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Biochemistry and Application of Exopolysaccharide Production in Mozzarella Cheese Starter Cultures

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BIOCHEMISTRY AND APPLICATION OF EXOPOLYSACCHARIDE PRODUCTION IN MOZZARELLA CHEESE STARTER CULTURES

by

Brent Petersen

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ABSTRACT

Biochemistry and Application of Exopolysaccharide Production in Mozzarella Cheese Starter Cultures

by

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Utah State University, 2001

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This study sought to investigate the role of the C_{55} undecaprenol lipid carrier in the production of exopolysaccharide (**EPS**), the effect of exopolysaccharide producing (**EPS$^+$**) starter cultures on the viscosity of Mozzarella cheese whey, and the possible protective characteristics of capsular EPS against freezing and freeze drying. Efforts to investigate the role of the lipid carrier in EPS production employed pAM*bac*A, a plasmid that encodes an enterococcal lipid kinase that confers bacitracin resistance by increasing intracellular levels of undecaprenol phosphate lipid carrier. Unfortunately, this avenue of study was thwarted by the inability to demonstrate *bac*A expression in a model dairy lactic acid bacterium, *Lactococcus lactis*.

To study the effect of EPS$^+$ cultures on cheese whey, Mozzarella cheese was made with starters consisting of *Lactobacillus helveticus* (LH100) paired with one of four *Streptococcus thermophilus* strains. These strains included a capsular EPS producer (**Cps$^+$**) MR-1C; a non-exopolysaccharide producing negative

mutant (**EPS**$^-$) of MR-1C, DM10; a ropy EPS producer, MTC360; and a non-EPS producing industrial strain, TA061. Results showed that Mozzarella cheese made with a Cps$^+$ or ropy EPS$^+$ *S. thermophilus* strain had significantly higher moisture levels than cheese made with non-exopolysaccharide producing (EPS$^-$) streptococci. Melt properties were also better in cheeses with higher moisture. Viscosity measurements of unconcentrated and ultrafiltered (5-fold concentrated) whey showed that ultrafiltered whey from cheeses made with *S. thermophilus* MTC360 was significantly higher in viscosity than whey from cheeses made with MR-1C, TAO61, or DM10. There was no significant difference in the viscosity of unconcentrated or concentrated whey from cheese made with *S. thermophilus* MR-1C and cheese made with the commercial starter culture TAO61. The results indicated that non-ropy, encapsulated exopolysaccharide-producing *S. thermophilus* strains can be used to achieve higher cheese moisture levels and to improve the melt properties of Mozzarella cheese without significantly increasing cheese whey viscosity.

Finally, *S. thermophilus* MR-1C and DM10 were subjected to freezing and freeze drying to test for possible protective effects of the capsular exopolysaccharide. Analysis of variance of cell counts taken before and after freezing or freeze drying cycles revealed there was no significant difference between the viability of these strains.

(65 pages)

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Brent Petersen

CONTENTS

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Many strains of dairy lactic acid bacteria manufacture extracellular polysaccharides. These compounds may be produced as capsules that are tightly associated with the cell wall (i.e., capsular or **Cps**$^+$), or as a loose slime that is secreted into the medium (i.e., ropy). The term exopolysaccharide (**EPS**) is commonly used to refer to both types of extracellular polysaccharide (2, 5, 14). Milk fermented with ropy EPS-producing (EPS$^+$) lactic acid bacteria generally develops a viscous texture, and EPS$^+$ strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* are commonly used by industry to enhance viscosity and reduce syneresis in yogurt (2, 12).

EPS BIOSYNTHESIS

The biochemical process of synthesizing EPS differs depending on whether the EPS is a homopolysaccharide (composed of a single type of sugar) or heteropolysaccharride (two or more sugars). Organisms such as *Leuconostoc* that synthesize a homopolysaccharide do so on the surface of the cell using extracellular precursors and peripheral proteins (2). Heteropolysaccharride-producing bacteria form their EPS at the inner surface of the cell membrane using precursors formed intracellularly (2). Heteropolysaccharide production is widespread among gram-positive (including lactic acid bacteria [**LAB**]) and gram-negative bacteria (3, 16). Although the biochemistry of heteropolysaccharide synthesis in LAB is not well understood, similarities in the enzymes and basic repeating structures suggest the process is probably very

similar to that in gram-negative bacteria. The process in many gram-negatives begins with the synthesis of activated phosphorylated nucleotide sugars critical to the polymerization of monosaccharrides within the cytoplasm. These sugar precursors are not directly linked to a growing polysaccharide, but instead are sequentially attached to a C_{55} polyisoprenoid alcohol derivative (undecaprenol phosphate). Sugar additions continue to the lipid carrier until the basic repeating unit of the polysaccharide has been assembled. Depending on the organism, new copies of the basic repeating unit may be polymerized inside the cell and exported, or they may be translocated to the outside of the cell before polymerization occurs (16).

Cell wall synthesis also involves the use of undecaprenol monophosphate (**C_{55}-P**) as the lipid carrier for assembly of disaccharide pentapeptide repeating units. This process begins with C_{55}-P being connected to a disaccharide pentapeptide, which in turn is linked to a growing peptidoglycan chain. Release and dephosphorylation of C_{55}-PP to C_{55}-P so it can be recycled for the assembly of additional repeating units is a critical step in both EPS and cell wall synthesis (13, 16). Bacitracin, a cyclic polypeptide antibiotic produced by several species of *Bacillus*, inhibits cell wall synthesis by binding to the C_{55}-PP lipid carrier (10). Binding forms a complex that inhibits lipid carrier dephosphorylation and thus prevents its recycling (1, 9, 13). Because the amount of lipid carrier available to the cells is limited, conditions that favor rapid growth (and thus require new cell wall synthesis) generally inhibit EPS production. Conversely, higher EPS yields are usually obtained when organisms are grown at suboptimal growth temperatures and at a neutral pH (2, 3, 16).

Because of the relationship between EPS biosynthesis and the availability of lipid carrier, one potential mechanism to increase EPS production in cells may be increase the amount of lipid carrier available. Cain et al. (1) showed *bac*A-mediated bacitracin resistance in *Escherica coli* is due to the production of a lipid kinase that phosphorlyates isoprenyl alcohol to form more C_{55}-P. Thus, research outlined in this thesis sought to introduce an *Enterococcus faecalis bac*A gene into EPS producing cells and determine whether the production of exopolysaccharides in LAB is limited by the availability of C_{55}-P.

EPS$^+$ STARTER BACTERIA IN DAIRY FOODS

The use of EPS$^+$ starter lactic acid bacteria to enhance the functional properties of fermented dairy foods has traditionally focused on yogurt and fermented milks, but our group has demonstrated that a Cps$^+$ starter, *S. thermophilus* MR-1C, significantly increased cheese moisture level and improved the functional properties of low-fat Mozzarella cheese (5, 8). However, one potentially serious limitation to the use of capsular or ropy EPS$^+$ starter bacteria in cheese is that the polysaccharide may partition into the whey and increase whey viscosity. This development would be undesirable because it can affect the efficiency of membrane processing and thereby retard the efficiency of whey concentration and drying (6, 7, 15). Accumulation of EPS in whey may also alter the functional properties of whey products (Bastian, E. 1999. Personal communication). Because Cps are tightly bound to the cell (14), however, it was the hypothesis of this study that Cps$^+$ starters were less likely than ropy EPS$^+$ strains to affect whey viscosity. To test this hypothesis, we characterized the

effect of Cps^+ and ropy *S. thermophilus* starter bacteria on Mozzarella cheese functionality and whey viscosity.

