Fat-free yogurt made using a galactose-positive exopolysaccharide-producing recombinant strain of *Streptococcus thermophilus*

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ABSTRACT

To prevent textural defects in low-fat and fat-free yogurts, fat substitutes are routinely added to milk. In situ production of exopolysaccharides (EPS) by starter cultures is an acknowledged alternative to the addition of biothickeners. With the aim of increasing in situ EPS production, a recombinant galactose-positive EPS+ *Streptococcus thermophilus* strain, RD-534-S1, was generated and compared with the parent galactose-negative EPS+ strain RD-534. The RD-534-S1 strain produced up to 84 mg/L of EPS during a single-strain milk fermentation process, which represented 1.3 times more than the EPS produced by strain RD-534. Under conditions that mimic industrial yogurt production, the starter culture consisting of RD-534-S1 and (EPS−) *Lactobacillus bulgaricus* L210R strain (RD-534-S1/L210R) led to an EPS production increase of 1.65-fold as compared with RD-534-S1 alone. However, the amount of EPS produced did not differ from that found in yogurts produced using an isogenic starter culture that included the parent *S. thermophilus* strain RD-534 and *Lb. bulgaricus* L210R (RD-534/L210R). Moreover, the gel characteristics of set-style yogurt and the rheological properties of stirred-style yogurt produced using RD-534-S1/L210R were similar to the values obtained for yogurts made with RD-534/L210R. In conclusion, it is possible to increase the production of EPS by ropy *S. thermophilus* strains reduces firmness and improves viscosity, water retention, and the mouthfeel of yogurt (Rohm and Kovac, 1994; Marshall and Rawson, 1999; Welman et al., 2003). These ropy strains are viewed as an alternative to hydrocolloids as texturing agents (Wacher-Rodarte et al., 1993; Dolevires et al., 2005). This also meets consumer demands for a reduction in the use of additives in foods as revealed by the fact that in many countries, the use of additives is strictly regulated or even prohibited.

Key words: exopolysaccharide, yogurt, *Streptococcus thermophilus*

INTRODUCTION

*S. thermophilus*, a gram-positive lactic acid bacterium, is used with lactobacilli in starter cultures for yogurt production. Some strains of *S. thermophilus* produce neutral exopolysaccharides (EPS). These polysaccharides may be assembled in a capsular structure that is tightly associated with the cell surface, or they may be secreted into the growth medium. Bacterial EPS are thought to play roles in protection against detrimental environmental conditions, in cell recognition, and in biofilm formation (Broadbent et al., 2003). Although EPS concentration in fermented milk is relatively low (De Vuyst and Degeest, 1999; Vaninigelgem et al., 2004), in situ production of EPS by ropy *S. thermophilus* strains reduces firmness and improves viscosity, water retention, and the mouthfeel of yogurt (Rohm and Kovac, 1994; Marshall and Rawson, 1999; Duboc and Mollet, 2001; Welman et al., 2003). These ropy strains are viewed as an alternative to hydrocolloids as texturing agents (Wacher-Rodarte et al., 1993; Dolevires et al., 2005). This also meets consumer demands for a reduction in the use of additives in foods as revealed by the fact that in many countries, the use of additives is strictly regulated or even prohibited.

Most of *S. thermophilus* strains are galactose-negative (Gal−) and are unable to efficiently metabolize galactose. This metabolic defect is due, in part, to the production of insufficient levels of galaktokinase, a key enzyme of the Leloir pathway encoded by *galK* (Vaillancourt et al., 2002). Indeed, transformation of *S. thermophilus* with a plasmid carrying a functional *galK* allows the bacterium to grow on galactose (Gal+) (Vaillancourt et al., 2002). However, the recombinant strains still secrete galactose when grown into lactose-rich medium (Vaillancourt et al., 2004; Robitaille et al., 2007) most likely because the LacS permease, which operates as a galactose/lactose antiport system, is very efficient to expel β-d-galactose from the surrounding medium (Gunniewijk and Poolman, 2000). Nevertheless, Levander and Radstrom (2001) have shown that EPS precursors can be formed from the galactose moiety of lactose via the Leloir pathway in

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Gal− S. thermophilus. It has also been demonstrated that recombinant S. thermophilus strains that produce enzymes of the Leloir pathway at high levels, together with enhanced phosphoglucomutase activity, can synthesize more EPS than the parental strain (Levander et al., 2002; Svensson et al., 2005). However, the conditions that were used did not mimic those applied by the industry for the production of yogurts, notably because the fermentation was carried out in a medium supplemented with yeast extract and peptone and in the absence of a Lactobacillus strain.

