Expression of Ropy and Mucoid Phenotypes in *Lactococcus lactis*

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ABSTRACT

Strains of *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* were cultured under aerobic and anaerobic conditions on plates of whey agar, Elliker agar, and M17L agar at 15, 20, and 30°C to determine the environmental conditions required for the expression of the ropy phenotype. Two strains, *L. lactis* ssp. *cremoris* Ropy 352 and *L. lactis* ssp. *cremoris* Hollandicus, exhibited two distinct polysaccharide phenotypes, ropy and mucoid. Expression of these phenotypes could be induced individually or simultaneously. The inducible nature of this response suggests that genetic regulators were present. Western blots were used to determine whether or not Lon protease and RcsA, two regulators of polysaccharide expression in *Escherichia coli*, were present in lactococci. Lon, a negative regulator, and RcsA, an unstable positive regulator, have been shown at the structural level to be conserved in a number of Gram-negative and Gram-positive microorganisms. The present study found evidence for structural conservation of Lon protease in lactococci. Less of the Lon-like protein was observed in the ropy strains than in the nonropy strains.
(**Key words**: ropy phenotype, mucoid phenotype, *Lactococcus lactis*, Lon protease)

Abbreviation key: **EPS** = exopolysaccharide, **LAB** = lactic acid bacteria, **TBST** = Tris-buffered saline plus 1% Tween 20.

INTRODUCTION

Lactic acid bacteria (**LAB**) have been used for centuries as starter cultures for fermented milk products. Strains with desirable characteristics, such as rapid production of acid, flavor, or aroma, have been selected for in the fermentation of specific products (42). A great amount of interest exists in identifying and developing strains that have improved or novel characteristics to enhance current products or in new applications. One characteristic that has been exploited in Scandinavian countries, but not in large-scale commercial applications elsewhere, is the synthesis of a ropy exopolysaccharide (**EPS**) by mesophilic LAB. The ropy polysaccharide that is produced during fermentation imparts desirable textural and rheological properties to Scandinavian milk products such as viili and langmjolk (31). Fermented milk products that are produced using ropy LAB have smooth body, high viscosity, and less syneresis than do products made using nonropy strains (58). Although ropy mesophilic strains could be used to produce smooth, fermented milk products with high viscosity without the need for thickeners or stabilizers, those strains have not been used extensively in commercial production because the phenotype is easily lost. Growth at high temperature or repeated serial transfer of a culture frequently has led to loss of the phenotype (10), and, in a number of strains, loss of a single plasmid has been associated with loss of the phenotype (35, 54, 55, 57). Other environmental influences also have been thought to affect the phenotype.

One approach in developing stable EPS-producing strains for use in large-scale fermentations would be to identify genetic regulators of EPS that have been shown to be conserved in other organisms and are amenable to manipulation for constitutive expression of the desired phenotype. Two such regulators of colanic acid capsular polysaccharide in *Escherichia coli*, Lon protease and RcsA, are conserved in a number of organisms. RcsA functions as a positive regulator of the *cps* (capsular polysaccharide synthesis) genes, and Lon negatively regulates *cps* expression, presumably by degrading RcsA. The stability of RcsA is enhanced in cells lacking Lon protease activity (e.g., cells contain a mutant *lon* gene), and, thus, the cells are very mucoid.

Lon protease has been identified in *Salmonella typhimurium* (17), and structural evidence suggests that Lon is present in a number of other Gram-negative microorganisms, including *Proteus vulgaris, Pseudomonas aeruginosa, Vibrio anginolyticus, Xan-*

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thomonas campestris, Alcaligenes viscolactis, Rhizobium spp., *Agrobacterium radiobacter* (15), *Erwinia amylovora* (18), *Myxococcus xanthus* (22, 36, 37), and *Caulobacter crescentus* (39). Structural evidence also suggests that Lon is present in Gram-positive microorganisms, including *Bacillus brevis* (26), *Bacillus subtilis* (4, 15, 40), *Bacillus stearothermophilus* (15), and *Streptococcus salivarius* (15). Lon has also been identified in yeasts (45) and in all human tissue types tested (1, 59), strongly suggesting its ubiquitous nature.