DESSICATION PROTECTION

Many different environments contain bacteria which produce EPS. One of numerous functions of EPS is to protect cells from desiccation in dry environments such as soil. The hydroscopic nature of EPS allows it to bind water, sometimes at levels higher than those present in the surrounding environment. This elevated state of hydration may decrease the rate at which water is lost from the cell and possibly impart hydration to dried cells, thus decreasing the amount of desiccation damage incurred by the cell (10).

Further evidence to suggest that capsular EPS may offer cells some protection against freezing and freeze drying is obtained from observations that, in dry environments, some bacteria shuttle a larger percentage of energy and nutrients into carbohydrate polymerization (10, 11). The protective effect of EPS on cell membranes has also been examined through the addition of glycolipids to cells before desiccation (11). Results showed that the glycolipid's carbohydrate end appeared to alter the organization of the cellular membrane. The acyl chains and carbonyl groups of the phospholipid bilayer were rearranged by intercalation among hydrophilic regions of the bilayer by the carbohydrate portion of the glycolipid. This imitates the effect of water on the lipid bilayer during nonhostile conditions, resulting in a protective effect from desiccation (11).

Because EPS protects cells from drying, EPS$^+$ bacteria may be suited to the development of highly viable freeze-dried starter cultures for the dairy industry. Such cultures would be attractive as alternatives to the frozen culture concentrates, that are currently in use, because they would not incur many of the overhead costs associated with low temperature (-20°C) shipping and storage. Because certain types of polymers have been shown to protect LAB during lyophilization (4), this study investigated the protective effect of the MR-1C Cps against freezing and freeze-drying conditions.

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CHAPTER 2

CLONING OF THE *ENTEROCOCCUS FAECALIS* BACITRACIN RESISTANCE GENE IN DAIRY LACTIC ACID BACTERIA

ABSTRACT

Five *Streptococcus thermophilus* exopolysaccharide-producing strains and one exopolysaccharide-negative industrially used strain were screened for bacitracin resistance. All strains showed a minimum inhibitory concentration of less than 10 μg/ml. To determine whether expression of a gene encoding undecaprenol kinase (***bacA***) might enhance bacitracin resistance in dairy lactic acid bacteria, the *Enterococcus faecalis bac*A gene was transformed into *Lactococcus lactis* LM0230. However, *Lactococcus lactis* LM0230: pAM*bacA* did not exhibit a level of bacitracin resistance above that seen in the wild-type strain. Efforts to confirm *bac*A expression in *Lactococcus lactis* were confounded by a native gene product that showed 58% DNA identity and 73% protein similarity to a 151 bp region of the *E. faecalis bac*A.

INTRODUCTION

Production of extracellular heteropolysaccharide (**EPS**) is prevalent among gram-positive and gram-negative bacteria (6, 16). The particular mechanisms used by lactic acid bacteria (**LAB**) to produce heteropolysaccharide are not known, but the enzymes and basic repeating structures used to produce the EPS are similar to that in gram-negative bacteria, suggesting the process is comparable.

EPS synthesis in many gram-negative organisms starts with the formation in the cytoplasm of activated phosphorylated nucleotide sugars. These activated precursors are sequentially linked to the C_{55} polyisoprenoid alcohol derivative (undecaprenol phosphate (**C_{55}-PP**)) until a complete basic repeating unit has been assembled. In some organisms, new copies of the basic repeating unit are polymerized inside the cell and exported; in others, they are transported to the outside of the cell before polymerization occurs (16).

Like EPS, bacterial cell wall synthesis also depends on an undecaprenol phosphate lipid carrier to assemble disaccharide pentapeptide repeating units. A disaccharide pentapeptide is connected to the C_{55}-P lipid carrier in the beginning of this process, after which the disaccharide pentapeptide is then linked to a growing peptidoglycan chain. Once the disaccharide pentapeptide is released from the C_{55}-PP carrier, dephosphorylation of the lipid carrier produces C_{55}-P, which can be reused to attach additional sugars to form another repeating unit (14, 16).

Because the quantity of lipid carrier that is available to cells is limited, EPS production is often hindered when conditions support rapid growth and cell wall synthesis. Conversely, higher EPS yields are usually obtained when organisms are grown under conditions that limit cell wall synthesis (e.g., suboptimal growth temperatures or neutral pH) (5, 6, 16). These data suggest that EPS production in LAB grown under optimal conditions may be limited by the availability of the C_{55}-P lipid carrier. To investigate this hypothesis, we sought to express an *Enterococcus faecalis* gene encoding undecaprenol kinase (*bac*A) in a dairy LAB. The *bac*A gene confers resistance to bacitracin, a cyclic

polypeptide antibiotic that inhibits cell wall formation by arresting the dephosphorylation of C_{55}-PP (4, 11, 13). This encoded undecaprenol kinase mediates resistance by phosphorlyating isoprenyl alcohol to form more C_{55}-P, consequently, circumventing the action of bacitracin.

MATERIALS AND METHODS

Bacterial Cultures and Plasmid DNA

Bacteria and plasmid DNA used in this study are described in Table 2-1. *Streptococcus thermophilus* strains were grown at 37°C in M17 broth (15) which contained 0.5% lactose, and *Lactococcus lactis* strains were propagated at 30°C in M17 that contained 0.5% glucose. *Escherichia coli* strains were grown in Lauria-Bertani broth (10) at 37°C. All bacteria were stored at 4°C and maintained by biweekly transfer.

Antibiotic Screening

Since the lipid carrier is likely involved with EPS synthesis, higher levels of carrier could be present in cells that produce EPS, and this attribute might be reflected through an increased resistance to bacitracin. Five EPS^+ (MR-1C, MR-2C, MTC360, MTC330 and MTC310) and one EPS^- (TAO61) *S. thermophilus* and two *Lactococcus lactis* (LM0230 and LM0230: pAM*bacA)* were screened for bacitracin resistance in broth that contained various concentrations of bacitracin. Each strain was inoculated at 1% from overnight cultures into fresh broth and incubated until the culture reached an OD at 600 nm ($\mathbf{A}_{600}$) of 0.5 to 0.7. These cultures were used for 1% inoculation into a series of broth tubes that contained

TABLE 2-1. Bacteria and plasmids used in the study.

Bacterium or plasmid	Relevant characteristics	Source or reference
Escherichia coli		
SURE: pAM*bac*A	Bacitracin-resistance transformant	*E.coli* SURE (Stratagene, La Jolla, Ca) transformed with pAM*bac*A
Lactococcus lactis		
LM0230	*bac*A-negative control	Rhodia, Inc. (Madison, WI)
LM0230: pAM*bac*A	LM0230 transformed with pAM*bac*A	(1)
Streptococcus thermophilus		
MR-1C	Capsular exopolysaccharide-producer (Cps^+)	(9)
MR-2C	Capsular exopolysaccharide-producer (Cps^+)	Rhodia, Inc. (Madison, WI)
MTC360	Ropy exopolysaccharide-producer (EPS^+)	Rhodia, Inc. (Madison, WI)
MTC330	Ropy exopolysaccharide-producer (EPS^+)	Rhodia, Inc. (Madison, WI)
MTC310	Ropy exopolysaccharide-producer (EPS^+)	Rhodia, Inc. (Madison, WI)
TAO61	EPS^- commercial Mozzarella cheese starter	(9)
Plasmids		
pAM*bac*A	*Enterococcus faecalis bac*A gene ligated into pAM401 shuttle vector	Beatti, D. 1999. Personal communication

0, 5, 10, 15, 20, 25, or 50 µg/ml of bacitracin. These tubes were incubated overnight in a water bath at 37°C or 30°C and then examined visually for growth.

