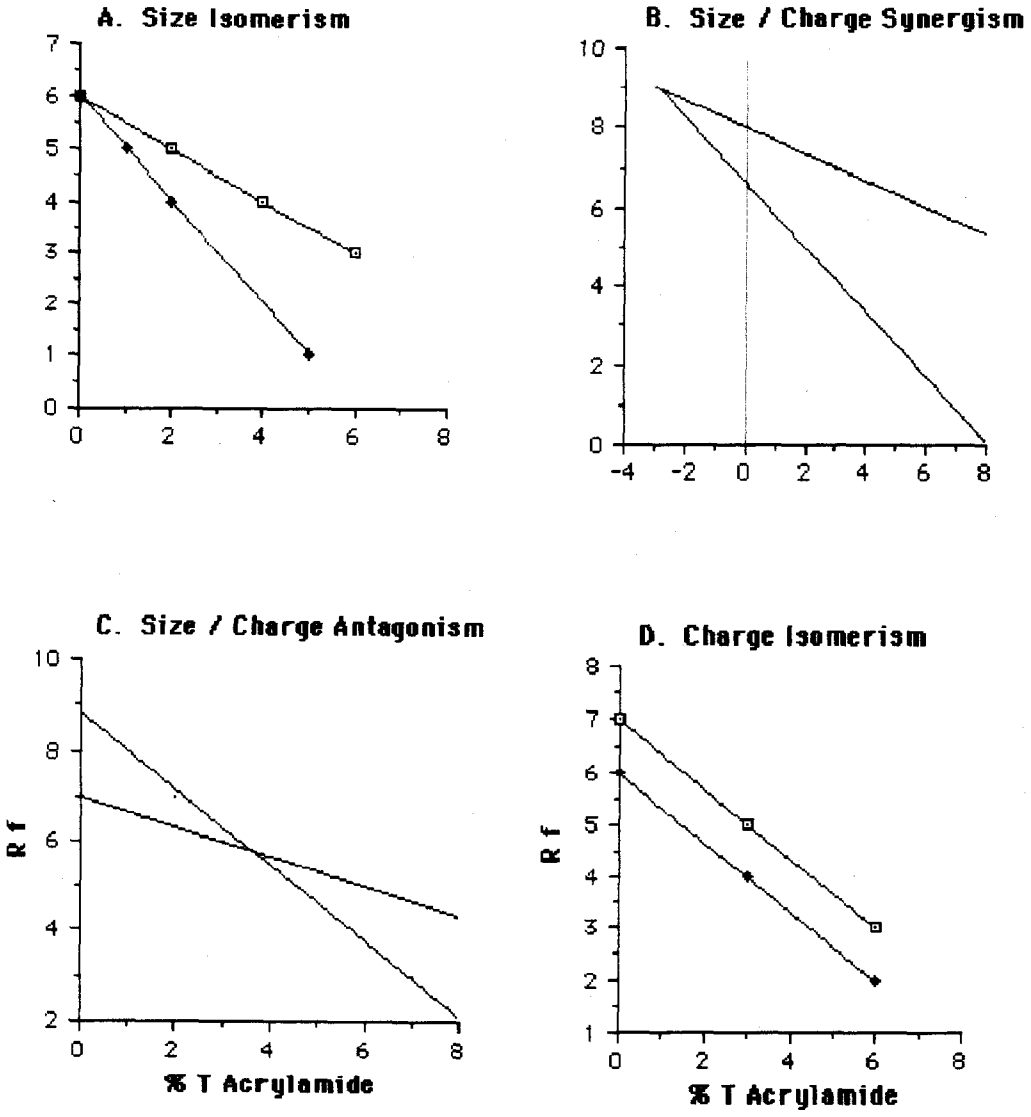


Figure 7. Classification of separation patterns encountered in polyacrylamide gel electrophoresis.

A. Typical migration pattern for SDS denatured proteins.
B. Protein with the higher free mobility has a lower retardation coefficient.
C. Protein with the higher free mobility has a higher retardation coefficient.
D. Proteins are the same size but have different free mobilities.

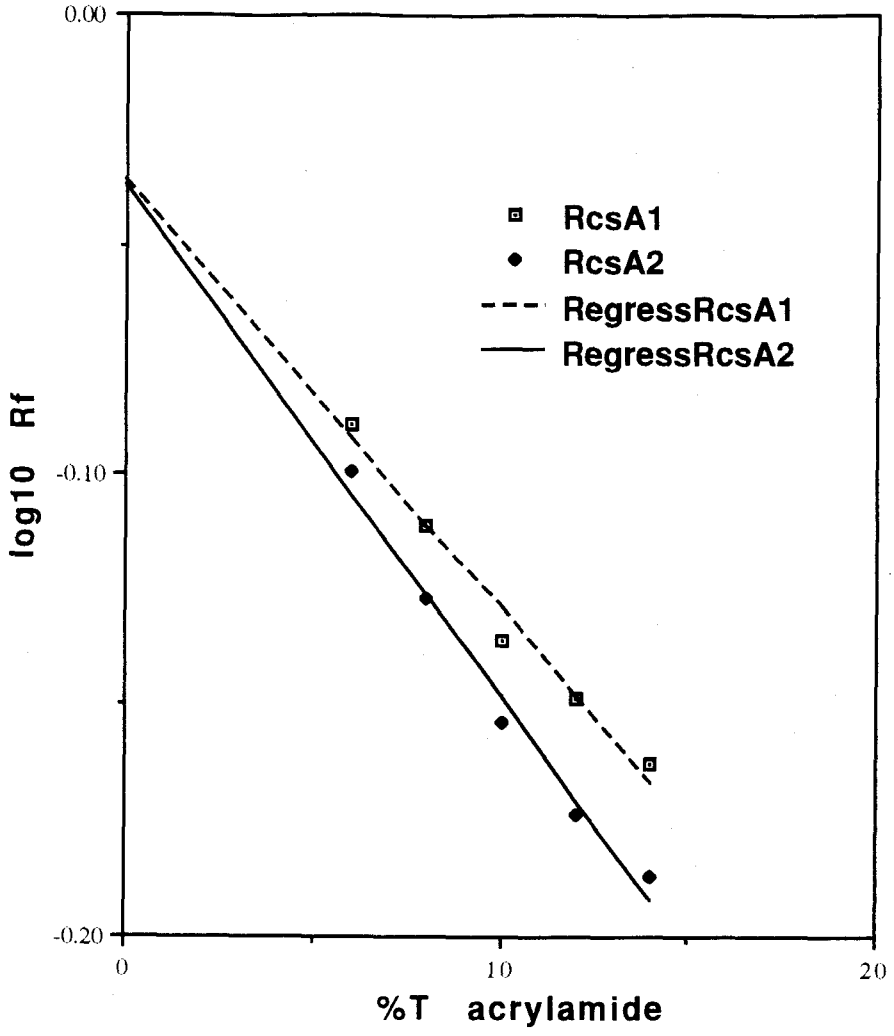


Figure 8. Ferguson Plots of each RcsA protein.

Equal amounts of protein from whole-cell extracts were boiled in sample buffer, fractionated on 6, 8, 10, 12, and 14% tricine SDS polyacrylamide gels, transferred to PVDF membrane, and analyzed by Western blotting with preabsorbed polyclonal antiserum specific to *E. coli* RcsA protein. Log_{10} of the relative mobility of each RcsA protein was plotted at each acrylamide concentration. The calculated values at each concentration acrylamide are shown overlaid with regression lines. The higher migrating protein on SDS-PAGE is designated RcsA1; the lower migrating form is designated RcsA2.

similar Y_0 values indicating similar relative free mobility due to identical charge densities. Finally, the molecular weight protein standards and the RcsA protein bands intersect the y-axis over a narrow range (data not shown). This further supports the assumption that the proteins have bound the same weight ratio of SDS and have assumed the same conformation as other SDS-protein complexes and are migrating according to size rather than charge differences. The relative difference in size (approximately 1000 Da.) of the two RcsA proteins was calculated from their migration distances relative to migration distances of molecular weight standards on four separate 14% SDS-PAGE gels (data not shown).

Two RcsA proteins expressed from a single *rcsA* gene.

The observation of two similarly sized RcsA proteins might be due to the presence of a second *rcsA* gene in *E. coli*, each gene encoding a protein of slightly different size. To test for the presence of a second gene, the chromosomal *rcsA* gene was interrupted by a *rcsA72*::Tn*10* insertion (13) transduced into two different Δ*lon* strains. In both Δ*lon* strains, when the chromosomal *rcsA* gene was interrupted, both RcsA protein bands disappeared (Figure 5, Panel B, lanes 2 and 6; refer to page 53). Both RcsA protein bands were detected in the Δ*lon rcsA*$^+$ isogenic counterparts (Figure 5, Panel B, lanes 1 and 5). To eliminate the possibility that a polar insertion had disrupted a downstream gene which might code for a second protein, pATC400 (128), a pBR322 based plasmid harboring a 2.4 kb fragment containing the *rcsA* gene was transformed into the two Δ*lon rcsA72*::Tn*10* strains. Both RcsA protein bands were detected in the two Δ*lon rcsA72*::Tn*10* strains harboring pATC400 (Figure 5, Panel B, lanes 4 and 7). The two RcsA proteins were not detected in the two Δ*lon rcsA72*::Tn*10* strains harboring pBR322 (Figure 5, Panel B, lanes 3 and 8). These data provided evidence that the two RcsA proteins were expressed from a single *rcsA* gene. This same result was observed with Δ*lon rcsA72*::Tn*10* strains containing a pBluescript II KS$^-$ (Stratagene)

plasmid harboring an 897 bp fragment carrying the *rcsA* gene but lacking *dsrA*, which encodes a small RNA implicated in *rcsA* regulation. The presence or absence of *dsrA* did not affect detection of either RcsA protein (data not shown).

Proteolytic processing is not responsible for generating the second RcsA protein.