RcsA has been identified in *Klebsiella aerogenes* (2), *Erwinia* spp. (14, 38), and *Salmonella typhi* (56). Structural evidence suggests that RcsA is also present in *P. vulgaris, Ps. aeruginosa, V. anginolyticus, X. campestris, Al. viscolactis, Rhizobium* spp., *Ag. radiobacter*, and *S. salivarius* (15). Mutations in the *lon* gene or deletion of the *lon* gene in *E. coli* (23), *Erwinia* spp. (18), or in *S. typhimurium* (17) increased expression of EPS. Complementation of an *E. coli* or *S. typhimurium rcsA* mutant with the *rcsA* gene from *Er. amylovora* restores EPS expression (51). These observations suggest that a common regulatory pathway for polysaccharide expression exists in these organisms. Conservation of Lon, RcsA, or both in lactococci would suggest that this common regulatory pathway exists in Gram-positive organisms as well.

Recently, Stingele et al. (43) reported identification of an *eps* (exopolysaccharide) gene cluster from *Streptococcus thermophilus* Sfi6 that, when expressed from a multicopy plasmid, is sufficient for expression of EPS in the non-EPS-producing heterologous host, *Lactococcus lactis* MG1363. Stingele et al. (43) found that the deduced amino acid sequences from the 13 genes in this cluster shared homology with gene products involved in synthesis of capsules or lipopolysaccharides from both Gram-positive and Gram-negative microorganisms. Their observations of conserved structural genes lent support to our hypothesis that conserved regulatory elements may be present in lactococci. Identification of such regulators may lead to the construction of stable EPS-producing strains. In the present study, Western blots were used to determine whether or not conservation of Lon and RcsA extends to the lactococci.

A second component of our study was to determine the environmental factors that were involved in EPS expression. We evaluated strains of *Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* under aerobic and anaerobic conditions at selected growth temperatures on common lactococcal media. The present study shows that strains of *L. lactis* express two distinct polysaccharide phenotypes, ropy and mucoid. Expression of one or both phenotypes is induced under defined environmental conditions, and the inducible nature of the phenotypes suggests the presence of genetic regulators.

TABLE 1. Bacterial strains used in the present study.

Species	Strain	Source
Lactococcus lactis ssp. *cremoris*	Hollandicus	OSU[1]
	Ropy 352	OSU
	LAPT 3001	OSU
	KTR II	OSU
Lactococcus lactis ssp. *lactis*	Strain B	OSU
	MG1363	(20)
Escherichia coli[2]	JT4000 Δ*lon*	(15)
	SG20250 *lon*$^+$	(50)

[1]OSU: Oregon State University Department of Microbiology Strain Collection.

[2]Strains derived from MC4100 ($\Delta lac U_{169}$ *ara*D *flb*B *rel*).

MATERIALS AND METHODS

Bacterial Strains and Media

The bacterial strains that were used in this study are presented in Table 1. Stock cultures of lactococci were maintained at –80°C in 11% reconstituted NDM containing 20% glycerol. Frozen stock cultures were streaked on whey agar (54) or M17-lactose (47) plates and incubated 24 h at 30°C; pure colonies were transferred to M17-lactose or M17-glucose broth. The M17 cultures were incubated under static conditions for 24 h at 30°C and used for subsequent inoculations and dilutions. *Escherichia coli* cultures were grown in Luria broth (41) at 32°C. Lactococcal cultures were assayed on Elliker (6, 19), M17, and whey agar plates.

Culture Conditions

Cultures that were grown in M17 broth were serially diluted using 0.1% (wt/vol) peptone water. Duplicate samples of dilutions yielding 25 to 250 colonies per plate were counted. Lactococcal strains were plated on M17-lactose agar, whey agar, and Elliker agar plates. Anaerobic incubations were carried out in BBL anaerobic jars using a BBL Gas Pak (Baltimore Biological Laboratory, Baltimore, MD) anaerobic system. Plates were incubated for 6 d at 15 and 20°C and for 3 d at 30°C. Duplicate plates containing 25 to 250 colonies were scored for mucoid properties (scale of ++++ = excess EPS to – = no visible mucoid). 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 (55).

Western Blot Conditions

Sterile 0.01 *M* $MgSO_4$ was added to whey agar plates containing lactococci or to Luria-Bertani agar plates containing *E. coli* to loosen colonies, which were harvested by scraping with a sterile glass rod. Cells were pelleted, washed twice in 0.01 *M* $MgSO_4$, and resuspended in Tris-EDTA-Laemmli buffer (30) containing the following protease inhibitors: N-tosyl-*L*-phenylalanine chloromethyl ketone (0.1 m*M*), Na-*p*-tosyl-*l*-lysine chloromethyl ketone (0.1 m*M*), phenylmethane sulfonylfluoride (1 m*M*), and aprotinin (0.03 m*M*) (25).