The objectives of this project were (i) to determine whether the introduction of a functional galK gene into S. thermophilus RD-534, a nonropy strain, would improve EPS synthesis and (ii) to study the effect of using a Gal+ recombinant strain (RD-534-S1) in combination with a Lactobacillus bulgaricus strain as a mixed starter culture on the rheological and chemical properties of fat-free set-style and stirred-style yogurts.

MATERIALS AND METHODS

Bacterial Strains

Streptococcus thermophilus RD534 (also known as S. thermophilus DGCC 7710; Barrangou et al., 2007; Deveau et al., 2008) is an EPS+ strain used for the industrial production of yogurt that was kindly provided by Danisco (Dangé Saint Roman, France; Lévesque et al., 2005). Streptococcus thermophilus SMQ-301 is a non-EPS-producing strain that is mainly used in large-scale cheese manufacture (Tremblay and Moineau, 1999). Lactobacillus delbrueckii ssp. bulgaricus L210R is a nonropy Gal− strain (Lamboley et al., 2003). Streptococcus thermophilus RD-534 was transformed with pTKRL2TK (Vaillancourt et al., 2004) by electroporation, as described previously (Robitaille et al., 2007), yielding the recombinant strain RD-534-S1. The bacterial strains were stored frozen at −80°C in milk-based medium made of 12% (wt/wt) reconstituted low-heat skim milk powder (RSM; Agropur, Granby, Quebec, Canada) in deionized water containing 5% (wt/vol) sucrose. Frozen cultures of S. thermophilus were transferred to M17 broth (Terzaghi and Sandine, 1975) supplemented with 0.5% (wt/vol) lactose (RD534) or with 0.5% (wt/vol) galactose (RD-534-S1) and grown at 40°C. Lactobacillus bulgaricus L210R was grown in acidic MRS broth at 37°C (De Man et al., 1960) before use. Two successive overnight growths were done in the RSM medium, and the second one was stopped at the early stationary growth phase, before their use in fermentation processes. All chemicals were obtained from Fisher Scientific (Nepean, Ontario, Canada), unless otherwise specified.

Milk Fermentation and Yogurt Production

Small-scale static milk fermentations were carried out at 40°C in RSM medium at 12% total solid, protein 4.4%, casein 3.3% (wt/wt). Inoculation of RSM was at 5% (wt/wt) with single strains of S. thermophilus. During fermentation, aliquots were withdrawn for compositional analysis. The culture media were diluted and spread on lactose supplemented M17 agar for cell count. Titratable acidity was determined by neutralization to pH 8.6 with 0.11 N NaOH (Titration Manager TIM800, Titralab, Radiometer Copenhagen, Copenhagen, Denmark). Sugars and organic acids were quantified by HPLC as described previously (Robitaille et al., 2007).

Fat-free laboratory-scale yogurts were produced in 12% (wt/wt) RSM. Before inoculation, RSM was homogenized at 20 MPa (Emulsiflex C-50, Avestin, Ottawa, Canada) at 23°C, heat-treated at 85°C for 20 min, and cooled to 40°C before use. Starters were prepared by mixing 4 volumes of S. thermophilus and 1 volume of Lb. bulgaricus L210R. Two ropy starters, RD-534/L210R and RD-534-S1/L210R, and a nonropy control starter, SMQ-301/L210R, were tested. One liter of RSM was aseptically inoculated at 2% (wt/wt) with starter culture, distributed in 175-ml plastic containers, and incubated at 42°C. Fermentation was stopped at a pH of 4.65 by rapid cooling. Fat-free set-style yogurts were cooled by transferring the containers in melted ice for 15 min, until the temperature in the middle of the sample reached 23°C and stored at 4°C for 2 days without disturbing the curd. For fat-free stirred-style yogurts, the yogurts were cooled in melted ice for 15 min, transferred in a water bath at 20°C for 10 min, broken with a spoon, manually forced to pass through a 60-ml syringe (1-mm-diameter orifice) with a flow rate of 70 ± 5 mL/min to mimic the industrial stirring process, and stored at 4°C for 5 d.