In all cases, under the conditions described above, either both RcsA proteins were observed or neither RcsA protein was observed. One possibility to explain the presence of two RcsA proteins, coded for by a single gene but differing in size by approximately 1000 Da. would be proteolytic processing. To address the possibility that the smaller RcsA protein was a proteolytic product of the larger protein, spectinomycin was added to Δ*lon rcsA*$^+$ cells to halt new protein synthesis. Samples were removed at the time points indicated (Figure 9, lanes 3 - 7), separated by tricine-SDS-PAGE and probed with polyclonal *E. coli* RcsA antisera. During the 60 minute incubation, there was no evidence of cleavage of a preprotein and a concomitant accumulation of the other protein as would be expected if proteolytic processing were occurring. There was some decrease in band intensity over time in both bands as predicted for a protein with a half-life of 30 minutes in Δ*lon* mutant cells.

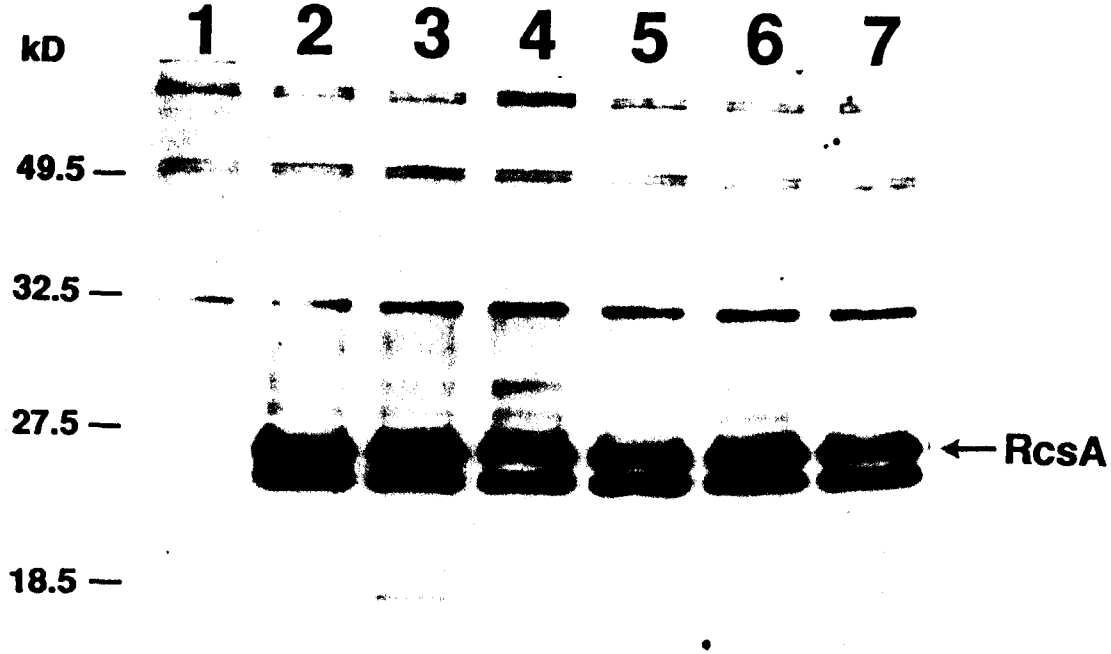
Neither RcsA protein is detected in the absence of RcsB in *lon* mutants.

RcsB and RcsA are proposed to interact to turn on transcription of *cps*. Stout and Gottesman demonstrated that RcsA, when expressed at high levels from a multicopy plasmid, had an increased half-life in a *lon*$^+$*rcsB*$^+$ strain, but in a *lon*$^+$*rcsB*$^-$ strain the half-life of RcsA was reduced. This observation suggested that the interaction of RcsA and RcsB leads to the stabilization of RcsA (116). However, nothing was known about this interaction in the

Figure 9. Analysis of the stability of the two forms of RcsA protein.

Cell cultures were grown to early log phase, samples were harvested before (lanes 1 and 2, T=0) and after addition of spectinomycin (lane 3, T= 2.5 min.; lane 4, T=5 min.; lane 5, T=10 min.; lane 6, T=30 min.; lane 7, T=60 min.), equal amounts of protein from the whole cell extracts were boiled in sample buffer, fractionated on a 14% tricine SDS polyacrylamide gel, transferred to PVDF membrane, and analyzed by Western blotting with preabsorbed polyclonal antiserum specific to *E. coli* RcsA protein. Immunoreactive proteins were visualized by enhanced chemiluminescence. Lane 1, SG20781 *lon*+ *rcsA*+ + pBR322; Lanes 2 - 7, SG20780 Δ*lon rcsA*+ + pBR322. The positions of molecular weight standards are indicated on the left.

Figure 9.



absence of Lon, nor had these studies been done with RcsA levels reflective of normal expression conditions: RcsA expressed from a single chromosomal copy of the *rcsA* gene. To examine the levels of RcsA protein in the absence of both Lon and RcsB, the *rcsB* gene was interrupted by an *rcsB62::*Tn10 insertion (116) transduced into a Δ*lon* strain. Neither RcsA protein was detected in Δ*lon rcsB*$^-$ cells (Figure 10, lane 4). This same result was also observed in *lon::*Tn*10* strains that are *rcsB*$^-$, and in *lon*$^-$ strains (constructed with point mutations in the *lon* gene) that are *rcsB*$^-$ (32).

Two possibilities existed to explain the absence of both RcsA proteins in Δlon *rcsB*$^-$ cells: (i) RcsB affects RcsA expression, or (ii) RcsA, a protein that is stabilized in the absence of Lon, is highly unstable in the absence of both Lon and RcsB. To address whether expression of both RcsA proteins was affected by RcsB, multiple copies of *rcsA* on either pVS103, a pACYC184 based plasmid, or pATC400, a pBR322 based plasmid, were added to Δ*lon rcsB*$^-$ cells. If RcsB was required for expression of *rcsA*, RcsA should not be detectable, even when overexpressed from a multicopy plasmid. As illustrated in Figure 10 (lanes 9 and 10), both RcsA proteins were readily detected in a Δ*lon rcsB*$^-$ background. This result suggested RcsB was not essential for *rcsA* expression. In support of my observation, Stout and Gottesman reported, using a *rcsA::lacZ* transcriptional fusion expressed in a *lon*+ strain, that there was no effect of RcsB on expression of *rcsA* (116).

To address whether RcsB stabilized both forms of RcsA in cells lacking Lon protease, multiple copies of *rcsB* on pJB100 (13), a pBR322 based multicopy plasmid, were added to Δ*lon rcsB*$^-$ cells. Both immunoreactive RcsA proteins were readily detected in the presence of RcsB expressed from a plasmid (Figure 10, lane 6). Interestingly, in *lon*+ *rcsB*$^-$ cells containing the *rcsB* plasmid, RcsA was not detected (Figure 10, lane 5). Whereas, RcsA was readily detected in *lon*+ *rcsB*$^-$ cells containing the *rcsA* plasmid (Figure 10, lanes 7 and 8). This strongly suggests that RcsB is not absolutely required for expression of *rcsA* but RcsB appears necessary for protection or stabilization of both RcsA proteins in Δ*lon* cells.

Figure 10. Analysis of expression of RcsA in the absence of RcsB.

Cell cultures were grown to late log phase, pelleted, boiled in sample buffer, fractionated on a 14% tricine SDS polyacrylamide gel, transferred to PVDF membrane, and analyzed by Western blotting with preabsorbed polyclonal antiserum specific to *E. coli* RcsA protein. Lanes: 1, SG20250 *lon*+ *rcsA*+ *rcsB*+; 2, *E. coli* JT4000 Δ*lon* *rcsA*+ *rcsB*+; 3, KD317 (SG20250 *rcsB*62::Kan); 4, KD318 (JT4000 *rcsB*62::Kan); 5, KD317 + pJB100 (p*rcsB*+); 6, KD318 + pJB100; 7, KD317 + pVS103 (p*rcsA*+); 8, KD317 + pATC400 (p*rcsA*+); 9, KD318 + pVS103; 10, KD318 + pATC400. The positions of molecular weight standards are indicated on the left.

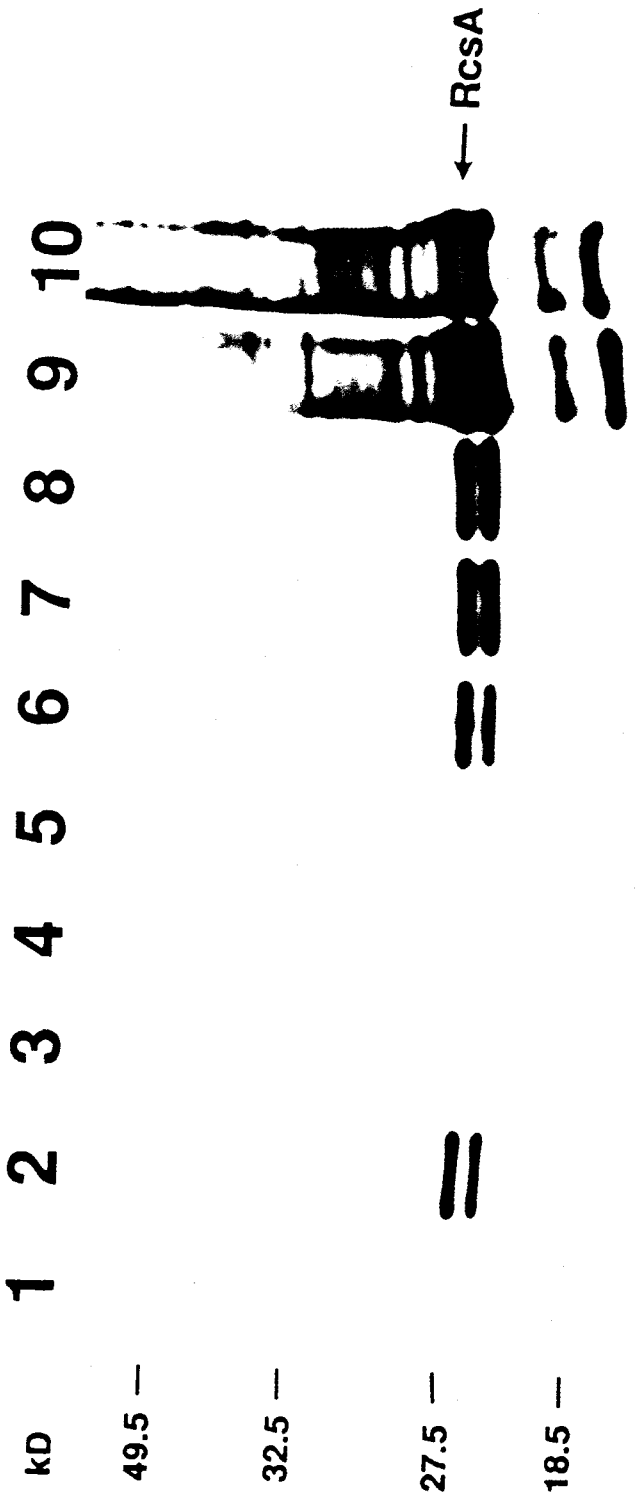


Figure 10.