Cells were lysed by bead-beating following the instructions of the manufacturer (Biospec Products minibeadbeater, Bartlesville, OK). The supernatant was recovered, and an equal volume of SDS gel loading buffer was added (41). The samples were boiled for 10 min and then stored at –80°C. Forty micrograms (determined by bicinchoninic protein assay using a BCA kit; Pierce Co., Rockford, IL) of cellular protein were loaded per well and separated on an 8% (Lon blots) or 14% SDS-PAGE gel (RcsA blots). Samples were electroblotted to nitrocellulose membrane in Towbin transfer buffer (53) (Lon blots) or to polyvinylidene difluoride membrane (DuPont, Wilmington, DE) in CAPS [3-(cyclohexylamine)1-propanesulfonic acid] transfer buffer (RcsA blots) (46). The membranes were blocked with Tris-buffered saline plus 1% Tween 20 (**TBST**) containing 5% (wt/vol) NDM (52), incubated with a 1:2000 (vol/vol) dilution of *E. coli* Lon antiserum or 1:3000 (vol/vol) dilution of RcsA antiserum, washed in TBST-NDM, incubated with a 1:3000 (vol/vol) dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (HyClone, Logan, UT), and washed in TBST. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL) and autoradiography film (Hyperfilm-ECL, Amersham).

RESULTS

Expression of Two Distinct Polysaccharide Phenotypes

The descriptive terms ropy, mucoid, and slime-producing have been used interchangeably in reports to describe viscous isolates of LAB (10). 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 (55), and, in sensory evaluations, ropy cultures have been shown to produce fermented milk products that have smooth body with higher viscosity and lower susceptibility to syneresis than those produced by nonropy strains (10, 28, 58). This study was undertaken to determine the culture conditions necessary for expression of the ropy phenotype. Lactococcal strains that were originally isolated from fermented milk cultures as ropy variants were grown under a set of defined environmental conditions to determine whether or not environmental conditions affected expression of the ropy phenotype. A surprising result was the observation of two distinct phenotypes, a ropy phenotype and a mucoid phenotype (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. lactis* ssp. *cremoris* LAPT, *L. lactis* ssp. *lactis* strain B, and *L. lactis* ssp. *cremoris* KTRII—did not exhibit the ropy phenotype under any of the conditions selected (data not shown) and were no longer ropy in milk. These strains were scored for expression of the mucoid phenotype. Two strains, *L. lactis* ssp. *cremoris* Hollandicus and *L. lactis* ssp. *cremoris* Ropy 352, consistently exhibited the ropy phenotype under some of the defined conditions (Table 2). All Hollandicus and Ropy 352 colonies exhibited the ropy phenotype when grown on whey agar plates at 30°C under aerobic conditions; 83 and 100%, respectively, were ropy at 20°C under aerobic conditions; and 94 and 100%, respectively, were ropy at 15°C under aerobic conditions. 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, a higher percentage of ropy colonies were observed on Elliker agar plates under anaerobic conditions than under aerobic conditions; all Hollandicus and Ropy 352 colonies were ropy at 15°C, 23% of Hollandicus were ropy at 20°C, and 73 and 91%, respectively, were ropy at 30°C. Further, on M17-lactose agar plates, under aerobic conditions, a small percentage of Hollandicus colonies exhibited the ropy phenotype at 15 and 20°C (9 and 2%, respectively); under the same conditions, however, Ropy 352 did not exhibit the ropy phenotype. Under anaerobic conditions, neither strain was ropy when cultured on M17-lactose agar plates.

TABLE 2. Influence of culture medium, temperature, and atmosphere on the expression of ropy and mucoid phenotypes in *Lactococcus lactis* ssp. *cremoris*.