EPS Analyses

The EPS content was determined in duplicate in each fermented milk and in yogurts. Briefly, the fermented product was mixed with 1 volume of 20% (wt/vol) TCA, heated at 100°C for 5 min, and centrifuged at 3,700 × g for 10 min at 20°C. The supernatant was removed, and the pellet was suspended in 0.5 volume of 10% (wt/vol) TCA and centrifuged again. Aqueous phases were pooled and dialyzed at 4°C against deionized water for 4 days. The EPS contents were estimated using the phenol-sulfuric acid assay (Dubois et al., 1956) with glucose as the standard.

Rheological Parameters

Yield stress, the minimum stress required to initiate flow in the gel (in Pa), and yield strain, the deforma-
tion at failure (Rad), were evaluated in set-style type of yogurts by the vane method (Dzuy and Boger, 1985; Yoo et al., 1995). A Hamann torsion gelometer consisting of a Brookfield viscometer (Brookfield Engineering Laboratories, Stoughton, MA) and equipped with a 4-blade vane 16 mm in diameter and 40 mm long was used. The vane was gently lowered into the gel yogurt held at 4°C and rotated at 0.5 rpm. Measurements were made in duplicate in each container, and the mean value was used. Rheological measurements of stirred-style yogurt were carried out with a dynamic stress rheometer (SR-2000, Rheometric Scientific, Piscataway, NJ) using a concentric cylinder system with a 25-mm bob. The yogurt (13.4 g) was transferred to the cup with minimal disturbance, and measurements were carried out at 1°C in sweep stress mode. Shear stress and corresponding shear rate values were recorded. Experimental flow curves ranging from 10 to 100 s⁻¹ were fitted to the Oswald de Waele model: τ = Kγⁿ, where τ is the shear stress (Pa) and γ the shear rate (s⁻¹), to evaluate the fluid consistency index (K, Pa·sⁿ) and the flow behavior index (n, dimensionless). The K and n values were then used to calculate the apparent viscosity (η_app) of stirred-style yogurts at shear rates of 10 and 100 s⁻¹. Susceptibility to syneresis was evaluated by a centrifugation method. Yogurt samples (25 g) were gently poured into preweighed 50-mL polypropylene tubes and centrifuged at 500 × g (IEC Centra-GP8R, Thermo Scientific, Waltham, MA) for 10 min at 4°C. The supernatant whey was removed and weighed. The syneresis index was calculated as the weight of whey per weight of yogurt in grams per kilogram.

Statistical Analyses

An experimental block consisted of set-style and stirred-style yogurts made from the same batch of RSM fermented with the 3 starter cultures. Duplicates for each yogurt were prepared and analyzed. The experiment was repeated 3 times, and data were subjected to ANOVA using the general linear model of SAS (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Transformation of S. thermophilus RD-534 with pT-KRL2TK yielded the recombinant strain RD-534-S1, which was able, unlike the parental strain, to grow on galactose-supplemented M17 media. The integrity of the plasmid in the recombinant strain was verified by PCR using the method described previously (Robitaille et al., 2007), whereas the identity of the recombinant strain was confirmed by phage typing (data not shown).

In the first set of experiments, milk fermentations were carried out using single purified strains and were stopped after 12 h of fermentation, during the stationary growth phase, to maximize EPS secretion. The composition of RD-534- and RD-534-S1-fermented milk (12% RSM) is presented in Table 1. Cultures reached the late exponential growth phase in 3 h. Cell counts in RD-534- and RD-534-S1-fermented milk, at 3 and 12 h of fermentation, did not differ (P > 0.1). Titratable acidity of milk inoculated with RD-534-S1 increased faster, and the amount of lactic acid in RD-534-S1-fermented milk after 12 h was significantly greater (P < 0.05) compared with RD-534-fermented milk. The amounts of galactose in RD-534-S1-fermented milk increased with time but did not differ from the amount found in RD-534-fermented milk. However, the lactic acid/galactose molar ratio in RD-534-S1-fermented milk was significantly greater than in RD-534-fermented milk at h 3 and 12 of fermentation, indicating that the relative concentration of lactic acid was much greater than the concentration of galactose in RD-534-S1-fermented milk compared RD-534-fermented milk. This result showed that the recombinant strain used galactose more efficiently for metabolic purpose than the parent strain. Similar results were previously reported for the recombinant S. thermophilus strains SMQ-301-K01 (Vailancourt et al., 2004) and MR-AAC (Robitaille et al., 2007), transformed with the same plasmid. On the other hand, EPS production was increased by 1.3 times (P < 0.05) with the recombinant S. thermophilus strain RD-534-S1 as compared with the parent strain. This points out to the possibility of improving the textural properties of yogurt through an increase in EPS production, by using S. thermophilus RD-534-S1 as part of starter culture.