One possible candidate contributing to the disappearance of both RcsA proteins in the absence of Lon and RcsB was the energy dependent Clp (Ti) protease. Clp protease is composed of two functionally dissimilar subunits coded for by the *clpA* and *clpP* genes (reviewed by (48)). To test for the possibility of Clp involvement in the Lon independent disappearance of RcsA, mutant *clpA* and *clpP* chromosomal genes, interrupted by transposon insertions conferring antibiotic resistance to either tetracycline (*clpA::Δtet* ; source: S. Gottesman) or chloramphenicol [*clpP::cat* ; (81)] were transferred by P1 transduction into a Δ*lon rcsB*$^-$ strain, and Western blot analysis with polyclonal RcsA antiserum was performed on purified transductants. Neither RcsA protein was detected in Δ*lon rcsB*$^-$ *clpA*$^-$ *clpP*$^-$ mutant cells (data not shown).

DISCUSSION AND SUMMARY

Lactic acid bacteria have been used for centuries as starter cultures for fermented milk products. In recent years, DNA technologies have been used to enhance commercially desirable characteristics such as fast acid production, or bacteriophage resistance in producer strains. Another characteristic with potential for commercial application is the production of an EPS that confers "ropy" characteristics to fermented milk products. These EPS contribute to the textural and rheological properties of milk products by increasing viscosity, reducing susceptibility to syneresis, and producing a smooth "mouthfeel". These EPS may have properties of commercial usefulness in medical, environmental, and industrial applications and may represent alternatives as food additives. Scant information on the regulation of EPS or on environmental conditions required for expression of EPS currently exists.

Environmental conditions influence expression of the ropy and mucoid phenotypes

In the present study, environmental conditions required for production of the ropy EPS phenotype in isolates of *L. lactis* and *L. cremoris* were determined. Analysis revealed the presence of two distinct EPS phenotypes, ropy and mucoid, with environmental conditions affecting expression of both phenotypes. The terms ropy, mucoid, and slime-producing are currently used interchangeably in the literature to describe strains that confer the desirable EPS phenotype in milk. The present study demonstrates that the terms mucoid and ropy represent different and distinct phenotypes. Colonies on agar plates that produce strings of 5 mm or longer when touched with a wire inoculating loop or sterile toothpick are defined as ropy. Ropy colonies may be either mucoid or non-mucoid dependent on environmental conditions and on the particular strain.

Colonies exhibiting the ropy phenotype under one or more of the environmental conditions when plated on solid media produce a ropy fermented milk. Colonies that present a shiny, glistening, slimy appearance on agar plates but do not form strings when touched with a loop are defined as mucoid; they are considered slime-producing if copious amounts of EPS are visible. Colonies that are mucoid under one or more of the environmental conditions, but are not ropy under any of the conditions tested, do not produce a ropy fermented milk. The "ropy" bacteria i.e. those that confer the desirable properties in milk are not necessarily mucoid or slime-producers. Scanning electron micrographs of ropy and non-ropy strains in association with milk provide evidence that specific interactions of the ropy EPS with the milk protein matrix confer the desired properties to the fermented product (109a,125). Lactic acid bacteria can be mucoid or slime-producing on solid media yet not produce the "ropy" characteristic in milk.

L. cremoris Ropy 352 and *L. cremoris* Hollandicus, the two strains exhibiting a ropy phenotype on solid media, produce the characteristic thick, smooth body fermented milk; the three non-ropy strains produce a thinner fermented milk. The correlation between a ropy plate phenotype and production of a thick fermented milk suggests that isolation of new ropy lactococcal strains could be accomplished by plating cultures under conditions where string formation is produced (on whey agar under aerobic conditions or on Elliker under anaerobic conditions).

Ruthenium red milk agar plates (RMA) have been used successfully in other laboratories to differentiate ropy strains of *S. thermophilus* from non-ropy strains (114). The RMA assay appeared not to consistently differentiate between ropy lactococcal strains and non-ropy strains used in the present study. When grown at 20°C or 30°C under aerobic conditions, *L. cremoris* LAPT 3001(non-ropy strain), *L. cremoris* Ropy352 (ropy strain), and *L. lactis* MG1363(laboratory non-ropy strain), all presented pink or red colonies on RMA plates indicating that no EPS was being produced. On the other hand, *L. lactis* Strain B (non-ropy strain), and *L. cremoris* Hollandicus (ropy strain) were white on RMA plates

indicating that EPS was being produced. Addition of ruthenium red dye to whey or Elliker plates gave similar contradictory results. Bouzar *et al.* also found that ruthenium red plates gave contradictory results in their studies of colonial variants of the ropy strain, *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 1187. Since the ruthenium red staining method was identified using a ropy *S. thermophilus* strain, Bouzar *et al.* suggested that the EPS produced by *S. thermophilus* might be different from the one produced by *Lb.delbrueckii* subsp. *bulgaricus*, and that the assay may only be valid for specific EPS (12). If that hypothesis is correct, the opposite results for one of the ropy strains in the present study would indicate that although this strain produces a ropy EPS, the EPS may be structurally different from the EPS produced by the other ropy strain, and hence react differently with ruthenium red.

Other methods used to identify or quantify EPS did not allow for differentiation between the ropy EPS-producing strains in the present study and the mucoid EPS-producing strains. For example, cetylpyridinium chloride which precipitates EPS was added to cells grown on microscope slides for a visual estimation of EPS production by ropy and non-ropy strains. The EPS was subsequently stained with Congo red and the staining reaction intensified with Tween 80 (36). Stained cells from ropy and non-ropy strains viewed at 1000x under an oil immersion lens did not show any obvious differences in the amount of EPS produced. An analysis of total EPS, expressed in glucose equivalents, and assayed by the anthrone reaction (52, 134) gave similar results.

The expression of both mucoid and ropy phenotypes simultaneously by *L. cremoris* Ropy 352 suggests that more than one pathway may exist for polysaccharide synthesis. This ability to produce two different polysaccharide phenotypes has not been previously documented in *Lactococcus*, but has been noted in *Rhizobium* (87), *Lactobacillus* sp. (79) 18), *E. coli* K1 and K5 (62), *Alcaligenes faecalis* (119), and in *Zoogloea sp* (72). The expression of one or the other phenotype under defined conditions suggests that the signals for expression of each polysaccharide may differ as well.

Examination of the environmental data did not reveal one factor (temperature, media composition, or atmosphere) that predominates in induction of the ropy phenotype. On whey plates, both *L. cremoris* Hollandicus and *L. cremoris* Ropy 352 were ropy under aerobic conditions, and temperatures in the range of 15°C to 30°C did not affect expression. On whey plates under anaerobic conditions neither strain significantly exhibited the ropy phenotype. In contrast, atmosphere had the opposite effect when these two strains were grown on Elliker plates; the ropy phenotype was predominantly expressed under anaerobic conditions with limited or no expression of the ropy phenotype under aerobic conditions. The effect of aeration has been shown to cause a notable difference in optimal production of exopolysaccharides in different organisms. In some, high aeration is required, in others low aeration or anaerobic conditions are required (119). However, it was surprising to observe that on one medium (whey) *L. cremoris* Ropy 352 and Hollandicus were ropy under aerobic conditions and on another medium (Elliker) they were ropy under anaerobic conditions. Although whey and Elliker media differ in composition, no one component stands out as an obvious factor to account for the differential expression of the ropy phenotype under aerobic or anaerobic conditions. One possible explanation involves the switch from homolactic fermentation (end-product lactate) to heterolactic fermentation (end-products lactate, ethanol, acetate, and CO_2) under anaerobic conditions. The pyruvate-formate lyase branch of pyruvate metabolism is activated under anaerobic conditions and metabolic end-products acetate, formate, and ethanol are produced (6). Acetate present in Elliker agar might inhibit this pathway, and hence slow the growth rate. A reduction in growth rate might free more glycosyl lipid carriers for EPS biosynthesis.

In their studies, Gassem *et al.* reported greater polysaccharide production by ropy yogurt cultures in simulated whey permeate broth, an unbuffered medium, than in Elliker or MRS broth both of which are buffered (38). In the present study, all three media used were buffered, and no conclusions could be drawn for the effect of buffering on expression of the ropy phenotype. Previous studies have

also shown that ropy stains are not easily categorized, and that the viscous trait is not linked to one specific characteristic such as carbon source, proteolytic ability, acid production, or growth rate (16, 38).

Media composition was found to be a significant factor for induction of the mucoid phenotype when strains were cultured on M17L plates. The two ropy strains and the three non-ropy strains exhibited the mucoid phenotype under all of the conditions selected when plated on M17L. This observation suggested that M17L could be used as a selective medium in mutagenesis experiments designed to differentiate between genes involved in expression of the mucoid phenotype and those involved in expression of both phenotypes. Mutants of ropy strains which are no longer mucoid on M17L could be assayed on whey agar plates for retention or loss of the ropy phenotype. Genes identified as important in expression of the ropy phenotype could be identified and further characterized.

No generalizations could be made with respect to conditions important for expression of the mucoid phenotype on Elliker or whey plates. Expression of the mucoid phenotype did not follow any observable pattern on these media.

