**Diversity of Heteropolysaccharide-Producing Lactic Acid Bacterium Strains and Their Biopolymers**

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Thirty-one lactic acid bacterial strains from different species were evaluated for exopolysaccharide (EPS) production in milk. Thermophilic strains produced more EPS than mesophilic ones, but EPS yields were generally low. Ropiness or capsular polysaccharide formation was strain dependent. Six strains produced high-molecular-mass EPS. Polymers were classified into nine groups on the basis of their monomer composition. EPS from Enterococcus strains were isolated and characterized.

Certain lactic acid bacteria (LAB) produce exopolysaccharides (EPS), either capsular polysaccharides (CPS) that are tightly associated with the cell surface or slime EPS that are secreted into the extracellular environment. EPS from LAB can be divided into homopolysaccharides, which are polymers composed of one type of monosaccharide, and heteropolysaccharides (HePS), which are polymers of repeating units that are composed of two or more types of monosaccharides (5, 6, 7, 11). A large biodiversity of HePS exists regarding their composition and structure, molecular mass (MM), yield, and functionalities (5, 7, 35). Further, polymer formation is strongly influenced by culture conditions (1, 7, 22, 32). Recently, the molecular genetics of HePS biosynthesis have been studied for different LAB species (6, 25). Several glycosyltransferases involved in the assembly of the HePS repeating units have been discovered (17, 18, 35). EPS can act as viscosifying, stabilizing, gel-forming, and/or water-binding agents in various foods (6, 10). Additionally, they have been claimed to display anti-inflammatory and functionalities (5, 7, 35). Further, polymer formation is generally low. Ropiness or capsular polysaccharide formation was strain dependent. Six strains produced high-molecular-mass EPS. Polymers were classified into nine groups on the basis of their monomer composition. EPS from Enterococcus strains were isolated and characterized.

**EPS screening, isolation, and characterization.** Two hundred one thermophilic (11 strains of Lactobacillus acidophilus, 79 of Lactobacillus delbrueckii subsp. bulgaricus, 1 of L. delbrueckii subsp. lactis, 1 of Streptococcus macdonaldicus, and 42 of Streptococcus thermophilus) and mesophilic (23 strains of Enterococcus faecalis, 1 of Enterococcus faecium, 5 of Lactobacillus casei, 29 of Lactobacillus paracasei, and 9 of Lactobacillus rhamnosus) LAB strains (CERELA Culture Collection, Tucumán, Argentina) were used throughout this study. All bacteria were stored as previously described (22). Before experimental use, cultures were propagated twice in MRS (Britania, Buenos Aires, Argentina) for lactobacilli or in LAPt (27) for streptococci and enterococci.

Screening for EPS-producing LAB was performed with reconstituted skim milk (10%, wt/vol) at 37°C for 16 h using cultures propagated in milk as the inoculum (1%, vol/vol). Noninoculated medium was used as a control. Cell counts, expressed as numbers of CFU per milliliter, were determined by pour plating in MRS agar (MRS plus 15 g liter⁻¹ agar) after 48 h at 37°C. The final pH of the milk cultures was measured as well. Milk cultures were evaluated for ropiness (R) as described by Mozza et al. (23). CPS formation was determined by the India ink negative-staining technique (23), and slime EPS was isolated and quantified as described previously (1, 8), by using ethanol instead of acetone for EPS precipitation. EPS production was expressed as milligrams of polymer dry mass (PDM) per liter. EPS were purified by dialysis as described previously (34). The EPS isolations were performed in two independent experiments.

The MM of pure, freeze-dried EPS (10 mg ml⁻¹) was determined by gel permeation chromatography with a Waters Chromatograph (Waters Corp., Milford, Mass.) equipped with a Waters Ultrahydrogel column (500 to 8.0 x 10⁶ Da) that was calibrated with dextran standards (ranging from 4.9 x 10⁶ to 8.0 x 10⁶ Da; Sigma, St. Louis, Mo.) with 0.1 M NaNO₃ as the eluent at a flow rate of 0.6 ml min⁻¹. Polysaccharides were detected with a Waters refractive index detector. High-MM EPS (HMM-EPS, arbitrarily defined as >10⁶ Da) were distinguished from low-MM EPS (LMM-EPS, <10⁶ Da). The MM determinations were performed in duplicate.

Purified, freeze-dried EPS (1.0 mg ml⁻¹) were hydrolyzed in 4 N (final concentration) HCl at 100°C for 3 h, freeze-dried, and redissolved in ultrapure water (1.0 mg ml⁻¹). Monomer analysis was carried out by high-performance anion-exchange chromatography with pulsed amperometric detection as described previously (34). EPS monomer analysis was performed in triplicate.