For yogurt manufacture, the starter culture must contain at least one S. thermophilus strain and one Lb. delbrueckii ssp. bulgaricus strain, the Lactobacillus being added to facilitate the acidification process, and to improve the organoleptic properties of the final product. Characteristics and gel properties of laboratory-scale yogurts produced in this study using the 2 ropy starters, RD-534/L210R and RD-534-S1/L210R, and a nonropy starter, SMQ-301/L210R, are presented in Table 2. The fermentation process was stopped when pH reached pH 4.65, and postacidification activity during cold storage resulted in a decreased pH by 0.1 to 0.2 units. Titratable acidity was similar (P > 0.1) for all type of yogurts.

Although the fermentation period for yogurt production was more than 2 times shorter than for milk fermentation with single strains, the amount of EPS produced in yogurt by RD-534/L210R and RD-534-S1/L210R (Table 2) were, respectively, 2.3 and 1.65 times...
greater than those found in fermented milk with *S. thermophilus* rropy strains alone (Table 1). The protocooperation between *S. thermophilus* and *Lb. bulgaricus* enhances EPS production by *S. thermophilus*, a phenomenon previously observed by Bouzar et al. (1997) and Marshall and Rawson (1999). Unlike what was observed when the fermentation was conducted with *S. thermophilus* strains alone (RD534 or RD534-S1; Table 1), the EPS content in RD-534/L210R and RD-534-S1/L210R yogurts was similar (Table 2). The presence of *Lb. bulgaricus* promotes EPS production, but the overproduction observed when recombinant strain was used alone (Table 1) seems to disappear. This result suggests that the difference between parent and recombinant strains becomes significant only when fermentation is performed in nonoptimal conditions for EPS production.

The firmness of fat-free set-style yogurts was evaluated by the vane method. This method is valuable because the measurements are done in situ, which minimizes structural breakdown. Moreover, the stress on the vane required to initiate flow, the yield stress, is highly correlated with the sensory initial firmness as perceived by trained panelists (Harte et al., 2007). The yield stresses for RD-534/L210R and RD-534-S1/L210R yogurts were similar (Table 2), being significantly less (*P* < 0.05) than for SMQ-301/L210R yogurt. This result is consistent with the fact that EPS tend to segregate within pores (Hassan et al., 2003) and interfere with protein micelle interactions during the acidification process, which result in weaker protein matrix and a somewhat softer gel. In this study, apparent yield strain values for RD-534/L210R and RD-534-S1/L210R yogurts were similar, being significantly less than yogurt made with nonropy SMQ-301/L210R starter.

Fat-free stirred-style yogurt produced using EPS* starters remained homogeneous after 5 d at 4°C, whereas several samples made using the nonropy SMQ-301/L210R starter exhibited a macroscopically granular appearance. The apparent viscosity values of stirred-style yogurts at 10 and at 100 s⁻¹ are presented in Table 2. The apparent viscosity of RD-534/L20R and RD-534-S1/L210R yogurts was similar but significantly greater than the apparent viscosity of SMQ-301/L210R yogurt. This is in accordance with the results of Marshall and Rawson (1999). Because yogurt behaves like a pseudoplastic fluid, the viscosity tends to decrease with increasing rate of shear. This decrease was less pronounced in the case of RD-534/L20R and RD-534-S1/L210R yogurts compared with SMQ-301/L210R yogurt, indicating that EPS help to prevent textural defects due to mechanical damage from handling. Again, no difference between the recombinant and parent rropy *S. thermophilus* strains was observed.