Inducible nature of the EPS phenotypes

The ability of environmental conditions to induce or repress one or both viscous phenotypes in *L. cremoris* Ropy 352 and Hollandicus indicates that EPS production is not constitutive and therefore, regulation is occurring. Structural studies of EPS from lactococcal strains that produce a ropy EPS reveal no common features to correlate structure to rheological behavior (51, 85, 86, 124). This observation suggested that regulators rather than structural genes would be preferred targets in the construction of stable EPS producing strains. One approach for identifying such regulators would be to screen for conservation of known regulators present in other microorganisms. Lon protease and RcsA are present in a number of organisms, and both regulatory genes have been shown

to function in heterologous hosts. The *rcsA* gene from both *Erwinia stewartii* (127) and *K. aerogenes* (2) activates expression of colanic acid capsular polysaccharide in *E. coli*, although the polysaccharides which are regulated by RcsA in *Erwinia* and *Klebsiella* are structurally distinct from colanic acid. The *rcsA* gene from *E. amylovora* activates capsular polysaccharide synthesis in other *Erwinia* sp., in *E. coli*, and in *S. typhimurium* (8, 19). The *E. coli lon* gene when expressed in a *S. typhimurium lon* mutant suppresses the pleiotrophic phenotypes associated with the *lon* mutation: increased expression of capsular polysaccharide, sensitivity to DNA damaging agents, and a decreased ability to degrade abnormal proteins (29).

Homology to *lon* and *rcsA* genes was not observed

In the present study, laboratory and commercial strains of *Lactococcus* were probed for DNA sequences homologous to *E. coli lon* and *rcsA*. The *E. coli lon* and *rcsA* DNA probes did not hybridize to homologous sequences in lactococci. While gene expression signals in *L. lactis* sp. show considerable similarity to signals observed in *E. coli*, and lactococcal promoter sequences closely resemble consensus sequences for *E. coli*, considerable diversity in codon usage has been observed (133). Under the conditions of high stringency used in Southern blotting, differences in codon usage may have been enough so that hybridization did not occur. Using low stringency hybridization conditions, DNA sequences similar to *E. coli lon* and *rcsA* might be detected in lactococci.

Homology to Lon protease was observed

Although codon usage may differ between *E. coli* and *Lactococcus* sp., researchers have used antiserum against *E. coli* proteins (GroEL, DnaK, DnaJ, and GrpE) to identify structurally related proteins in *Lactococcus* (5, 11, 143). In the present study, antiserum specific to *E. coli* Lon and RcsA proteins was used in

Western blot analysis of strains of *L. lactis*. A protein similar in size to *E. coli* Lon protease (94 kDa.) and immunoreactive with *E. coli* Lon antiserum was detected in the laboratory strain MG1363 and in all the commercial strains of *L. lactis* and *L. cremoris* probed. An interesting observation was that *L. cremoris* Ropy 352 and Hollandicus, the two strains which produce a ropy polysaccharide, expressed significantly less of the 94 kDa. Lon-like protein than the non-ropy strains or the laboratory strain, MG1363. The possible involvement of this protein in expression of the ropy phenotype requires further examination. The ropy strains also expressed significantly less of an 86 kDa. protein that was immunoreactive with *E. coli* Lon antiserum. Although no evidence exists at this time to suggest the 86 kDa. protein is a Lon homolog, evidence for the presence of two *lon* genes in microorganisms exists. Two *lon* genes, encoding proteins essential for separate cellular functions, have been identified in *Myxococcus xanthus*. *lonV* encodes a 92 kDa. protein that is essential for vegetative growth (126), and *lonD* encodes a 90.4 kDa. protein that is essential for multicellular differentiation (42, 126). In the present study, evidence for the presence of more that one Lon protein was also observed in the Western blot analysis of *B. subtilus* and *Ps. aeruginosa*. In addition to the 94 kDa. protein observed in both organisms, an 86 kDa. protein was observed in *Ps. aeruginosa* and, under heat-shock conditions, an additional 120 kDa. protein was observed in *B. subtilus*. To date, only one *lon* gene has been identified in *B. subtilus*. However, it would not be surprising to identify a second *lon* gene in *B. subtilus* given that *B. subtilus*, a spore-forming organism, undergoes cellular differentiation, and that deletion of the *lon* gene in *B. subtilus* (100) does not result in the pleotrophic phenotypes seen in *E. coli*, *S. typhimurium*, and *E. amylovora lon* mutant cells.

Alternate explanations for detection of additional proteins immunoreactive to Lon antiserum are as follows: (i) the smaller immunoreactive proteins are Lon degradation products, (ii) Lon is proteolytically processed, and antiserum reacts with the precursor and the active protease, or (iii) proteins which share no functional relationship to Lon may contain one or more of the conserved

domains identified in Lon, and may react with antiserum derived from epitopes to these domains. These conserved domains include an ATP-binding domain, two basic regions proposed to be involved in DNA binding, one acidic region (20), and a highly conserved seven amino acid region bordering the serine residue implicated in catalytic activity (3). Analysis of the amino acid sequenced deduced from the *sms* (sensitivity to methylmethane sulfonate) gene by Neuwald *et al.*, revealed similarity of Sms (55 kDa.) to the region of Lon protease containing the proposed catalytically active serine residue. Sms did not contain amino acid sequences similar to other conserved domains present in Lon (88). Although these alternate explanations cannot be ruled out entirely, evidence to date suggests that Lon is highly stable, and no evidence for proteolytic processing has been reported (80). Although the observance of weakly-reactive proteins might be due to the presence of one or more conserved domains in proteins sharing no functional relationship to Lon, the strongly-reactive proteins suggest that many epitopes and therefore, many domains are similar in these proteins. It remains to be determined whether *Lactococcus* contains one or more *lon* genes and whether one or both genes are involved in regulation of EPS.

Conservation of Lon and RcsA in other organisms

Analysis of the structural conservation of Lon and RcsA was extended to a number of Gram negative and Gram positive microorganisms. As observed with *Lactococcus*, none of the other Gram positive organisms contained DNA sequences that hybridized to the *lon* DNA probe under high stringency conditions. This included *B. subtilis*, an organism where the *lon* sequence is now known and in which the deduced amino acid sequence shares significant homology to the *E. coli* Lon protein sequence. Under conditions of low stringency the *B. subtilis lon* sequence may have hybridized to the *E. coli lon* probe. At the time of the present study, the *B. subtilis lon* sequence was not known, and conditions of low stringency were not used.

As observed in *Lactococcus*, all of the other Gram positive organisms except *S. aureus* expressed at least one high molecular weight protein strongly reactive with *E. coli* Lon antiserum. In *S. aureus*, the result was ambiguous, probably due to the presence of Staph A protein in the whole cell extract. Staph A protein which is used in immunoprecipitation reactions would strongly bind antibodies in both the primary and the secondary antiserum.

In the Gram negative organisms, the *lon* and *rcsA* DNA probes hybridized with sequences from organisms classified by 16S rRNA analysis (144) as γ-purple or β-purple bacteria. At least one high molecular weight protein reacted strongly to *E. coli* Lon antiserum, and one lower molecular weight protein reacted strongly to *E coli* RcsA antiserum. In addition, two α-purple bacteria, *Rhizobium* sp. and *Agrobacterium* sp., had proteins immunoreactive with Lon and RcsA antiserum. A surprising result of this study was the ability to detect an RcsA-like protein in strains expressing a lon-like protein. In *E. coli*, RcsA is not detected in a *lon*$^+$ strain, and under normal laboratory conditions *lon*$^+$ *E. coli* are not mucoid. The other Gram negative organisms in this study and the Gram positive organism expressing an RcsA-like protein (*S. salivarius*) are all mucoid under normal laboratory conditions. Several possibilities exist to explain the presence of an RcsA-like protein in the mucoid organisms, all of which contain a lon-like protein. (i) Laboratory-adapted *E. coli* have evolved mechanisms for regulation of polysaccharide expression not seen in the other organisms probed. (ii) In the mucoid organisms RcsA is expressed at higher levels, is resistant to Lon-dependent degradation, or is protected from Lon-dependent degradation through protein-protein interactions. (iii) Other factors, not present in *E. coli*, are involved in regulation of EPS expression in the mucoid organisms. Evidence for the later hypothesis has been reported in *Erwinia stewartii*, an organism in which EPS is implicated in virulence (140). In addition to regulation by the *rcs* system, EPS expression in this organism is controlled by an N-acylhomoserine lactone autoinducer that is identical to the autoinducer used by *Vibrio fischeri* in bioluminescence. The relationship between autoinduction and the *rcs* regulatory system has not yet been

determined. It remains to be seen whether autoinduction is involved in the *rcs* system, dominates the *rcs* system, or operates by a distinct pathway that overrides the *rcs* system (139). Other mucoid organisms in which EPS has a role in virulence may be regulated in an acyl-HSL-dependent manner. A comparison of the stability of RcsA in mucoid organisms to the stability of RcsA in *E. coli* might reveal how regulation of polysaccharide expression differs in these organisms.

Identification of two RcsA proteins in *E. coli*

In the course of the present study, the presence of two RcsA proteins in *E. coli* was revealed. The two RcsA proteins differ in size by approximately 1000 Da. Strains lacking Lon protease activity, either by the deletion of *lon*, by transposon insertion into *lon*, or by point mutations in *lon*, expressed both RcsA proteins, whereas neither RcsA protein was detected in isogenic *lon*$^+$ strains. Both RcsA proteins were expressed from the same *rcsA* gene. The *rcsA*$_{62}$ mutation (116), which stabilizes RcsA in the presence of Lon, did not impact the detection of one or the other RcsA protein: both RcsA proteins were detected in *lon*$^+$ cells carrying the *rcsA*$_{62}$ dominant allele (data not shown). Additional classes of *rcsA* point mutations which stabilize RcsA in the presence of Lon have been isolated; these mutant RcsA strains express both RcsA proteins (31). Electrophoretic artifact does not explain the two RcsA proteins, as evident by the ability to detect both RcsA proteins in whole cell extracts subjected to complete denaturing conditions in a Laemmli SDS-PAGE system using either glycine or tricine in the cathode buffer. Peptide digestion patterns were almost identical for both proteins except each protein had one peptide band not seen in the other protein demonstrating that the proteins exhibit a high degree of primary amino acid sequence homology. Finally, a construction of Ferguson plots revealed that anomalous migration was not involved, and that the two RcsA proteins fractionated by virtue of size and not charge on a SDS-PAGE gel. Taken together,

these data argued strongly against the possibility that detection of the two RcsA proteins was due to electrophoretic artifact, anomalous migration, or was due to charge differences, while these data argued strongly for the conclusion that *E.coli* expresses two differently sized RcsA proteins.