		Whey agar		Elliker agar		M17-Lactose agar	
Condition	Strain[1]	Ropy[2]	Mucoid[3]	Ropy	Mucoid	Ropy	Mucoid
		(%)		(%)		(%)	
Aerobic							
15°C	Holl	94	–	57	–	9	++
	Ropy	100	–	14	–	0	++
20°C	Holl	83	–	21	–	2	++
	Ropy	100	+	0	–	0	++
30°C	Holl	100	–	0	+	0	++
	Ropy	100	++	0	+	0	++
Anaerobic							
15°C	Holl	0	+	100	–	0	++
	Ropy	6	+	100	–	0	++
20°C	Holl	0	+	23	–	0	++
	Ropy	0	–	0	–	0	++
30°C	Holl	29	–	73	–	0	++
	Ropy	0.7	++	91	–	0	++

[1]Full strain designations: Holl = *Lactococcus lactis* ssp. *cremoris* strain Hollandicus, and Ropy = *Lactococcus lactis* ssp. *cremoris* strain Ropy 352.

[2]Plates at 15 and 20°C were incubated for 6 d; plates at 30°C were incubated for 3 d. 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.

[3]Plates at 15 and 20°C were incubated for 6 d; plates at 30°C were incubated for 3 d. Plates with 25 to 200 colonies were evaluated. Colonies were scored visually for mucoidy and rated on the following scale: – = no observable capsule; + = mucoid to ++++ = copious capsular material visible.

In the course of this study, some colonies were observed with a second EPS phenotype that was distinct from the ropy phenotype. 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 the mucoid phenotype, was observed under certain environmental conditions in the two ropy strains (Hollandicus and Ropy 352), as well as in the three nonropy strains (*L. lactis* ssp. *cremoris* LAPT 3001 and KTR II and *L. lactis* ssp. *lactis* strain B). When the cultures were grown on M17-lactose agar plates, all five strains exhibited the mucoid phenotype under the temperature and oxygen conditions of our study (Tables 2 and 3). On whey agar plates, the mucoid phenotype was expressed primarily under anaerobic conditions at 15 and 20°C. On Elliker plates, the mucoid phenotype was expressed by all five strains at 30°C under aerobic conditions, by strain B and KTRII at 15°C under anaerobic conditions, and strain B at 20°C under anaerobic conditions (Table 3).

When greater than 10% of Hollandicus colonies were ropy, the mucoid phenotype was not expressed, regardless of media, temperature, or atmospheric conditions. In contrast, the Ropy 352 strain exhibited both phenotypes at 20 and 30°C when plated on whey agar plates under aerobic conditions. Neither phenotype was exhibited by Ropy 352 at 20°C under anaerobic conditions on whey agar plates and 20°C under aerobic or anaerobic conditions on Elliker plates (Table 2).

Structural Evidence for Conservation of Lon Protease

A number of regulators of polysaccharide synthesis have been identified in *E. coli*. Two of these regulators, Lon, a negative regulator, and RcsA, an unstable positive regulator, have been shown to be conserved at the structural level in a number of Gram-negative and Gram-positive microorganisms, especially Lon, which appears to be present in all living organisms. In this study, a Western blot approach was used to probe lactococcal strains with a polyclonal antiserum that was specific to *E. coli* Lon and RcsA proteins. In the five lactococcal strains probed, a protein was observed that was immunoreactive to *E. coli* Lon antiserum and that migrated at the reported SDS-PAGE molecular mass of *E. coli* Lon (94 kDa) (Figure 1, lane 1). The intensity of the signal for the protein band was lower in the two ropy strains, *L. lactis* ssp. *cremoris* Ropy 352 (lane 3) and *L. lactis* ssp. *cremoris* Hollandicus (lane 4), than in the nonropy strains, *L.*

TABLE 3. Influence of culture medium, temperature, and atmosphere on the expression of the mucoid phenotype in *Lactococcus lactis* ssp. *cremoris* and ssp. *lactis*.

Condition[1]	Strain[2]	Whey agar	Elliker agar	M17-lactose agar
Aerobic				
15°C	LAPT 3001	–	–	++
	Strain B	–	–	++
	KTR II	–	–	++
20°C	LAPT 3001	–	–	++
	Strain B	–	–	++
	KTR II	–	–	++
30°C	LAPT 3001	–	+	++
	Strain B	–	+	++
	KTR II	–	+	++
Anaerobic				
15°C	LAPT 3001	++	–	++
	Strain B	+	+	++
	KTR II	+	+	++
20°C	LAPT 3001	+	–	++
	Strain B	+	+	++
	KTR II	–	–	++
30°C	LAPT 3001	–	–	++
	Strain B	–	–	++
	KTR II	–	–	++

[1]Plates at 15 and 20°C were incubated for 6 d; plates at 30°C were incubated for 3 d. Plates with 25 to 200 colonies were evaluated. Colonies were scored visually for mucoidy and rated on the following scale: – = no observable capsule; + = mucoid to ++++ = copious capsular material visible.