### Table 1. The composition of RD-534 and RD-534-S1-inoculated 12% (wt/wt) reconstituted skim milk during fermentation at 40°C

<table>
<thead>
<tr>
<th>Item</th>
<th>0 h</th>
<th>3 h</th>
<th>12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RD-534</td>
<td>RD-534-S1</td>
<td></td>
</tr>
<tr>
<td>Cell count (log cfu/mL)</td>
<td>7.12b</td>
<td>7.04b</td>
<td>9.02a</td>
</tr>
<tr>
<td>pH</td>
<td>6.61a</td>
<td>6.61a</td>
<td>5.55b</td>
</tr>
<tr>
<td>Titratable acidity (g/kg)</td>
<td>2.11e</td>
<td>2.11e</td>
<td>5.25b</td>
</tr>
<tr>
<td>Lactic acid (g/kg)</td>
<td>0.6d</td>
<td>0.6d</td>
<td>2.9c</td>
</tr>
<tr>
<td>Galactose (g/kg)</td>
<td>0.5e</td>
<td>0.5e</td>
<td>2.47c</td>
</tr>
<tr>
<td>Lactic acid/galactose (mol/mol)</td>
<td>2.36e</td>
<td>2.36e</td>
<td>31c</td>
</tr>
<tr>
<td>Exopolysaccharides (mg/L)</td>
<td>0</td>
<td>0</td>
<td>31c</td>
</tr>
</tbody>
</table>

*–eValues with different superscripts in the same row are significantly different (*P* < 0.05).

### Table 2. Characteristics and gel properties of yogurts produced using *Streptococcus thermophilus* RD-534 (RD-534/L210R), RD-534-S1 (RD-534-S1/L210R), and SMQ-301 (SMQ-301/L210R) as starters, in combination with *Lactobacillus delbrueckii* ssp. *bulgaricus* L210R

<table>
<thead>
<tr>
<th>Item</th>
<th>RD-534/L210R</th>
<th>RD-534-S1/L210R</th>
<th>SMQ-301/L210R</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation process</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH after storage</td>
<td>4.46b</td>
<td>4.53b</td>
<td>4.43b</td>
<td>0.01</td>
</tr>
<tr>
<td>Titratable acidity (g/kg)</td>
<td>10.34a</td>
<td>9.79a</td>
<td>10.28a</td>
<td>1.23</td>
</tr>
<tr>
<td>Fermentation (h)</td>
<td>4.31b</td>
<td>5.19a</td>
<td>3.34c</td>
<td>0.09</td>
</tr>
<tr>
<td>Exopolysaccharides production (mg/L)</td>
<td>147c</td>
<td>144c</td>
<td>—</td>
<td>13</td>
</tr>
<tr>
<td>Firmness of set-style yogurt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield stress (mPa)</td>
<td>162.3c</td>
<td>159.4c</td>
<td>179.3c</td>
<td>4.8</td>
</tr>
<tr>
<td>Yield strain (Rad)</td>
<td>0.256b</td>
<td>0.261b</td>
<td>0.277c</td>
<td>0.006</td>
</tr>
<tr>
<td>Rheology of stirred-style yogurt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apparent viscosity at 10 s⁻¹ (Pa·s)</td>
<td>0.776a</td>
<td>0.697a</td>
<td>0.205c</td>
<td>0.04</td>
</tr>
<tr>
<td>Syneresis index at 500 × g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whey expelled from yogurt (g/kg)</td>
<td>232b</td>
<td>244b</td>
<td>377a</td>
<td>12</td>
</tr>
</tbody>
</table>

*–cValues with different superscripts in the same row are significantly different (*P* < 0.05).
Syneresis is an important defect in yogurt production. It has been shown that the use of a ropy strain as starter was more prone to reduce yogurt syneresis than a nonropy strain (Hess et al., 1997; Folkenberg et al., 2006). The ability of the recombinant and parent S. thermophilus strains to decrease syneresis was evaluated here by centrifugation. The syneresis indexes of yogurts made with RD-534/L20R and RD-534-S1/L210R were similar ($P > 0.1$), indicating that the Gal$^+$ phenotype associated with RD-534-S1 did not change water retention in yogurt. On the other hand, these 2 ropy starters were more efficient than the nonropy strain to minimize syneresis. In fact, the syneresis indexes of yogurts made with RD-534/L20R and RD-534-S1/L210R were 35% less than the syneresis index of yogurt fermented with a nonropy SMQ-301/L210R starter culture.

In conclusion, this study shows that it is possible to increase the production of EPS by ropy S. thermophilus strains through genetic engineering by increasing GalK activity. However, when used in combination with Lb. bulgaricus for yogurt manufacture, the EPS overproduction of recombinant strain is not significant. Nevertheless, the recent finding that simultaneous increase of GalK and mutarotase (GalM) activities of EPS production during milk fermentation using a mixed starter culture.

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