What mechanism(s) might account for the generation of two differently sized RcsA proteins? The present study suggests that proteolytic processing is not responsible for generating the smaller RcsA protein. Sledjeski and Gottesman used primer extension and ribonuclease protection assays to determine the transcriptional start site of *rcsA* (112). They identified a single start point of transcription 132 nucleotides upstream of the RcsA translation start codon. In view of their results, alternate transcriptional start sites appears unlikely. Examination of a list of approximately 200 experimentally confirmed or potential transcriptional terminators in the *E. coli* database collection at the University of Giessen, Germany revealed that a potential or experimentally confirmed transcriptional termination site for *rcsA* has not been reported. Thus, alternate transcriptional terminators as a method for generating alternate mRNA's remains a possibility. However, alternate mRNA's would require alternate translational stop codons if two different sized RcsA proteins were translated. Analysis of the *rcsA* coding region did not reveal any additional in-frame codons to code for a larger protein or an alternate internal stop codon to code for a smaller RcsA protein.

Examination of the *rcsA* gene sequence for an alternate translational initiation site revealed a potential AUG initiation codon at position number six of the *rcsA* open reading frame and a potential ribosomal binding site (RBS) at the -10 position relative to this AUG codon. The sequence of the potential RBS (AACGA) is complementary to a region upstream of and including the CU nucleotides of the most-favored region (CUCC) of the 16S rRNA for mRNA binding (70). The difference in five amino acids might be sufficient to account for the size differences of the two RcsA proteins.

Regulating protein activity by expressing differently sized proteins from the same ORF (open reading frame) by the use of alternate translational initiation sites has been proposed in a number of systems (30, 33, 37, 61, 71, 95, 102). In the McrB restriction system of *E. coli* K-12, Ross *et al.* have proposed that the smaller protein may play a regulatory role in the restriction of DNA containing 5-methylcytosine (102). In DNA replication of bacteriophage f1, Gene II protein is required for phage DNA synthesis and the Gene X product which is translated from an internal initiation site and is identical to the C-terminal one-third of the Gene II protein is a strong inhibitor of phage-specific DNA synthesis in vivo (37). In both examples, evidence suggests that the smaller protein acts as an inhibitor of the mechanism which the larger protein is mediating. What would be the advantages of expressing two differently sized RcsA proteins, and how might this impact *cps* expression? One possibility is that binding of one of the RcsA proteins to RcsB leads to a non-functional interaction, thus resulting in no *cps* expression. This tactic would insure that the cell conserves energy during certain favorable growth conditions when capsular polysaccharide is not required. Another possibility is that one of the RcsA proteins titrates Lon protease, thereby permitting the other RcsA protein to remain intact and functional for activation of *cps* expression. This tactic would insure that high levels of functional RcsA would be available during certain unfavorable growth conditions when high levels of *cps* expression were required. And in this case, the non-functional RcsA protein might be a preferred substrate of Lon.

Preliminary data from site-directed mutagenesis of the start codon(s) indicate alternate translation initiation may not be the mechanism involved in generating two RcsA proteins. However, a more in-depth analysis of the region will be necessary before this mechanism can be ruled out completely.

Other mechanisms such as peptide tagging should be considered. Keiler *et al.* have recently identified a peptide tagging system in *E. coli* whereby an 11-residue peptide tag is added to the carboxy terminus of proteins translated from incomplete or damaged

mRNA's (63). Other peptide tagging systems that exert a role in the control of unstable regulatory proteins such as RcsA might exist. Although no evidence exists implicating a tagging system in the generation of the second RcsA protein, it is interesting to note that in the *ssrA* tagging system an eleven amino acid tag is added to targeted proteins; the size difference for the two RcsA proteins is approximately 1000 Da.

One of the surprising observations in the present study was the inability to detect RcsA in cells mutant for *lon* and *rcsB*. Detection of RcsA could be restored when multiple copies of the *rcsA* gene were added to cells mutant for *lon* and *rcsB*. This result suggested that RcsB was not required for the expression of *rcsA*, but was required for the protection or stabilization of RcsA, even in the absence of Lon. Why would RcsB be needed to protect or stabilize RcsA if Lon was absent? An obvious possibility is that an alternate protease, whose substrate specificity overlaps with Lon, is degrading RcsA. In support of this possibility, RcsA is still very unstable in *lon*$^-$ cells: its half-life is only increased from 2 minutes to 30 minutes (116). Compared to highly stable proteins, RcsA disappears at a fairly fast rate in *lon*$^-$ cells, thus implicating an alternate proteolytic mechanism. The Clp protease system did not appear to be involved in this Lon-independent disappearance of RcsA leaving open the possibility that a previously unidentified proteolytic mechanism may be involved.

Summary

Two EPS phenotypes, ropy and mucoid, were observed in strains of *L. lactis*. Strains of *L. lactis* exhibiting the ropy milk phenotype were differentiated from non-ropy strains by their ability to form strings on agar plates. The ability to distinguish the two phenotypes might lead to the isolation of other ropy strains from milk products or from the environment, and might be useful as a means of differentiating genes involved in the mucoid phenotype from genes involved in the ropy phenotype.

A protein exhibiting structural homology to Lon protease, a negative regulator of polysaccharide expression in *E. coli*, was identified in all strains of lactococci probed. The ropy strains expressed significantly less of the lon-like protein than the non-ropy strains. Given the role of Lon in negatively regulating polysaccharide expression, this suggested the possible involvement of the Lon protein in expression of the ropy phenotype. Identification, characterization, and inactivation of this protein may provide greater understanding of regulation of EPS in lactococci and, in turn, lead to the construction of stable EPS-producing strains. A detailed genetic examination of these organisms is needed to identify mechanisms involved in regulating expression of both polysaccharides and in switching from one polysaccharide phenotype to the other.

This study provided strong evidence for the cellular expression of two RcsA proteins in *E. coli,* and that the two RcsA proteins differ by size rather than by charge. The physiological significance of these two RcsA proteins and their impact on the regulation and expression of capsule in both *lon*+ and Δ*lon* strains remains to be determined. Defining the functions of the two RcsA proteins should provide the necessary insight into understanding their roles in the expression of capsular polysaccharide.

BIBLIOGRAPHY

1. **Alifano, P., R. Fani, P. Lio, A. Lazcano, M. Bazzicalupo, M. S. Carlomagno, and C. B. Bruni.** 1996. Histidine biosynthetic pathway and genes: structure, regulation, and evolution. Microbiol. Rev. **60**:44-69.

2. **Allen, P. A., A. C. Hart, and J. R. Saunders.** 1987. Isolation from *Klebsiella* and characterization of two *rcs* genes that activate colanic acid capsular biosynthesis in *Escherichia coli.* J. Gen. Microbiol. **133**:331-340.

3. **Amerik, A. Y., V. K. Antonov, A. E. Gorbalenya, S. A. Kotova, T. V. Rotanova, and E. V. Shimbarevich.** 1991. Site-directed mutagenesis of La protease. FEBS. **287**:211-214.

4. **Arnosti, D. N., V. Singer, and M. J. Chamberlin.** 1986. Characterization of heat shock in *Bacillus subtilis.* J. Bacteriol. **168**(3):1243-1249.

5. **Auffray, Y., X. Gansel, B. Thammavongs, and P. Boutibonnes.** 1992. Heat shock-induced protein synthesis in *Lactococcus lactis* spp. *lactis.* Curr Microbiol. **24**:281-284.

6. **Axelsson, L. T.** 1993. Lactic acid bacteria: classification and physiology. *In* S. Salminen and A. vonWright (ed.), Lactic acid bacteria. Marcel Dekker, Inc, New York.

7. **Barach, J. T.** 1979. Improved enumeration of lactic acid streptococci on Elliker agar containing phosphate. Appl. Environ. Microbiol. **38**:173-174.

8. **Bernhard, F., K. Poetter, K. Geider, and D. L. Coplin.** 1990. The *rcs*A gene from *Erwinia amylovora*: identification, nucleotide sequence analysis, and regulation of exopolysaccharide biosynthesis. Mol. Plant-Microbe Interac. **3**:429-437.

9. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513-1523.

10. **Bolivar, F., R. L. Rodrigues, M. C. Betlach, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. I. Ampicillin-resistant derivatives of the plasmid pMB9. Gene. **2**:75.

11. **Boutibonnes, P., C. Tranchard, A. Hartke, B. Thammavongs, and Y. Auffray.** 1992. Is thermotolerance correlated to heat-shock protein synthesis in *Lactococcus lactis* subsp. *lactis* ? Int. J. Food Microbiol. **16**:227-236.

12. **Bouzar, F., J. Cerning, and M. Desmazeaud.** 1996. Exopolysaccharide production in milk by *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ 1187 and by two colonial variants. J. Dairy Sci. **79**:205-211.

13. **Brill, J. A., C. Quinlan-Walshe, and S. Gottesman.** 1988. Fine-structure mapping and identification of two regulators of capsule synthesis in *Escherichia coli* K-12. J. Bacteriol. **170**:2599-2611.

14. **Broadbent, J. R., and J. K. Kondo.** 1993. Biotechnology of dairy starter cultures, p. 77-104. *In* Y. H. Hui (ed.), Dairy science and technology handbook, vol. 3. VCH Publishers, Inc., New York.

15. **Bukhari, A. I., and D. Zipser.** 1973. Mutants of *Escherichia coli* with a defect in the degradation of nonsense fragments. Nature. **243**:238-241.

16. **Cerning, J.** 1990. Exocellular polysaccharides produced by lactic acid bacteria. FEMS Microbiol. Lett. **87**:113-130.

17. **Cerning, J., C. Bouillanne, M. Landon, and M. Desmazeaud.** 1992. Isolation and characterization of exopolysaccharides from slime-forming mesophilic Lactic acid bacteria. J. Dairy Sci. **75**:692-699.