Plasmid Isolation and Transformation of *E. coli* and *L. lactis*

Plasmid DNA was isolated from *Lactococcus lactis* LM2301: pAM*bac*A by the method of Anderson and McKay (1), followed by cesium chloride-ethidium bromide density gradient centrifugation for plasmid purification (10). The presence of the plasmid in the cell lysates was detected by agarose gel electrophoresis as outlined by Maniatis et al. (10).

Transformation of *E. coli* SURE with pAM*bac*A was performed using a Bio-Rad Gene Pulser™(Bio-Rad Laboratories, Richmond, CA). Preparation of competent cells and electroporation procedures were as outlined by Smith (12). Electroporation parameters were set at 25 µF capacitance, 400 ohms resistance, and a field strength of 2.5kV/cm. A chilled 0.1 cm electrode gap electroporation cuvette was used with 40 µl of competent cells and 2 µl of plasmid (pAM*bac*A) DNA. Selection of pAM*bac*A transformants was performed using Lauria-Bertani agar that contained 25 µg/ml of chloramphenicol (10).

Electrotransformation of *Lactococcus lactis* LM0230 with pAM*bac*A was performed by the method of Holo and Nes (7). Electroporation-competent cells of *Lactococcus lactis* LM0230 were prepared in M17-0.5% glucose broth containing 3% glycine and 0.5M sucrose and frozen at -70°C until needed.

For transformation, 40 µl of competent cell suspension and 2 µl of pAM*bac*A were transferred to a chilled 0.1 cm electrode gap electroporation cuvette. Electroporation parameters were 25 µF capacitance, 400 ohms resistance, and a field strength of 2.5 kV/cm. Immediately after the electrical pulse, the cells were mixed with 0.96 ml of 0.5 M sucrose + M17-0.5% glucose containing 20 mM $MgCl_2$ and 2 mM $CaCl_2$ and chilled on ice for 5 min. The mixture was then incubated at 30°C for 1 h, after which 0.1 ml of cells was plated on 0.5 M sucrose + M17-0.5% glucose agar that contained 10 µg/ml of chloramphenicol. Plates were incubated at 30°C for 48 h; then, Cm^r colonies were picked and grown in 10 ml of M17-0.5% glucose broth with 10 µg/ml of chloramphenicol. Chloramphenicol-resistant colonies were confirmed to contain the pAM*bac*A plasmid by agarose gel electrophoresis as outlined by Maniatis et al. (10).

Northern Dot Blot

Northern dot blots were performed in an effort to detect expression of the *bac*A gene in *L. lactis* LM0230: pAM*bac*A. Total RNA was isolated from *E. coli* SURE, *E. coli* SURE: pAM*bac*A, *L. lactis* LM0230, and *Lactococcus lactis* LM0230: pAM*bac*A by the method of Arnau (2) omitting the heat shock step, with the use of LB (10) and M17-0.5% glucose (15) media for *E. coli* or *L. lactis*, respectively. *L. lactis* LM0230 and *E. coli* SURE were included in each experiment as negative controls.

Restriction digestion of pAM*bac*A with *Sal*I was performed as described in Maniatis et al. (10), and a 1.1 kb fragment containing the *bac*A gene was isolated

and purified from agarose gels with a Bio-Rad (Hercules, Calif.) Prep-a-Gene kit. The *bac*A gene probe was end-labeled using the digoxigenin (DIG) DNA Labeling Kit (Boehringer Mannheim, Germany); then, Northern hybridization and chemiluminescent detection were performed as directed by the kit supplier (Boehringer Mannheim Genius™ system, Boehringer Mannheim, Germany).

RT-PCR

*bac*A gene expression in *Lactococcus lactis* LM0230: pAM*bac*A was also invesigated RT-PCR. RNA was isolated from *E. coli* SURE, *E. coli* SURE: pAM*bac*A, *Lactococcus lactis* LM0230, and *Lactococcus lactis* LM0230: pAM*bac*A using a Qiagen (Valencia, Calif.) Rneasy Mini Kit. For first strand cDNA synthesis, a Qiagen (Valencia, Calif.) Omniscript reverse transcriptase kit was used. Oligomeric DNA primers (5′-CCTCTTGGTATTATCGAAGG-3′ and 5′-AACAACCGCCATAATCGCT-3′) were designed to generate a 151 base pair DNA amplicon from the *bac*A cDNA. Thirty cycles of the following RT-PCR steps were performed using a model 480 DNA thermal cycler (Perkins-Elmer, Foster City, Calif.): denaturation at 96°C for 10 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min. Analysis of PCR amplicons was performed using agarose gel electrophoresis as described by Maniatis et al. (10).

Genomic DNA Isolation

To control against the possibility that the RT-PCR fragments might be obtained from expression of a native *bac*A homology, genomic DNA was isolated from *E. coli* SURE as described by Ausubel et al. (3) and from *Lactococcus lactis* LM0230 as outlined by Low et al. (9). Samples from each genomic DNA

preparation were then used in a PCR reaction with the *bac*A primers described in the previous section, and any amplicons obtained from these reactions were sequenced from the 5′ and 3′ ends by Big Dye termination on a Perkin-Elmer Applied Biosystems automated DNA sequencer (model 373A) following parameters set by Innis et al. (8).

RESULTS

Bacitracin Resistance and *bac*A Expression

As shown in Table 2-2, all six *S. thermophilus* strains tested showed minimal growth in broth that contained 5 μg/ml bacitracin and no growth at bacitracin concentrations above 5 μg/ml. Not surprisingly, *E. coli* SURE: pAM*bac*A exhibited a 16-fold increase in resistance to bacitracin as compared to the nontransformed wild-type strain. In contrast, *L. lactis* LM0230: pAM*bac*A did not express higher bacitracin resistance than the wild-type *L. lactis* LM0230. This observation suggested that *bac*A gene expression might not occur in *L. lactis.*

To explore this possibility, we initially performed Northern dot with RNA collected from *E. coli* SURE, *E. coli* SURE: pAM*bac*A, *Lactococcus lactis* LM0230, and *Lactococcus lactis* LM0230: pAM*bac*A. However, no hybridization to a *bac*A gene probe was detected using hybridization temperatures of 42 or 38°C. Additional attempts to detect *bac*A expression by RT-PCR also proved inconclusive. Agarose gel electrophoresis of RT-PCR products from *E. coli* SURE, *E. coli* SURE: pAM*bac*A, *Lactococcus lactis* LM0230, and *Lactococcus lactis* LM0230:

TABLE 2-2. Bacitracin resistance screening.