[2]Full strain designations: LAPT 3001 = *Lactococcus lactis* ssp. *cremoris* strain LAPT 3001, strain B = *Lactococcus lactis* ssp. *lactis* strain B, and KTR II = *Lactococcus lactis* ssp. *cremoris* strain KTRII.

lactis ssp. *cremoris* KTRII (lane 5) and *L. lactis* ssp. *lactis* St. B (lane 6), or the *lac*$^-$, plasmid-free laboratory strain, *L. lactis* ssp. *lactis* MG1363 (lane 7). The Lon protein band was absent in the Δ*lon E. coli* strain (lane 2). An immunoreactive protein migrating at a molecular mass of 86 kDa was observed in the *E. coli* *lon*$^+$ strain and in the lactococcal strains. The intensity of the signal for this protein band was also lower in the two ropy strains than in the nonropy strains and the laboratory strain. The differences in signal intensity for the 94- and 86-kDa proteins in the ropy strains were not due to unequal protein loading; each lane contained 40 μg of 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 intensity in all five lactococcal strains.

A similar Western blot approach was used to probe lactococcal strains with polyclonal antiserum that was 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).

DISCUSSION

Many organisms—from archaebacteria, to algae, to dairy bacteria—produce a variety of EPS with high molecular mass. Many of these EPS have unique properties of commercial usefulness for medical, environmental, and industrial applications. In recent years, interest has grown in the EPS that are produced by LAB because these EPS contribute to the textural and rheological properties of milk products. These EPS may represent alternatives to food additives for other applications as well. In this study, we determined the environmental conditions that were required for production of two viscous phenotypes in isolates of *L. lactis* ssp. *lactis* and ssp. *cremoris*. Analysis revealed the presence of two distinct EPS phenotypes, ropy and mucoid; expression of both phenotypes were affected by environmental conditions. The present study demonstrated that mucoid and ropy represent different and distinct phenotypes. The ropy bacteria (those that confer the desirable properties in milk) are not necessarily mucoid or slime-producing. The expression of both phenotypes

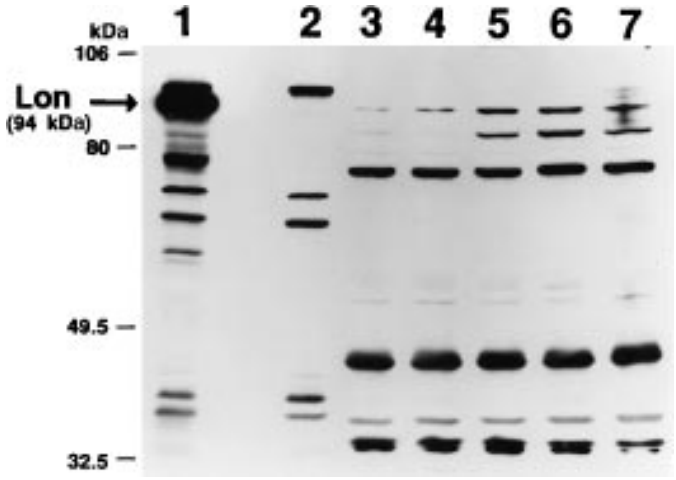

Figure 1. Western blot analysis of *Lactococcus lactis* ssp. using *Escherichia coli* Lon antiserum. Equal amounts of protein from whole cell extracts were boiled in sample buffer, fractionated on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and analyzed by Western blotting with preabsorbed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

simultaneously by Ropy 352 suggests that more than one pathway may exist for polysaccharide synthesis. The expression of one or the other phenotype under defined conditions suggests that the signals for expression of each polysaccharide may differ as well. The ability to produce two different polysaccharide phenotypes has not been previously documented in lactococci, but has been noted for *Rhizobium* sp. (34), *Lactobacillus casei* CG11 (11), *E. coli* K1 and K5 (27), *Alcaligenes faecalis* (44), and *Zoogloea* spp. (29).