18. **Charette, M. F., G. W. Henderson, and A. Markovitz.** 1981. ATP hydolysis-dependent activity of the *lon* (*capR*) protein of *E. coli* 12. Proc. Natl. Acad. Sci. USA. **78**:4728-4732.

19. **Chatterjee, A., W. Chun, and A. K. Chatterjee.** 1990. Isolation and characterization of an *rcsA*-like gene of *Erwinia amylovora* that activates extracellular polysaccharide production in *Erwinia* species, *Escherichia coli*, and *Salmonella typhimurium*. Mol. Plant-Microbe Interact. **3**:429-437.

20. **Chin, D. T., S. A. Goff, T. Webster, T. Smith, and A. L. Goldberg.** 1988. Sequence of the *lon* gene in *Escherichia coli:* A heat-shock gene which encodes the ATP-dependent protease La. J. Biol. Chem. **263**:11718-11728.

21. **Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102-1106.

22. **Coffey, A. G., C. Daly, and G. Fitzgerald.** 1994. The impact of biotechnology on the dairy industry. Biotech. Adv. **12**:625-633.

23. **Crescenzi, V.** 1995. Microbial polysaccharides of applied interest: Ongoing research activities in Europe. Biotechnol. Prog. **11**:251-259.

24. **Daeschel, M. A.** 1989. Antimicrobial substances from lactic acid bacteria for use as food preservatives. Food Tech. **43**:164-167.

25. **Delorme, C., S. D. Ehrlich, and P. Renault.** 1992. Histidine biosynthesis genes in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. **174**:6571-6579.

26. **Delorme, C., J. J. Godon, S. D. Ehrlich, and P. Renault.** 1994. Mosaic structure of large regions of the *Lactococcus lactis* subsp. *cremoris* chromosome. Microbiol. **140**:3053-3060.

27. **deMan, R., and J. Sharpe.** 1960. J. Appl. Bacteriol. **23**:130.

28. **Difco, L.** 1953. Difco Manual of dehydrated culture media and reagents for microbiological and clinical laboratory procedures, 9th ed. , Detroit, Michigan.

29. **Downs, D., L. Waxman, A. L. Goldberh, and J. Roth.** 1986. Isolation and characterization of *lon* mutants in *Salmonella typhimurium*. J. Bacteriol. **165**:193-197.

30. **Dunn, J. J., and F. W. Studier.** 1983. Complete nucleotide sequence of Bacteriophage T7 DNA and the locations of T7 genetic elements. J. Mol. Biol. **166**:477-535.

31. **Ebel, W., J. L. Claycomb, and J. E. Trempy.** 1995. Defining substrate parameters for the *Escherichia coli* Lon protease, abstr. H-95, p. 509. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology 1995 American Society for Microbiology, Washington, D.C.

32. **Ebel, W., K. P. Dierksen, and J. E. Trempy.** 1996. Unpublished data.

33. **Eisenberg, S., and M. Finer.** 1980. Cleavage and circularization of single-stranded DNA: a novel enzymatic activity of ø174 A* protein. Nucleic Acids Res. **8**(22):5305-5315.

34. **Elliker, P. R., A. Anderson, and G. H. Hannessen.** 1956. An agar culture medium for lactic streptococci and lactobacilli. J. Dairy Sci. **39**:1611-1612.

35. **Ferguson, K. A.** 1964. Starch-gel electrophoresis-application to the classification of pituitary proteins and polypeptides. Metabolism. **13**:985-1002.

36. **Fletcher, M.** 1990. Methods for studying adhesion and attachment to surfaces., p. 251-283. *In* R. Grigorova and J. R. Norris (ed.), Methods in Microbiology. Academic Press.

37. **Fulford, W., and P. Model.** 1988. Regulation of Bacteriophage f1 DNA replication. J. Mol. Biol. **203**:49-62.

38. **Gassem, M. A., K. A. Schmidt, and J. F. Frank.** 1995. Exopolysaccharide production in different media by lactic acid bacteria. Cultured Dairy Products J. **30**:18-21.

39. **Geller, B. L., R. G. Ivey, J. E. Trempy, and B. Hettinger-Smith.** 1993. Cloning of a chromosomal gene required for phage infection of *Lactococcus lactis* subsp. *lactis* C2. J. Bacteriol. **175**:5510-5519.

40. **Gervais, F. G., and G. R. Drapeau.** 1992. Identification, cloning, and characterization of *rcs*F, a new regulator gene for exopolysaccharide synthesis that suppresses the division mutation *fts*Z84 in *Escherichia coli* K-12. J. Bacteriol. **174**:8016-8022.

41. **Geuskens, V., A. Mhammedi-Alaoui, L. Desmet, and A. Toussaint.** 1992. Virulence in bacteriophage Mu: a case of trans-dominant proteolysis by the *Escherichia coli* Clp serine protease. EMBO J. **11**:5121-5127.

42. **Gill, R. E., M. Karlok, and D. Benton.** 1993. *Myxococcus xanthus* encodes an ATP-dependent protease which is required for developmental gene transcription and intercellular signaling. J. Bacteriol. **175**:4538-4544.

43. **Godon, J. J., C. Delorme, S. D. Ehrlich, and P. Renault.** 1992. Divergence of genomic sequences between *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. Appl. Environ. Microbiol. **58**:4045-4047.

44. **Goldberg, A. L.** 1992. The mechanism and functions of ATP-dependent proteases in bacterial and animal cells. Eur. J. Biochem. **203**:9-23.

45. **Goldberg, A. L., R. P. Moerschell, C. H. Chung, and M. R. Maurizi.** 1994. ATP-dependent protease La (Lon) from *Escherichia coli*, Methods in Enzymology, vol. 244. Academic Press.

46. **Gottesman, S.** 1989. Genetics of proteolysis in *Escherichia coli*. Annu. Rev. Genet. **23**:163-198.

47. **Gottesman, S.** 1995. Regulation of Capsule Synthesis: Modification of the Two-Cpmponent Paradigm by an Accessory Unstable Regulator, p. 253-262. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-Component Signal Transduction. ASM, Wash. D.C.

48. **Gottesman, S., and M. R. Maurizi.** 1992. Regulation by proteolysis: Energy-dependent proteases and their targets. Microbiol. Rev. **56**:592-621.

49. **Gottesman, S., P. Trisler, and A. Torres-Cabassa.** 1985. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: Characterization of three regulatory genes. J. Bacteriol. **162**:1111-1119.

50. **Grant, W. D., I. W. Sutherland, and J. F. Wilkinson.** 1969. Exopolysaccharide colanic acid and its occurrence in the *Enterobacteriaceae*. J. Bacteriol. **100**:1187-1193.

51. **Gruter, M., B. Leeflang, J. Kuiper, K. Kamerling, and J. Vliegenhart.** 1992. Structure of the exopolysaccharide produced by *Lactococcus lactis* subspecies *cremoris* H414 grown in a defined medium or skimmed milk. Carbohydr. Res. **231**:273-291.

52. **Hanson, R. S.** 1981. Metabolism, p. 333. *In* P. Gerhardt (ed.), Manual of methods for general bacteriology. ASM, Wash. D. C.

53. **Harlow, E., and D. Lane.** 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory.

54. **Harrigan, W. F., and M. E. McCance.** 1976. Laboratory Methods in Food and Dairy Microbiology. Academic Press.

55. **Hill, C.** 1993. Bacteriophage and bacteriophage resistance in lactic acid bacteria. FEMS Microbiol. Rev. **12**:87-108.

56. **Howard-Flanders, R., E. Simson, and L. Theriot.** 1964. A locus that controls filament formation and sensitivitiy to radiation in *Escherichia coli* K-12. Genetics. **49**:237-246.

56a. **Huggins, A. R., and W. E. Sandine.** 1984. Differentiation of fast and slow milk-coagulating isolates in strains of lactic streptococci. J. Dairy Sci. **67**:1674-1679

57. **Hwang, B. J., K. M. Woo, A. L. Goldberg, and C. H. Chung.** 1986. Protease Ti, a new ATP-dependent protease in *Escherichia coli*, contains protein-activated ATPase and proteolytic functions in distinct subunits. J. biol. Chem. **263**:8727-8734.

58. **Ito, K., S. Udaka, and H. Yamagata.** 1992. Cloning, characterization and inactivation of the *Bacillus brevis lon* gene. J. Bacteriol. **174**:2281-2287.

59. **Jann, K., and B. Jann.** 1990. Bacterial capsules. Crrr. Top. Microbiol. **150**:19-42.

60. **Jay, J. M.** 1982. Antimicrobial properties of diacetyl. Appl. Environ. Microbiol. **44**:525.

61. **Johnson, R. C., and W. S. Reznikoff.** 1984. Role of the IS*50*R proteins in the promotion and control of Tn5 transposition. J. Mol. Biol. **177**:645-661.

62. **Keenlyside, W. J., P. Jayarante, P. R. Maclachlan, and C. Whitefield.** 1992. The *rcs*A gene of *Escherichia coli* 09:K30:H12 is involved in the expression of the serotype specific Group 1 K (capsular) antigen. J. Bacteriol. **174**:8-16.

63. **Keiler, K. C., P. R. H. Waller, and R. T. Sauer.** 1996. Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. Science. **271**:990-993.

64. **Kim, S. G., and C. A. Batt.** 1991. Antisense mRNA-mediated bacteriophage resistance in *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **57**:1109-1113.

65. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12**:39-86.

66. **Klaenhammer, T. R., and W. D. Sing.** 1991. A novel rotation strategy using different phage defenses in a single-strain starter culture system. J. Dairy Sci. **74**:120.

67. **Kojic, M., M. Vujcic, A. Banina, P. Cocconcelli, J. Cerning, and L. Topisirovic.** 1992. Analysis of

exopolysacchaide production by *Lactobacillus casei* CG11, isolated from cheese. Appl. Environ. Microbiol. **58**:4086-4088.

68. **Kok, J.** 1990. Genetics of the proteolytic system of lactic-acid bacteria. FEMS Microbiol. Rev. **87**:15.

69. **Kontusaari, S., and R. Forsen.** 1988. Finnish fermented milk "Viili": Involvement of two cell surface proteins in production of slime by *Streptococcus lactis* spp. *cremoris*. J. Dairy Sci. **71**:3197-3202.