	Bacitracin concentration (µg/ml) [1]						
Bacterium	0	5	10	15	20	25	50
Lactococcus lactis							
LM0230	+++	+	-	-	-	-	-
LM0230: pAM*bac*A	+++	+	-	-	-	-	-
Streptococcus thermophilus							
MR-1C	+++	+	-	-	-	-	-
MR-2C	+++	+	-	-	-	-	-
MTC360	+++	+	-	-	-	-	-
MTC330	+++	+	-	-	-	-	-
MTC310	+++	+	-	-	-	-	-
TAO61	+++	+	-	-	-	-	-

[1]Growth of bacteria in selected broth, at various bacitracin concentrations (-, no growth; +, slight growth; ++, moderate growth +++, robust growth)

pAM*bac*A all contained a cDNA amplicon of the expected size (approximately 151 bp).

To determine whether this fragment was an artifact or a legitimate *bac*A product, chromosomal DNA was isolated from *E. coli* SURE and *Lactococcus lactis* LM0230 and used in conventional PCR with *bac*A primers. Agarose gel electrophoresis revealed that chromosomal DNA from *Lactococcus lactis* LM0230 yielded a PCR product of approximately 151 bp in size. DNA sequence analysis of this fragment showed that it was 58% identical at the DNA level and more than 70% similar in deduced amino acid sequence to a corresponding region of the *E. faecalis bac*A gene. This observation suggests that problems with RT-PCR assays were due in part to a native *bac*A homology in *L. lactis* LM0230.

DISCUSSION

The initial goal of this study was to investigate a possible role for C_{55} undecaprenol-P in the synthesis of EPS by LAB. To accomplish this, we sought to determine whether the *E. faecalis bac*A gene could be expressed in dairy LAB. Preliminary screening of *S. thermophilus* or *L. lactis* strains revealed that all of the organisms tested were completely inhibited by 10 µg/ml of bacitracin. This result suggested that none of these organisms were likely to possess a native *bac*A homolog, and supported our hypothesis that heterologous *bac*A expression may facilitate increased EPS production. Unfortunately, efforts to further evaluate this hypothesis were stymied by the finding that pAM*bac*A did not enhance bacitracin resistance in a lactococcal host strain. This particular *bac*A gene originates from *Enterococcus faecalis*, so it would then be expected not to

have expression problems in *L. lactis*. Therefore, if there is expression of *bac*A within *L. lactis*, then this should increase the levels of the lipid carrier and thus increase bacitracin resistance, unless bacitracin is antagonistic to *L. lactis* by another unknown pathway. This unexpected outcome prompted further investigation into whether this gene was being expressed in *L. lactis*.

Northern dot blots failed to detect any hybridization between host RNA and a *bac*A gene probe. The basis for this observation is unclear, but it may have been due to suboptimal hybridization conditions, RNA degradation, or other factors. A second attempt to detect *bac*A expression using RT-PCR also proved unsuccessful because *L. lactis* LM0230 chromosomal DNA produced PCR fragments of the expected size. DNA sequence analysis of this fragment showed it had 58% DNA identity and more than 70% amino acid similarity to a corresponding region of the *E. faecalis bac*A gene. This suggested that *L. lactis* LM0230 already carries a native form of the *bac*A gene that is apparently expressed at a low level (since elevated bac^r was not observed). In summary, the results of this study were inconclusive regarding the role of the lipid carrier in EPS manufacture and the influence the *bac*A gene has on LAB.

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CHAPTER 3

INFLUENCE OF CAPSULAR AND ROPY EXOPOLYSACCHARIDE-PRODUCING *STREPTOCOCCUS THERMOPHILUS* ON MOZZARELLA CHEESE AND CHEESE WHEY

ABSTRACT

This study investigated the effect of capsular and ropy exopolysaccharide-producing *Streptococcus thermophilus* starter bacteria on Mozzarella cheese functionality and whey viscosity. Mozzarella cheeses were manufactured using *Lactobacillus helveticus* LH100 paired with one of four *S. thermophilus* strains: MR-1C, a bacterium that produces a capsular exopolysaccharide; MTC360, a strain that secretes a ropy exopolysaccharide; TAO61, a nonexopolysaccharide-producing commercial cheese starter; and DM10, a nonencapsulated, exopolysaccharide-negative mutant of strain MR-1C. As expected, cheese moisture levels were significantly higher in Mozzarella cheeses made with exopolysaccharide-positive versus exopolysaccharide-negative streptococci, and melt properties were better in the higher moisture cheeses. Whey viscosity measurements showed that unconcentrated and ultrafiltered, 5-fold concentrated whey from cheeses made with *S. thermophilus* MTC360 were significantly more viscous than whey from cheeses made with MR-1C, TAO61, or DM10. No significant differences were noted between the viscosity of unconcentrated or concentrated whey from cheeses made with *S. thermophilus* MR-1C versus the

[1] Reprinted from B.L. Petersen, R.I. Dave, D.J. McMahon, C.J. Oberg, and J.R. Broadbent. 2000. Influence of Capsular and Ropy Exopolysaccharide-Producing Streptococcus thermophilus on Mozzarella Cheese and Cheese Whey. J. Dairy Sci. 83:1952-1956.

industrial cheese starter TAO61. These data indicate that encapsulated, but not ropy, exopolysaccharide-producing *S. thermophilus* strains can be utilized to increase cheese moisture level and to improve the melt properties of Mozzarella cheese without adversely affecting whey viscosity.

INTRODUCTION

Many strains of dairy lactic acid bacteria synthesize extracellular polysaccharides. These compounds may be tightly associated with the cell wall (i.e., capsular), or as a loose slime that is secreted into the medium (i.e., ropy). The term exopolysaccharide (**EPS**) is commonly used to refer to both types of extracellular polysaccharide (3, 8, 14). Milk fermented with ropy EPS-producing (**EPS$^+$**) lactic acid bacteria generally develops a more viscous texture, and EPS$^+$ strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* are commonly used to enhance viscosity and reduce syneresis in yogurt (3, 13).

In the dairy industry, the application of EPS$^+$ starter lactic acid bacteria to enhance product functionality has traditionally been focused on the manufacture of yogurt and fermented milks. Recent research by our group, however, showed that a capsule-producing (**Cps$^+$**) starter, *S. thermophilus* MR-1C, can be used to significantly increase cheese moisture level and improve the functional properties of low fat Mozzarella cheese (8, 11). While the ability to enhance cheese yield (via an increased moisture content) and functionality are attractive incentives to the application of EPS$^+$ starter bacteria in Mozzarella cheese, widespread use of these bacteria for cheesemaking may be limited by the fact that EPS can partition into the whey and increase whey viscosity. The

accumulation of EPS in cheese whey is undesirable because it will retard the efficiency of membrane processing and thereby slow whey concentration and drying processes (9, 10, 16). In addition, the presence of EPS in whey products may alter the functional properties of these goods (Bastian, E. 1999. Personal communication).

Exopolysaccharide accumulation in whey is an obvious concern for cheese-making applications that employ ropy EPS$^+$ starters, but Cps$^+$ bacteria produce an EPS that typically is covalently anchored to the cell surface (14). Thus, it was the hypothesis of this study that Cps$^+$ starters were less likely than ropy EPS$^+$ strains to affect whey viscosity. To test this hypothesis, we characterized the effect of Cps$^+$ and ropy EPS$^+$ *S. thermophilus* starter bacteria on Mozzarella cheese functionality and whey viscosity.