Examination of the environmental data did not reveal the predominance of one particular factor (temperature, media composition, or atmosphere) in induction of the ropy phenotype. On whey agar plates, both *L. lactis* ssp. *cremoris* Hollandicus and *L. lactis* ssp. *cremoris* Ropy 352 were ropy under aerobic conditions, and temperatures in the range of 15 to 30°C did not affect expression. On whey agar plates under anaerobic conditions, however, 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; the expression of the ropy phenotype was limited or nonexistent under aerobic conditions. The effect of aeration has been to cause a notable difference in optimal production of EPS in different organisms. For some, high aeration is required; in others, low aeration or anaerobic conditions are required (44). However, surprisingly, *L. lactis* ssp. *cremoris* Ropy 352 and Hollandicus were ropy under aerobic conditions on whey but were ropy under anaerobic conditions on Elliker agar. Although whey and Elliker agars differ in composition, no one component stands out as an obvious factor to account for the different expression of the ropy phenotype under aerobic or anaerobic conditions.

Gassem et al. (20) reported greater polysaccharide production by ropy yogurt cultures in simulated whey permeate broth, an unbuffered media, than in Elliker agar or MRS broth, both of which are buffered. In our study, all three media are buffered, and no conclusions can be drawn for the effect of buffering on the expression of the ropy phenotype. Previous studies (10, 20) have also shown that ropy strains are not easily categorized and that the viscous trait has not been linked to one specific characteristic, such as carbon source, proteolytic ability, acid production, or growth rate.

Media composition was a significant factor for the induction of the mucoid phenotype when strains were cultured on M17-lactose agar plates. The two ropy strains and the three nonropy strains that were cultured on M17-lactose plates exhibited the mucoid phenotype under all of the conditions selected, which suggests that M17-lactose could be used as a selective medium in mutagenesis experiments designed to differentiate between genes involved in the expression of the mucoid phenotype and those involved in expression of both phenotypes. Mutants of ropy strains, which are no longer mucoid on M17-lactose, could be assayed on whey agar plates for retention or loss of the ropy phenotype. No generalizations could be made with respect to conditions that are important for the expression of the mucoid phenotype on Elliker or whey agar plates; expression did not follow any observable pattern.

Lactococcus lactis ssp. *cremoris* Ropy 352 and Hollandicus, the two strains exhibiting a ropy phenotype on solid media, also produced a viscous, fermented milk with smooth body; the three nonropy strains produced a less viscous fermented milk (our unpublished observation). The correlation between a ropy plate phenotype and production of a viscous fermented milk suggested that new ropy lactococcal strains could be isolated if cultures were plated on whey agar under aerobic conditions or on Elliker agar under anaerobic conditions and then scored for string formation. Ruthenium red-milk agar plates, which have been used successfully in other laboratories to differentiate ropy strains of *Strep. thermophilus* from nonropy strains (43), varied in the ability to differentiate the ropy lactococcal strains from the nonropy strains used in this study (E. Knoshaug, K. P. Dierksen, and J. E. Trempy, 1996, unpublished data). In their studies of colonial variants of *Lactobacillus delbrueckii* ssp. *bulgaricus* CNRZ 1187, Bouzar et al. (9) reported that ruthenium red agar plates gave inconsistent results. Because the ruthenium red staining method was identified for a ropy *S. thermophilus* strain, Bouzar et al. (9) suggested that the EPS may have been different from the EPS produced by their strain and that the assay might only be valid for specific EPS. Further studies are in progress to sort out the discrepancies in these observations (E. Knoshaug and J. E. Trempy, 1996, unpublished observations).

The ability of Ropy 352 and Hollandicus to induce or repress one or both viscous phenotypes suggests that EPS production is not constitutive and that regulation is occurring. Many organisms produce EPS, and the biosynthetic genes in many cases are conserved. Although EPS structures vary, many contain the same sugars and are synthesized and regulated similarly (44). Lactococcal strains have been isolated

that produce a ropy polysaccharide. Structural studies of EPS from these strains reveal no common features to correlate structure to rheological behavior (24, 32, 33, 48). Thus, regulators rather than structural genes would likely 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 that are 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 *E. coli lon* gene, when expressed in a *S. typhimurium lon* mutant, suppressed the pleiotrophic phenotypes that were associated with the *lon* mutation: increased expression of colanic acid capsular polysaccharide, sensitivity to DNA damaging agents, and a decreased ability to degrade abnormal proteins (17). The *rcsA* gene from both *Erwinia stewartii* (49) and *K. aerogenes* (2) activated expression of colanic acid capsular polysaccharide in *E. coli*, although the polysaccharides that were regulated by RcsA in *Erwinia* and *Klebsiella* spp. were structurally distinct from colanic acid. The *rcsA* gene from *Er. amylovora* activated capsular polysaccharide synthesis in other *Erwinia* spp., in *E. coli*, and in *S. typhimurium* (7, 12).