70. **Kozak, M.** 1983. Comparison of initiation of protein synthesis in Procaryotes, Eucaryotes, and Organelles. Microbiol. Rev. **47**:1-45.

71. **Kruger, T., C. Grund, C. Wild, and M. Noyer-Weidner.** 1992. Characterization of the *mcrBC* region of *Escherichia coli* K-12 wild-type and mutant strains. Gene. **114**:1-12.

72. **Kwon, K. J., K. J. Park, J. D. Kim, J. Y. Konh, and I. S. Kong.** 1994. Isolation of two different polysaccharides from halophilic *Zoogloea* sp. Biotech. Letters. **16**:783-788.

73. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227**:680-685.

74. **Leigh, J. A., and D. L. Coplin.** 1992. Exopolysaccharides in plant-bacterial interactions. Annu. Rev. Microbiol. **46**:307-346.

75. **Lohmann, D.** 1990. Structural diversity and functional versatility of polysaccharides. *In* E. A. Dawes (ed.), Novel biodegradable microbial polymers, vol. 186. Kluwer Academic Publishers, Dordrecht.

76. **Lopez-Lara, I. M., G. Orgamlide, F. B. Duzzo, J. Olivares, and N. Toro.** 1993. Characterization and symbiotic importance of acidic extracellular polysaccharides of *Rhizobium* sp. strain GRH2 isolated from acacia nodules. J. Bacteriol. **175**:2826-2832.

77. **Markovitz, A.** 1977. Genetics and regulation of bacterial capsular polysaccharide biosynthesis and radiation sensitivity., p. 415-462. *In* I. Sutherland (ed.), Surface carbohydrates of the procaryotic cell. Academic Press, New York.

78. **Markovitz, A.** 1964. Regulatory mechanisms for the synthesis of capsular polysaccharide in mucoid mutants of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA. **51**:239-246.

79. **Marshall, V. M. E., E. N. Cowie, and R. S. Moreton.** 1995. Analysis and production of two exopolysaccharides from *Lactobacillus lactis* subsp. *cremoris* LC330. J. Dairy Res. **62**:621.

80. **Maurizi, M. R.** 1992. Proteases and protein degradation in *Escherichia coli.* Experientia. **48**:178-201.

81. **Maurizi, M. R., W. P. Clark, Y. Katayama, S. Rudikoff, J. Pumphrey, B. Bowers, and S. Gottesman.** 1990. Sequence and structure of Clp P, the proteolytic component of the ATP-dependent Clp protease of *Escherichai coli.* J. Biol. Chem. **265**:12536-12545.

82. **Maurizi, M. R., P. Trisler, and S. Gottesman.** 1985. Insertional mutagenesis of the lon gene in *Escherichia coli*: *lon* is dispensable. J. Bacteriol. **164**:1124-1135.

83. **Mayra-Makinen, A., and M. Bigret.** 1993. Industrial Use and production of lactic acid bacteria, p. 65-95. *In* S. Salminen and A. vonWright (ed.), Lactic acid bacteria. Marcel Dekker Inc., New York.

84. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Springs Harbor Laboratory, Cold Springs Harbor, N.Y.

85. **Nakajima, H., T. Hirota, T. Toba, T. Itoh, and S. Adachi.** 1992. Structure of the extracellular polysaccharide from slime-forming *Lactococcus lactis* subsp. *cremoris* SBT 0495. Carbohydr. Res. **224**:245-253.

86. **Nakajima, H., S. Toyoda, T. Toba, T. Itoh, T. Mukai, H. Kitazawa, and S. Adachi.** 1990. A Novel

phosphopolysaccharide from slime-Forming *Lactococcus lactis* subspecies *cremoris* SBT 0495. J. Dairy Sci. **73**:1472-1477.

87. **Navarini, L., A. Cesaro, and S. B. Ross-Murphy.** 1992. Exopolysaccharides from *Rhizobium meliloti* YE-2 grown under different osmolarity conditions: viscoelastic properties. Carbohydr. Res. **223**:227-234.

88. **Neuwald, A. F., D. E. Berg, and G. V. Stauffer.** 1992. Mutational analysis of the *Escherichia coli serB* promoter region reveals transcriptional linkage to a downstream gene. Gene. **120**:1-9.

89. **Neve, H., A. Geis, and M. Teuber.** 1988. Plasmid encoded functions of ropy lactic strains from Scandinavian fermented milk. Biochimie. **70**:437-442.

90. **Niven, C. F., K. L. Smiley, and J. M. Sherman.** 1942. The hydrolysis of arginine by streptococci. J. Bacteriol. **43**:651-660.

91. **Nobuki, T., S. Inouye, and T. Komano.** 1993. Cloning and nucleotide sequence of the *Myxococcus xanthus lon* gene: indispensability of *lon* for vegetative growth. J. Bacteriol. **175**:2271-2277.

92. **Nobuki, T., S. Inouye, and T. Komano.** 1993. The *lonD* gene is homologous to the *lon* gene encoding an ATP-dependent protease and is essential for the development of *Myxococcus xanthus*. J. Bacteriol. **175**:4545-4549.

93. **Olsen, G. J., C. R. Woese, and R. Overbeek.** 1994. The winds of (evolutionary) change: breathing new life into microbiology. J. Bacteriol. **176**:1-6.

94. **Ophir, T., and D. L. Gutnick.** 1994. A role for exopolysaccharides in the protection of microorganisms from desiccation. Appl. and Environ. Microbiol. **60**(2):740-745.

95. **Park, S. K., K. I. Kim, K. M. Woo, J. H. Seol, K. Tanaka, A. Ichihara, D. D. Ha, and C. H. Chung.** 1993. Site-directed mutagenesis of the dual translational initiation sites of the

clpB gene of *Escherichia coli* and characterization of its gene products. J. Biol. Chem. **268**:20170-20174.

96. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. in Applied Microbiol. **8**:151-156.

97. **Reuter, S. H., and L. Shapiro.** 1987. Asymmetric segragation of heat-shock proteins upon cell division in *Caulobacter crescentus*. J. Mol. Biol. **194**:653-662.

98. **Reynolds, J. A., and C. Tanford.** 1970. Binding of dodecyl sulfate to proteins at high binding ratios. Possible implications for the state of proteins in biological membranes. Proc. Natl. Acad. Sci. USA. **66**(3):1002-1007.

99. **Reynolds, J. A., and C. Tanford.** 1970. The gross conformation of protein-sodium dodecyl sulfate complexes. J. Biol. Chem. **245**(19):5161-5165.

100. **Riethdorf, S., U. Volker, U. Gerth, A. Winkler, S. Engelmann, and M. Hecker.** 1994. Cloning, nucleotide sequence, and expression of the *Bacillus subtilis lon* gene. J. Bacteriol. **176**:6518-6527.

101. **Rodbard, D., A. Chrambach, and G. H. Weiss.** 1974. Optimization of resolution in analytical and preparative polyacrylamide gel electrophoresis, p. 62-105. *In* R. C. Allen and H. R. Maurer (ed.), Electrophoresis and isoelectric focusing in polyacrylamide gel. deGruyter, Walter, NY.

102. **Ross, T. K., E. C. Achberger, and D. Braymer.** 1989. Nucleotide sequence of the McrB region of *Escherichia coli* K-12 and evidence for two independent translational initiation sites at the *mcrB* locus. J. Bacteriol. **171**:1974-1981.

103. **Salama, M., W. Sandine, and S. Giovannoni.** 1991. Development and application of oliognucleotide probes for identification of *Lactococcus lactis* subsp. *cremoris*. Appl. Environ. Microbiol. **57**:1313-1318.

104. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A laboratory manual, 2nd ed. , Cold Spring Harbor, NY.

105. **Sanders, M. E., P. J. Leonhard, W. D. Sing, and T. R. Klaenhammer.** 1986. Conjugal strategy for construction of fast acid-producing, bacteriophage resistant lactic streptococci for use in dairy fermentations. Appl. Environ. Microbiol. **52**:1001-1007.

106. **Sandine, W. E.** 1979. Lactic starter culture technology. Pfizer Inc, New York.

107. **Sandine, W. E.** 1985. The Streptococci: Milk products, p. 6-19. *In* S. Gilliland (ed.), Bacterial starter cultures for foods. CRC Press, Boca Raton.

108. **Sandine, W. E., P. C. Radich, and P. R. Elliker.** 1972. Ecology of the lactic streptococci. A review. J. Milk and Food Tech. **35**(3):176-185.

109. **Schagger, H., and G. von Jagow.** 1987. Tricine-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis for the Separation of Proteins in the Range from 1 to 100 kDa. Anal. Biochem. **166**:368-379.

109a. **Schellhaass, S. M., and H. A. Morris.** 1985. Rheological and scanning electron microscopic examination of skim milk gels obtained by fermenting with ropy and non-ropy strains of lactic acid bacteria. Food Microstruc. **4**:279-287

110. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

111. **Sinskey, A., S. Jamas, D. Easson, Jr., and C. Rha.** 1986. Biopolymers and modified polysaccharides, p. 73-114. *In* S. K. Harlander and T. P. Labuza (ed.), Biotechnology in Food Processing. Nova, New Jersey.

112. **Sledjeski, D., and S. Gottesman.** 1995. A small RNA acts as an antisilencer of the H-NS-silenced *rcsA* gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA. **92**:2003-2007.

113. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophorsis. J. Mol. Biol. **98**:503.

114. **Stingele, F., J. Nesser, and B. Mollet.** 1996. Identification and characterization of the *eps* (exopolysaccharide) gene cluster from *Streptococcus thermophilus* Sfi6. J. Bacteriol. **178**:1680-1690.

115. **Stout, V., and S. Gottesman.** 1990. RcsB and RcsC: A Two-component regulator of capsule synthesis in *Escherichia coli*. J. Bacteriol. **172**:659-669.

116. **Stout, V., A. Torres-Cabassa, M. R. Maurizi, D. Gutnick, and S. Gottesman.** 1991. RcsA, an unstable positive regulator of capsular polysaccharide synthesis. J. Bacteriol. **173**:1738-1747.

117. **Sutcliffe, J. G.** 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Springs Harbor Symp Quant. Biol. **43**:77.