MATERIALS AND METHODS

Bacterial Cultures and Growth Conditions

The bacteria used in this study are described in Table 3-1. The presence or absence of capsular EPS was determined by the Duguid stain procedure (5) with cultures grown in 9% reconstituted skim milk, and production of ropy EPS was defined by a strain's ability to increase the viscosity of fermented skim milk (Romero, D. 2000. Personal communication). The streptococci were grown at 37°C in M17 broth with 0.5% lactose (15), and *Lactobacillus helveticus* LH100 was propagated at 37°C in MRS broth (4). All bacteria were stored at 4°C and maintained by biweekly transfer.

TABLE 3-1. Bacteria used in the study.

Strain	Relevant characteristics	Source or reference
Streptococcus thermophilus MR-1C	Capsular exopolysaccharide-producer (Cps^+)	(11)
Streptococcus thermophilus DM10	Cps^- mutant of MR-1C	(8)
Streptococcus thermophilus MTC360	Ropy exopolysaccharide-producer (EPS^+)	Rhodia, Inc. (Madison, WI)
Streptococcus thermophilus TAO61	EPS^- commercial Mozzarella cheese starter	(11)
Lactobacillus helveticus LH100	EPS^- commercial Mozzarella cheese starter	(11)

Cheese Manufacture

Part-skim Mozzarella cheeses were manufactured on three separate occasions using *Lactobacillus helveticus* LH100 individually paired with each of the four *S. thermophilus* strains listed in Table 3-1. Bulk starter for cheesemaking was prepared by three successive overnight transfers of individual strains in 500 ml of sterilized 11% reconstituted skim milk. The cheese was manufactured using 10kg of milk for each vat as described by Fife et al. (7), except that the curd was set at 33.3°C, the cook temperature was 43.3°C, and the curd was milled and dry salted (2%, wt/wt).

Cheese Composition and Functionality

Each cheese was analyzed for fat, moisture, and protein by the modified Babcock, vacuum oven, and Kjeldahl methods, respectively (1, 12). The sodium chloride and calcium contents of each cheese were also determined. To measure the sodium chloride content, grated cheese was homogenized 1:20 (wt/vol) in double deionized water and the slurry was filtered through number 1 filter paper (Whatman International Ltd., Maidstone, England). The concentration of sodium chloride in the filtrate was then determined using a chloride analyzer (model 926 salt analyzer; Corning, Medfield, MA) as directed by the equipment supplier. The calcium content of each cheese was determined by atomic emission spectroscopy (6). Cheese pH was measured on d 1 after pressing using an Ionalyzer (model 811; Orion Research Inc., Cambridge, MA). Cheese melt was determined within 48 h of make by the method of Bogenrief and Olson (2) as modified by Fife et al. (7).

Whey Concentration

Approximately 2.5 L of whey was collected from each vat during cheese manufacture and filtered through cheese cloth to remove curd particles. A fraction (0.5 L) of each fresh whey sample was placed at 4°C for starter enumeration and viscosity tests; then, the remainder was concentrated 5-fold using a Minitan ultrafiltration system (Millipore, Bedford, MA) with 10 kDa membranes. The pump speed was set at 7, and a constant pressure of 10547 kg/m^2 was maintained during the UF process. The whey was kept at 4°C during concentration to minimize microbial growth, and the membrane was cleaned and sanitized between each sample run as directed by the equipment manufacturer. After UF, concentrated whey samples were stored at 4°C until needed.

Whey Viscosity

The viscosity of unconcentrated and concentrated whey samples was determined using a Brookfield viscometer (Model DV II+; Brookfield Engineering Laboratories, Stoughton, MA) with UL adapter. The speed of the spindle was set to 30 rpm, and the sample temperature was maintained at 20°C using a water bath. At least 20 readings were recorded at 5-s intervals for each sample.

Determination of Starter Numbers in Cheese and Whey

The number of *L. helveticus* LH100 and *S. thermophilus* colony forming units in cheese and whey was determined by the pour plate method using Rogosa SL (Difco Laboratories, Detroit, MI) and M17 agars, respectively, with

anaerobic incubation for 2 d. Cheese for microbiological sampling was collected from each vat before and after stretch. The samples were shredded, homogenized in 99 ml of 2% sodium citrate at 40°C with a stomacher (model 400; Seward, London, England), then serially diluted in 99 ml of sterile 2% sodium citrate immediately before plating. Fresh whey samples collected during cheese manufacture were stored overnight at 4°C before plating. Numbers of viable starter bacteria in concentrated whey samples were determined by the pour plate method immediately after UF.

Statistics

The influence of the *S. thermophilus* starter on the viscosity of unconcentrated and concentrated whey was analyzed by ANOVA. The least significant difference (LSD) method was also performed using SAS® (SAS Inst., Inc., Cary, NC) software to compare the effect of individual *S. thermophilus* strains on whey viscosity, cheese moisture content, and cheese melt.

RESULTS AND DISCUSSION

Cheese Composition and Yield

Statistical ANOVA showed the pH and the fat, calcium, and sodium chloride contents of the cheeses were not significantly ($P > 0.05$) affected by the strain of *S. thermophilus* used in the starter blend (Table 3-2). The percent protein of each cheese was significantly ($P < 0.001$) affected, and this value decreased when cheese moisture level increased. Thus, this value was lowest in cheeses

TABLE 3-2. Mozzarella cheese composition.

	Streptococcus thermophilus[1]			
Component	DM10	TAO61	MR-1C	MTC360
Moisture	49.88 ± 0.38	51.11 ± 0.42	53.81 ± 0.21	57.22 ± 1.33
Fat	20.25 ± 0.14	20.42 ± 0.08	20.25 ± 0.14	20.08 ± 0.08
Protein	26.16 ± 0.13	24.88 ± 0.74	24.19 ± 0.17	22.52 ± 0.24
Calcium	0.56 ± 0.03	0.56 ± 0.03	0.53 ± 0.02	0.57 ± 0.02
Salt	1.41 ± 0.02	1.44 ± 0.04	1.41 ± 0.01	1.32 ± 0.03
pH	5.34 ± 0.08	5.23 ± 0.02	5.28 ± 0.02	5.33 ± 0.02

[1]Cheeses were manufactured using *Lactobacillus helveticus* LH100 paired with each *S. thermophilus* strain listed. Values represent the mean percent of the total cheese composition from three replicate experiments SE.

made with the ropy *S. thermophilus* strain MTC360 and highest in cheese made with the EPS$^-$ strain DM10 (Table 3-2).

The ANOVA also showed cheese moisture level and curd yield were significantly ($P < 0.0001$) affected by the *S. thermophilus* strain used in the starter pair. As expected, cheeses made with the EPS$^+$ *S. thermophilus* strains MR-1C or MTC360 contained significantly ($P < 0.05$) more moisture than cheeses made with the nonexopolysaccharide-producing (EPS$^-$) cocci DM10 or TAO61 (Table 3-2). The LSD tests also showed that part-skim Mozzarella made with the ropy EPS$^+$ culture MTC360 had a significantly ($P > 0.05$) higher moisture level than cheese made with the Cps$^+$ strain MR-1C. Finally, it may be worthwhile to note that the 3% difference in the moisture level of part-skim Mozzarella made in this study with MR-1C versus TAO61 was consistent with previous observations for low fat (6% fat) Mozzarella cheese (8, 11). This result suggested that the ability of the *S. thermophilus* MR-1C Cps to increase cheese moisture content was not dependent upon the final fat content of the cheese.