In an earlier study (16), laboratory and commercial strains of lactococci were probed for DNA sequences that were homologous to the DNA sequences comprising the *E. coli lon* and *rcsA* genes. No homologous DNA sequences were detected. However, our DNA probes did not hybridize to DNA sequences in other Gram-positive organisms, including *B. subtilis*, an organism for which the *lon* gene sequence is known and in which the deduced amino acid sequence shares significant homology to the *E. coli* Lon protein sequence. Although our DNA probes did not hybridize, similar amino acid epitopes might be present that would react with *E. coli* polyclonal antiserum. Our laboratory and others have used *E. coli* antiserum successfully to detect immunoreactive proteins in Gram-positive microorganisms including lactococci. Proteins that were immunoreactive to *E. coli* Lon antiserum have been detected in *B. subtilis*, *B. stearothermophilus*, and *S. salivarius* (15). Under heat shock conditions, proteins that were immunoreactive to *E. coli* Lon antiserum have been detected in *B. subtilis* (4, 15). Antiserum against the *E. coli* heat shock proteins, GroEL, DnaK, DnaJ, and GrpE, have been used to identify structurally related proteins in lactococci (5, 8, 60). In this study, *E. coli* Lon and RcsA polyclonal antisera was used to probe for structural conservation of Lon and RcsA in *L. lactis*. A protein that was similar in size to *E. coli* Lon protease (94 kDa) and immunoreactive with *E. coli* Lon antiserum was detected in MG1363 and in all of the commercial strains of *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* that were probed.

Interestingly, Ropy 352 and Hollandicus, the two strains that produce a ropy polysaccharide, expressed less of the 94-kDa Lon-like immunoreactive protein than did the nonropy strains or the laboratory strain, MG1363. The ropy strains also expressed less of an 86-kDa protein that is immunoreactive with *E. coli* Lon antiserum. Although no evidence exists at this time to suggest that the 86-kDa protein is a Lon homolog, evidence exists for the presence of two *lon* genes in microorganisms. 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 (49), and *lonD* encodes a 90.4-kDa protein that is essential for multicellular differentiation (22, 49). Under heat shock conditions, a 120-kDa protein was seen in *B. subtilus* in addition to the 94-kDa protein observed under conditions of physiological growth (15). To date, only one *lon* gene has been identified in *B. subtilis*. However, it would not be surprising to identify a second *lon* gene in *B. subtilis* given that *B. subtilis*, a spore-forming organism, undergoes cellular differentiation and that deletion of the *lon* gene in *B. subtilis* (40) did not result in the pleiotrophic phenotypes found in *lon* mutant cells of *E. coli*, *S. typhimurium*, and *Er. amylovora*. Although identification of a *lon* gene has not been reported to date in *Pseudomonas aeruginosa*, 94- and 86-kDa proteins that were immunoreactive to *E. coli* Lon antiserum have been detected (15).

An alternate explanation for detection of additional immunoreactive proteins is that proteins in lactococci that share no functional relationship to Lon may contain one or more of the conserved domains identified in Lon and may react with antibodies derived from epitopes to these domains. These proposed domains include an ATP-binding domain, two basic regions proposed to be involved in DNA binding, one acidic region (13), and a highly conserved seven amino acid region bordering the serine residue implicated in catalytic activity (3). 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.

The reduced expression of the 94-kDa protein in the ropy strains suggests the potential for involvement of this *E. coli* Lon-like protein in expression of the ropy phenotype. In *E. coli*, mutations in *lon* or

deletion of the *lon* gene result in increased production of capsular polysaccharide. To test this hypothesis by examining the function of the Lon-like lactococcal protein, a λGT11 library has been constructed using chromosomal DNA from a lactococcal strain and screened with *E. coli* Lon antiserum for candidates expressing the Lon protein homolog or screened with a DNA probe that is specific to the *B. subtilis lon* gene sequence. Candidates are in the process of being characterized (H. K. Peters, W. Ebel, and J. E. Trempy, 1996, unpublished data). Further examination of mechanisms that regulate the expression of EPS, such as those governed by Lon, is currently in progress.

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