118. **Sutherland, I. W.** 1972. Bacterial exopolysaccharides. Adv. Microbial. Physiol. **8**:143-212.

119. **Sutherland, I. W.** 1982. Biosynthesis of microbial exopolysaccharides. Adv. Microb. Physiol. **23**:79-150.

120. **Swamy, K. H. S., and A. L. Goldberg.** 1981. *E. coli* contains eight soluble proteolytic activities, one being ATP-dependent. Nature. **292**:652-654.

121. **Szewczyk, B., and L. M. Kozloff.** 1985. A method for the efficient blotting of strongly basic proteins from sodium dodecyl sulfate-polyacrylamide gels to nitrocellulose. Anal. Biochem. **150**:403-407.

122. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29**:807-813.

123. **Teuber, M., and A. Geis.** 1981. The Family Streptococcaceae (Nonmedical Aspects), p. 1614-1630. *In* M. P. Starr, Stolp, H., Trueper, H. G., Balows, A., Schlegel, H. G. (ed.), The Prokaryotes, vol. 2. Springer-Verlag, Berlin.

124. **Toba, T., T. Kotani, and S. Adachi.** 1991. Capsular polysaccharide of a slime-forming *Lactococcus lactis* ssp. *cremoris* LAPT 3001 isolated from Swedish fermented milk "langfil". Int. J. Food Microbiol. **12**:167-172.

125. **Toba, T., H. Nakajima, A. Tobitani, and S. Adachi.** 1990. Scanning electron microscopic and texture studies on characteristic consistency of Nordic ropy sour milk. Int. J. Food Microbiol. **11**:313-320.

126. **Tojo, N., S. Inouye, and T. Komano.** 1993. Cloning and nucleotide sequence of the *Myxococcus xanthus lon* gene: indispensability of *lon* for vegetative growth. J. Bacteriol. **175**(8):2271-2277.

127. **Torres-Cabassa, A. S., S. Gottesman, R. D. Frederick, P. J. Dolph, and D. L. Coplin.** 1987. Control of extracellular polysaccharide biosynthesis in *Erwinia stewartii* and *Escherichia coli* K12. J. Bacteriol. **160**:184-191.

128. **Torres-Cabassa, A. S., and S. Gottesman.** 1987. Capsule synthesis in *Escherichia coli* K-12 is regulated by proteolysis. J. Bacteriol. **169**(3):981-989.

129. **Tovey, E. R., S. A. Ford, and B. A. Baldo.** 1989. Enhanced immunodetection of blotted house dust mite protein allergen on nitrocellulose following blocking with Tween 20. Electrophoresis. **10**:243-249.

130. **Towbin, J., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA. **76**:4350-4354.

131. **Trisler, P., and S. Gottesman.** 1984. *lon* transcriptional regulation of genes necessary for capsular polysaccharide synthesis in *Escherichia coli* K-12. J. Bacteriol. **160**:184-191.

132. **Troy, F. A. I.** 1979. The chemistry and biosynthesis of selected bacterial capsular polymers. Ann. Rev. Microbiol. **33**:519-560.

133. **van de Guchte, M., J. Kok, and G. Venema.** 1992. Gene expression in *Lactococcus lactis.* FEMS Microbiol. Rev. **88**:73-92.

134. **Vandevivere, P., and D. L. Kirchman.** 1993. Attachment stimulates exopolysaccharide synthesis by a bacterium. Appl. and Environ. Microbiol. **59**:3280-3286.

135. **Vedamuthu, E. R., and J. M. Neville.** 1986. Involvement of a plasmid in production of ropiness (mucoidness) in milk cultures by *Streptococcus cremoris* MS. Appl. Microbiol. **51**(4):677-682.

136. **Vedamuthu, E. R., W. E. Sandine, and P. R. Elliker.** 1966. Flavor and texture in cheddar cheese. 1. Role of mixed strain lactic starter cultures. J. Dairy Sci. **49**:144-150.

137. **Vedamuthu, E. R., W. E. Sandine, and P. R. Elliker.** 1966. Flavor and texture in cheddar cheese. II. Carbonyl compounds produced by mixed strain lactic starter cultures. J. Dairy Sci. **49**:151-157.

138. **Vescovo, M., G. L. Scolari, and V. Bottazzi.** 1989. Plasmid-Encoded Ropiness Production in *Lactobacillus casei* ssp. *casei.* Biotechnology Letters. **II**(10):709-712.

139. **Von Bodman, S. B., and S. K. Farrand.** 1995. Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an N-acylhomoserine lactone autoinducer. J. Bacteriol. **177**:5000-5008.

140. **von Wright, A., and S. Tynkkynen.** 1987. Construction of *Streptococcus lactis* subsp. *lactis* strains with a single plasmid associated with mucoid phenotype. Appl Environ Microbiol. **53**(6):1385-1386.

141. **von Wright, A., and M. Sibakov.** 1993. Genetic modification of lactic acid bacteria. *In* S. Salminen and A. vonWright (ed.), Lactic acid bacteria. Marcel Dekker, Inc, New York.

142. **Wacher-Rodarte, C., M. Galvan, A. Farres, F. Gallardo, V. Marshall, and M. Garcia-Garibay.** 1993. Yogurt production from reconstituted skim milk powders using different polymer and non-polymer forming starter cultures. J. Dairy Res. **60**:247-254.

143. **Whitaker, R. D., and C. A. Batt.** 1991. Characterization of the Heat Shock Response in *Lactoccus lactis* subsp. *lactis*. Appl. Microbiol. **57**:1408-1412.

143a. **Willrett, D. L.** 1982. Development of internally pH-controlled bulk starter media for the propagation of lactic acid bacteria. Ph.D. dissertation. Oregon State University Department of Microbiology.

144. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**:221-271.

145. **Yamamoto, Y., S. Murosaki, R. Yamauchi, K. Kato, and Y. Sone.** 1994. Structural study on an exopolysaccharide produced by *Lactobacillus helveticus* TY1-2. Carbo. Res. **261**:67-78.

146. **Zeigler, D. R., and D. H. Dean.** 1989. Bacillus Genetic Stock Center strains and data, 4th ed. .

147. **Zinkewich, K., and J. M. Fraser.** 1988. New locus for exopolysaccharide overproduction in *Escherichia coli* K-12. J. Bacteriol. **170**:1405-1407.

APPENDIX

BIOGRAPHICAL INFORMATION

Karen Patricia Dierksen (maiden name Smith) was born October 14, 1954 at Edwards Air Force Base in Kern, California to Joe R and Anne Frances Smith. She was raised in Japan, New Hampshire, and Texas. After graduation from high school, she moved to Merced, California where she earned an A.S. in Biology at Merced Community College. She was employed by the State of California as a Communications Operator for a number of years prior to her return to college to pursue her interest in the Biological Sciences. She earned a B. A. in Biology with a concentration in Microbiology from California State University, Stanislaus June 1991. Fall term, 1991, she began graduate training in bacterial genetics in the laboratory of Dr. Janine E. Trempy at Oregon State University (Corvallis). She was a recipient of a Tartar award for research in the summer of 1992. She was a predoctoral fellow of the Eckelman Foundation from Fall 1992 through Spring 1996.

PUBLICATIONS:

Dierksen, K. P., J. Marks, D. D. Chen, and J. E. Trempy. 1994. Evidence for structural conservation of Lon and RcsA. J. Bacteriol. **176** (16) : 5126-5130.

Dierksen, K. P., W. Ebel, J. Marks, W. E. Sandine, and J. E. Trempy. 1995. Polysaccharide expression in *Lactococci*, p. 469-480. *In* J. J. Ferretti, M. S. Gilmore, T. R. Klaenhammer, and F. Brown (ed.), Genetics of *Streptococci, Enterococci* and *Lactococci*, vol. 85. Karger, Basel.

Dierksen, K. P., and J. E. Trempy. 1996. Identification of a second RcsA protein, a positive regulator of colanic acid capsular polysaccharide genes, in *Escherichia coli*. In press J. Bacteriol.

Dierksen, Karen P., William E. Sandine, and Janine E. Trempy. 1996. Expression of Ropy and Mucoid Phenotypes in *Lactococcus lactis*. Submitted to J. Dairy Sci.

ABSTRACTS:

Capsule synthesis regulators in lactic acid bacteria. K.P. Dierksen, J. Marks, W.E. Sandine and J.E. Trempy. Presented at American Dairy Science Association Annual Meeting at the University of Maryland, College Park. June 13 - 16, 1993.

Capsule synthesis regulators in lactic acid bacteria. K.P. Dierksen, J. Marks, W.E. Sandine and J.E. Trempy. Presented at Center for Gene Research and Biotechnology Annual Retreat, Newport, Oregon. October 8 - 9, 1993.

Polysaccharide synthesis regulators in *Lactococcus lactis*. K.P. Dierksen, M.J. Nather, W. Ebel, W.E. Sandine and J.E. Trempy. Presented at American Society for Microbiology General Meeting at Las Vegas Convention Center. May 23 - 27, 1994.

Identification of two immunological forms of RcsA. K.P. Dierksen and J.E. Trempy. Presented at American Society for Microbiology General Meeting in Washington, D.C. May 21 - 25, 1995.

A *Bacillus subtilis* gene product suppresses polysaccharide synthesis and sensitivity to DNA damaging agents in *Escherichia coli* Δ*lon* mutant cells. W. Ebel, K.P. Dierksen and J.E. Trempy. Presented by W. Ebel at American Society for Microbiology General Meeting at Las Vegas Convention Center. May 23 - 27, 1994

Bacillus subtilis ORF5 gene product functions in a nonproteolytic manner to restore a *lon*$^+$ phenotype to *Escherichia coli* Δ*lon* cells. R. Yarwood, W. Ebel, K.P. Dierksen and J.E. Trempy. Presented by R. Yarwood at American Society for Microbiology General Meeting at Las Vegas Convention Center. May 23 - 27, 1994.

Polysaccharide Synthesis in *Lactococci*. K.P.Dierksen, W.Ebel and J.E.Trempy. Presented by J. E. Trempy at the Fourth International ASM Conference on Streptococcal Genetics. Santa Fe, New Mexico. May 15 - 18, 1994.