Statistical evaluation of the mean curd yield (grams of cheese from 10 kg milk) from each cheese produced results that were parallel to the cheese moisture data. Thus, curd yield was significantly ($P < 0.05$) higher when cheeses were made with MR-1C or MTC360 (1083 3 and 1130 15 g, respectively) versus the EPS$^-$ cocci DM10 or TAO61 ((993 7 and 1013 7 g, respectively). In addition, mean curd was significantly ($P > 0.05$) higher if cheese was made with the ropy EPS$^+$ culture MTC360 versus the Cps$^+$ strain MR-1C.

Cheese Melt Properties

The choice of *S. thermophilus* strain also had a significant ($P < 0.0001$) effect on cheese melt properties at d 2. The average melt distance of cheese made with *S. thermophilus* MTC360 (12.5 0.6 cm) was significantly ($P < 0.05$) longer than that of cheeses made with MR-1C, TAO61 or DM10 (10.4 0.5, 9.3 0.4, and 8.8 0.2, respectively). Use of MR-1C as the starter coccus gave cheese with an average melt distance that was significantly ($P < 0.05$) longer than that from cheese made with DM10, but not TAO61. Our finding that part-skim Mozzarella cheese made with capsular or ropy EPS^+ *S. thermophilus* had significantly higher moisture levels and better melt properties than cheese made with EPS^- cocci was in good agreement with the observations of Perry et al. (11) for low fat Mozzarella cheese.

Whey Viscosity

The mean viscosity of individual whey samples in centipoise are presented in Table 3-3. The ANOVA showed whey viscosity was significantly ($P < 0.001$) affected by the *S. thermophilus* used in the cheese starter pair, and LSD tests confirmed that the mean viscosity of unconcentrated whey from cheese made with the ropy EPS^+ strain, MTC360, was significantly ($P < 0.05$) more viscous than whey from cheeses made with MR-1C, TAO61, and DM10. This result was expected because whey from cheese made with MTC360 formed distinct, string-like (ropy) structures during packing, piling, re-piling, and milling of the curd. Ropiness was not observed in whey from cheese made with any of the other starter cocci. Five-fold UF concentrated whey from MTC360 cheese was also significantly ($P < 0.05$) more viscous than concentrated whey from cheeses

TABLE 3-3. Mean viscosity of concentrated and unconcentrated whey.

	Streptococcus thermophilus[1]			
Sample	DM10	TAO61	MR-1C	MTC360
Concentrated	2.37 ± 0.046	2.51 ± 0.067	2.56 ± 0.059	0.04 ± 0.040
Unconcentrated	1.69 ± 0.007	1.93 ± 0.056	1.97 ± 0.073	2.20 ± 0.074

[1]Collected from cheeses manufactured using *Lactobacillus helveticus* LH100 individually paired with each *S. thermophilus* strain listed. Values represent the mean centipoise SE.

made with MR-1C, TAO61, or DM10. In addition, whey from MTC360 cheese required more time for 5-fold UF concentration. The average time required to concentrate 2 L of whey from cheese made with DM10, TAO61, or MR-1C was 4 to 4.5 h, but whey from MTC360 cheese required approximately 5.5 h.

An unexpected finding from this part of the study was that while the viscosity of unconcentrated whey from MR-1C and TAO61 cheese were not significantly different from one another, both were significantly ($P < 0.05$) more viscous than whey from cheese made with DM10. Similar results were obtained for the concentrated whey samples; whey from MR-1C and TAO61 cheese were not significantly different from one another, but MR-1C cheese whey was significantly more viscous than that obtained from its Cps^- mutant, DM10. The basis for this observation is unknown, but one possibility is that even though TAO61 is phenotypically EPS^-, this strain may produce some other type of cell surface carbohydrate (8). This hypothesis is supported by results from this study and from the work of Low et al. (8), which showed cheese made with TAO61 contained more moisture than cheese made with DM10.

Starter Numbers

As shown in Table 3-4, the *S. thermophilus* strain used in the starter pair did not appear to affect the total numbers of lactobacilli and streptococci in each cheese. In contrast, unconcentrated whey samples from cheese made with TAO61 contained populations of rods and cocci that were approximately one order of magnitude higher than those found in whey from cheeses made with the other *S. thermophilus* starters. This difference was apparently lost during UF treatment as numbers of viable starter bacteria, especially *L. helveticus* LH100,

TABLE 3-4. Numbers of starter bacteria in Mozzarella cheese and whey.

	Streptococcus thermophilus[1]							
	DM10		TAO61		MR-1C		MTC360	
Sample	M17	Rogosa	M17	Rogosa	M17	Rogosa	M17	Rogosa
Cheese before stretch	1.1×10^9	1.3×10^8	4.2×10^9	4.0×10^8	8.6×10^8	1.2×10^8	1.0×10^9	1.4×10^8
Cheese after stretch	1.3×10^9	2.1×10^8	2.9×10^9	2.2×10^8	1.1×10^9	1.4×10^8	9.5×10^8	1.6×10^8
Unconcentrated whey	2.2×10^7	1.2×10^6	8.1×10^8	8.7×10^7	1.7×10^8	2.2×10^6	7.3×10^7	2.1×10^6
Concentrated whey	4.3×10^4	$<10^2$	5.4×10^4	$<10^2$	1.2×10^4	$<10^2$	9.6×10^4	3.0×10^3

[1]Cheeses were manufactured using *Lactobacillus helveticus* LH100 individually paired with each *S. thermophilus* strain listed.

were considerably reduced in concentrated whey samples. An interesting exception to this effect was noted in concentrated whey from cheese made with MTC360, which contained approximately 10-fold higher numbers of lactobacilli (Table 3-4). This observation suggested that accumulation of the MTC360 ropy EPS in the whey may somehow protect *L. helveticus* LH100 cells during the UF process.

CONCLUSIONS

This study confirmed that both ropy and capsular EPS$^+$ *S. thermophilus* can be utilized to significantly increase cheese moisture content and to improve the melt properties of part-skim Mozzarella cheese. The ropy EPS$^+$ starter coccus MTC360 produced cheese with significantly ($P < 0.05$) higher moisture, yield, and melt properties than cheeses made with any of the other *S. thermophilus* starters, including the Cps$^+$ strain MR-1C. Although cheese made with MTC360 contained nearly 7% more moisture than cheese made with the EPS$^-$ commercial starter TAO61, an increase in cheese moisture of this magnitude may not be desirable. For example, part-skim Mozzarella cheese made with MTC360 expelled serum during the melt test, and this cheese was also sticky, soft, and difficult to shred. In contrast, part-skim Mozzarella made with the Cps$^+$ starter MR-1C melted and shredded in a manner that was very similar to cheese made with the commercial starter coccus TAO61.

Another important consideration in the use of EPS$^+$ cheese starters is their potential to deleteriously affect whey processing. Unconcentrated and UF concentrated whey from cheese made with the ropy strain MTC360 was

significantly ($P < 0.05$) more viscous than comparable whey samples from cheeses made with any of the other three *S. thermophilus* starters, and it also required a longer UF processing time. In contrast, the viscosities of unconcentrated or concentrated whey from MR-1C and TAO61 cheese were not significantly different, and the time required for 5-fold UF concentration of these whey samples was very similar. These results indicate that Cps^+, but not ropy, *S. thermophilus* can be used as Mozzarella cheese starters to enhance cheese moisture level, yield, and melt properties without deleteriously affecting whey viscosity or UF concentration time.

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CHAPTER 4

EFFECT OF CAPSULAR AND ROPY EXOPOLYSACCHARIDES ON CELL VIABILITY DURING FREEZING AND FREEZE DRYING

ABSTRACT

This study investigated a possible protective role for capsular exopolysaccharides (**Cps**) against freezing and freeze drying. Statistical analysis of variance (**ANOVA**) of cell counts before and after these challenges showed that the difference in *Streptococcus thermophilus* MR-1C (**EPS**$^+$) and DM10 (**EPS**$^-$) viability after a freeze-thaw cycle was not significant ($P > 0.05$). Microscopic examination of cells rehydrated after lyophilization revealed that chain lengths were reduced by this treatment, and that the chain length of the capsule producer MR-1C did not decrease as much after freeze drying as did that of DM10. However, even when chain length was taken into account, there was no significant difference ($P > 0.05$) in survivability between the two strains after lyophilization.

INTRODUCTION

Many cheesemakers propagate bulk starters containing organisms such as *Streptococcus thermophilus*, which is used extensively in Mozzarella cheese and yogurt production (3, 4). However, some processors have started using frozen culture concentrates that can be added directly to cheese milk (Bastian, E. 1999. Personal communication). Although frozen cultures are very convenient, shipping and storage costs of frozen cultures are rather high (1). Alternatives

such as a highly viable freeze-dried starter culture may be very appealing to the dairy industry because they provide the same favorable benefits without the burden of these additional costs.

Lyophilization is a very hostile and sometimes lethal process in which cells may suffer damage to nucleic acids, proteins, and the plasma membrane (6). Many organisms produce exocellular polysaccharides, and some of these molecules are thought to offer protection to bacteria against drying (6). The innate ability of EPS to bind water may offer protection from desiccation, because water loss from the cell will be much more gradual. This hypothesis is supported by the fact that some bacteria transport a large percentage of energy and nutrients into carbohydrate polymerization during desiccation (6, 7). The protective effect of EPS on the cellular membrane has been further verified by the addition of glycolipids to cells before desiccation. This resulted in an organizational change of acyl chains and carbonyl groups within the membrane due to intercalation among the hydrophilic regions of the lipid bilayer by the carbohydrate portion of the glycolipid. This imitates the effect of water on the lipid bilayer during nonhostile conditions, resulting in a protective effect from desiccation (6). In addition, certain types of polymers have been shown to protect *Streptococcus thermophilus* during lyophilization (2). From these observations, it was our hypothesis that the capsular polysaccharide produced by MR-1C may offer protection to the adverse conditions that cells are subjected to during freeze-drying.

MATERIALS AND METHODS

Bacterial Cultures and Growth Conditions

The streptococci used in this study are described in Table 4-1. All strains were propagated at 37°C in M17 broth (8) that contained 0.5% lactose, stored at 4°C, and maintained by biweekly transfer.

Freeze Challenge

S. thermophilus strains were prepared for cryotolerance tests by overnight growth in M17 broth that contained 0.5.% lactose. Cells were then inoculated at 1% into 10 ml of fresh M17-0.5% lactose broth and incubated at 37°C until they reached an absorbance at 600 nm ($\mathbf{A}_{600}$) of 0.35. The bacteria were collected by centrifugation at 3,450*g* for 10 min at room temperature, then suspended in 10 ml of fresh broth and incubated at 37°C for an additional 20 min (1). Plate counts were performed in duplicate; then, the cultures were placed overnight (approximately 20 h) at -20°C. The following day, frozen cultures were thawed quickly in a 37°C water bath, and duplicate plate counts were performed again. Colonies were counted after anaerobic incubation overnight at 37°C in Gaspak jars (Becton-Dickinson Microbiological Systems, Cockeyville, MD).

Freeze-Drying

The resistance of *S. thermophilus* strains to freeze-drying was determined by preparing the cells in the manner described above, except that freezing was performed overnight at -80°C. The frozen cells were then desiccated in a

TABLE 4-1. Streptococci used in the study.

Streptococcus thermophilus	Relevant characteristics	Source or reference
MR-1C	Capsular exopolysaccharide-producer (Cps^+)	(4)
DM10	Non-exopolysaccharide producer (EPS^-)	(4)
MTC360	Ropy exopolysaccharide-producer (EPS^+)	Rhodia, Inc. (Madison, WI)
TAO61	EPS^- commercial Mozzarella cheese starter	(4)

benchtop freeze dryer (VirTis Co., Gardiner, NY) for 45 h. Levels of residual moisture in the lyophilized cultures were determined as described by Pomeranz et al. (5) using a Karl Fischer titrator (model E547; Metrohm Ltd., Herisau, Switzerland). After a 45-h lyophilization, MR-1C and DM10 freeze-dried cultures were both rehydrated at various time intervals (d 1, d 7 with 4°C storage, and d 7 with 24°C storage) in 5 ml of fresh M17-0.5% lactose broth, vortexed at full-speed for 30 s, and then allowed to sit for 12 min. Samples of *S. thermophilus* MTC360 and TAO61 were also lyophilized and rehydrated at similar intervals. Plate counts were prepared in duplicate after rehydration for each time interval and colonies were counted after overnight anaerobic incubation at 37°C in Gaspak jars (Becton-Dickinson Microbiological Systems, Cockeyville, MD).

Microscopic Analysis

To determine whether the chain length of *S. thermophilus* MR-1C or DM10 might be affected by the freeze-drying process, samples of each culture were taken before and after (rehydrated in 5 ml of fresh M17-0.5% lactose broth) the lyophilization and examined under a phase contrast microscope (Olympus model BH-2 microscope, Olympus Corp., New Hyde Park, NY.). A random count of 20 cell chains was taken for each strain before and after lyophilization, and the average chain length for each sample was calculated. Any change in chain length after freeze drying was calculated into the results from the CFU counts and statistically analyzed.

Statistics

The influence of the capsular exopolysaccharide on the freeze resistance of *S. thermophilus* MR-1C and DM10 was assessed by ANOVA using SAS® (SAS Inst., Inc., Cary, NC) software. ANOVA tests were also used to determine whether or not capsule production increased viability after freeze-drying at d 1, d 7 stored at 24°C, or d 7 stored at 4°C.

RESULTS AND DISCUSSION

Freeze Challenge

The mean viability of each strain after freeze challenge is shown in Table 4-2. The strain that showed the highest survivability after freezing was MR-1C (82%), while the lowest was TAO61 (49%). Despite the fact that MR-1C viability was numerically higher than that of DM10, ANOVA revealed the difference was not significant ($P > 0.05$) (Table 4-3). Although one might expect MTC360 to have higher freeze resistance than DM10, due to its production of ropy EPS, the viability percentages for these two strains were very close. Another interesting outcome was that the two non-EPS producers, DM10 and TAO61, did not have similar survivability percentage. As a whole, data presented in Table 4-2 suggested that strain differences affected the survivability of *S. thermophilus* strains far more than did the presence or absence of exopolysaccharides (4).

Freeze-Drying

Mean viability values for each strain after lyophilization are shown in Table 4-4. Microscopic observation revealed that the chain length of DM10 was

TABLE 4-2. Viability after freeze challenge.

Streptococcus thermophilus	Viability[1]
MR-1C	0.82 ± 0.08
DM10	0.72 ± 0.07
TAO61	0.48 ± 0.01
MTC360	0.70 ± 0.15

[1] Values represent the mean fraction (±SE) of the initial cell population recovered after freeze challenge.

TABLE 4-3. Analysis of variance of viability after freeze challenge for *Streptococcus thermophilus* MR-1C vs. DM10.

Source	df	Sum of squares	Mean square	F	*P*
Model	1	162.8	162.8	1.03	0.36
Error	4	635.2	158.8		
Corrected total	5	798.0			

TABLE 4-4. Viability after freeze drying.

	Viability[1]		
Streptococcus thermophilus	Day 1	Day 7 (4°C)	Day 7 (24°C)
MR-1C	0.37 ± 0.08	0.41 ± 0.06	0.31 ± 0.04
DM10	0.32 ± 0.03	0.24 ± 0.03	0.24 ± 0.01
TAO61	0.27 ± 0.06	0.32 ± 0.04	0.36 ± 0.06
MTC360	0.91 ± 0.07	0.87 ± 0.12	0.81 ± 0.08

[1] Values represent the mean fraction (±SE) of the initial cell population recovered after freeze drying.

highly affected by freeze-drying. The random count of 20 chain clusters showed that MR-1C had a mean chain length of 12.4 ± 2.59 cells before freeze drying and 12.1 ± 2.33 after, and DM10 had a mean chain length of 8.6 ± 0.93 cells before and 5.5 ± 0.67 after. As expected, MR-1C had a higher survivability percentage than DM10 did at d 1, d 7 stored at 4°C, and d 7 stored at 24°C, but these differences were not statistically significant ($P > 0.05$), even when chain length differences were taken into account (Tables 4-5, 4-6, and 4-7). The largest difference between the two was at d 7 (storage at 4°C) where the percentage of viable cells was almost double (40.5% and 23.8%). The ropy EPS producer MTC360 had considerably the highest survivability values at all three time intervals than the other three starters. The industrial used starter TA061 possessed viability values similar to MR-1C and DM10. In conclusion, data suggest capsule production does not significantly increase the resistance of *S. thermophilus* to freezing or lyophilization.

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TABLE 4-5. Analysis of variance of viability after freeze drying, day 1 for *Streptococcus thermophilus* MR-1C vs. DM10.

Source	df	Sum of squares	Mean square	F	*P*
Model	1	41.0	41.0	0.35	0.58
Error	4	473.8	118.4		
Corrected total	5	3724.0			

TABLE 4-6. Analysis of variance of viability after freeze drying, day 7 (4°C) for *Streptococcus thermophilus* MR-1C vs. DM10.

Source	df	Sum of squares	Mean square	F	*P*
Model	1	418.5	418.5	6.02	0.07
Error	4	278.1	69.5		
Corrected total	5	696.6			

TABLE 4-7. Analysis of variance of viability after freeze drying, day 7 (24°C) for *Streptococcus thermophilus* MR-1C vs. DM10.

Source	df	Sum of squares	Mean square	F	*P*
Model	1	74.0	74.0	3.32	0.14
Error	4	89.2	22.3		
Corrected total	5	163.2			

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Extracellular polysaccharides (**EPS**) are manufactured by many strains of lactic acid bacteria. These compounds may be produced as capsules that are tightly associated with the cell wall (**Cps**$^+$) or as a loose slime that is secreted into the medium. EPS is a term that is commonly used to designate both types of extracellular polysaccharide. The objectives of this study were to investigate: 1) the role of the C_{55} undecaprenol lipid carrier (used in cell wall synthesis) in the production of exopolysaccharide; 2) the effect of capsular exopolysaccharide-producing starter cultures on Mozzarella cheese and cheese whey; and 3) the possible protective characteristics capsular EPS may offer cells against freezing and freeze-drying.

Unfortunately, the role of the C_{55} undecaprenol lipid carrier in EPS production could not be determined due to problems associated with the pAM*bac*A plasmid. This plasmid encodes a lipid kinase that increases levels of the lipid carrier and thus confers resistance to bacitracin in *E. coli*. When *Lactococcus lactis* LM0230 was transformed with pAM*bac*A, no increase in resistance was noted. If the *bacA* gene were expressed in that host, then one would expect that the increased availability of lipid carrier would manifest in enhanced resistance to bacitracin and, if the lipid carrier is the rate-limiting compound in EPS synthesis, in increased EPS production. However, expression of *bac*A was not confirmed by Northern blots or RT-PCR, thus precluding our ability to assess its role in EPS production by LAB.

Studies to characterize the effect of capsular exopolysaccharide-producing starter cultures on Mozzarella cheese and cheese whey proved more successful. As expected, moisture levels were significantly higher in Mozzarella cheese made with an exopolysaccharide-producing streptococci strain versus cheese made with a non-exopolysaccharide-producing strain. The cheeses with higher moisture levels also possessed better melt properties. Viscosity of 5-fold ultrafiltered whey from cheeses made with the ropy EPS producer, *S. thermophilus* MTC360, were significantly higher than whey from cheeses made with MR-1C , TAO61, or DM10. This demonstrated that encapsulated exopolysaccharide-producing *S. thermophilus* strains such as MR-1C can be used to raise moisture levels and improve meltability in Mozzarella cheese without significantly increasing cheese whey viscosity.

Finally, the protective characteristics of capsular and ropy exopolysaccharides against freezing and lyophilization were studied. Cell counts taken before and after each freeze and freeze-drying trial showed higher survivability for the capsule producer MR-1C values than for DM10, but ANOVA showed the difference was not significant ($P > 0.05$).

In summary, this study presents evidence that encapsulated exopolysaccharide-producing *S. thermophilus* strains can be used in Mozzarella production to increase cheese moisture levels and to improve meltability without significantly increasing cheese whey viscosity, as ropy strains do. In addition, the capsule-producing *S. thermophilus* strain exhibited higher cell viability values when challenged with freezing and freeze-drying than did the non-encapsulated

mutant. However, the differences in viability values were not statistically significant and probably require further study.

APPENDIX

March 12, 2001

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Department of Microbiology
Ogden, UT 84408-2506

Dear Dr. Oberg,

I am in the process of completing the requirements for my MS degree and would like your permission as a co-author to reprint the article listed below as a chapter in my thesis.

> Petersen, B. L., R. I. Dave, D. J. McMahon, C. J. Oberg, and J. R. Broadbent. 2000. Influence of capsular and ropy exopolysaccharide-producing *Streptococcus thermophilus* on Mozzarella cheese and cheese whey. J. Dairy Sci. 83:1952-1956.

Please sign this letter to indicate your consent.

Thank you,

Brent Petersen

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Permission granted: Craig J Oberg

Date: 14 March 2001

March 12, 2001

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Permission granted: RJ Dave

Date: 03/12/2001.

March 12, 2001

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Dear Dr. McMahon,

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Thank you,

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