**PCR conditions, DNA sequencing, and analyses.** Genomic DNA from LAB was isolated as described previously (24). The presence of eps genes (HePS) was verified by using four primer...
pairs (Invitrogen Life Technologies, Karlsruhe, Germany) designed from eps genes described before (Table 1). Strains harboring these genes were used as positive controls after confirmation of the DNA sequences of the amplified genes. The PCR mixtures (25 μl) contained 15 ng of DNA, 2.5 mM MgCl₂, the four deoxynucleoside triphosphates at 100 μM each, each primer at 1 μM in Taq buffer, and 2.5 U of Taq polymerase. The PCRs were performed with a programmable heating incubator (Perkin-Elmer Corp., Norwalk, Conn.) by following the programs detailed in Table 1. Amplicons were analyzed by electrophoresis in 1% (wt/vol) agarose gels in 1× Tris-acetate-EDTA buffer. The 200-bp PCR products obtained with the epsD/E primers were run in 2% (wt/vol) agarose gels. A 100-bp ladder (Invitrogen Life Technologies) was used to identify the molecular sizes of the bands. PCR products were purified with the GFX PCR, DNA/gel band purification kit (Amersham Biosciences, Buckinghamshire, United Kingdom). Sequencing was performed with an ABI Prism 3100 genetic analyzer. Next, nucleotide and amino acid sequence analyses were performed as described recently (25).

Diversity of HePS from LAB. This is the first report on an extensive phenotypic and genotypic screening for EPS-producing strains from different LAB species. Thirty-one out of 201 LAB strains belonging to different species were capable of producing EPS (EPS⁺) (Tables 2 and 3). No HePS were found for L. acidophilus and S. macedonicus strains. The growth and acidification capacity in milk of the LAB assayed was strain dependent. Vaningelgem et al. (34) investigated the growth and acidification capacity in milk of the LAB assayed that were ropy synthesized less than 10 mg of PDM liter⁻¹.

In general, the HePS yields were low, varying from 10 to 166 mg of PDM liter⁻¹. Seven out of 31 strains synthesized HePS in large amounts (>100 mg of PDM liter⁻¹). Also, Vaningelgem et al. (34) found a limited number of S. thermophilus strains producing HePS in large amounts. All thermophilic LAB strains produced HePS in larger amounts than mesophilic strains. The ability to synthesize CPS (CPS⁺) or to display ropiness (R⁺) by LAB was variable and strain dependent (Tables 2 and 3). Although CPS or ropy phenotypes are not usually sought in LAB, 14 LAB strains were CPS⁺. Only two thermophilic LAB strains were both CPS⁺ and R⁺, while most of the mesophilic HePS-producing LAB strains were CPS⁺ and R⁻. E. faecium CRL210 was both CPS⁺ and R⁻. Two LAB strains (L. delbrueckii subsp. bulgaricus CRL861 and L. paracasei CRL208) that were ropy synthesized less than 10 mg of PDM liter⁻¹. In contrast, S. thermophilus CRL638 was the only R⁺ strain among the LAB producing large amounts of HePS. Thus, ropiness was not correlated with the amount of EPS produced. Whether ropiness is associated with CPS or slime-EPS formation is still unclear. Hassan et al. (15) have classified EPS⁺ LAB strains into three groups, CPS⁺ R⁻, CPS⁻ R⁻, and CPS⁻ R⁺; no report of CPS⁻ R⁺ strains, as was the case for S. thermophilus CRL863 (this study), was made. The ability to form CPS was much more widespread among the mesophilic HePS-producing LAB strains than among the thermophilic HePS producers. A positive effect of CPS formation on the

**TABLE 1. Oligonucleotide primers used to detect eps genes containing sequences similar to the primers used in this study.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Gene target</th>
<th>Expected fragment size (bp)</th>
<th>PCR conditions</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>φpsA fw</td>
<td>AGAYGARYTNCCNCARYTNWKNAAYGT</td>
<td>Priming glycosyltransferase</td>
<td>1,600</td>
<td>30 cycles of 94°C (30 s), 49°C (45 s), 72°C (1 min)</td>
<td>19, this work</td>
</tr>
<tr>
<td>φpsA rev</td>
<td>TGCAGCYTCWGCCACATG</td>
<td>Priming glycosyltransferase</td>
<td>300</td>
<td>30 cycles of 94°C (30 s), 56°C (45 s), 72°C (1 min)</td>
<td>19, this work</td>
</tr>
<tr>
<td>φpsB fw</td>
<td>TCATTTTATTCGTAAAACCTCAATTGAY</td>
<td>Priming glycosyltransferase</td>
<td>1,150</td>
<td>35 cycles of 94°C (45 s), 46°C (1 min), 72°C (1 min)</td>
<td>20</td>
</tr>
<tr>
<td>φpsB rev</td>
<td>GARYTNCC</td>
<td>Priming glycosyltransferase</td>
<td>800</td>
<td>35 cycles of 94°C (30 s), 49°C (30 s), 72°C (1 min)</td>
<td>20</td>
</tr>
<tr>
<td>φpsC fw</td>
<td>TGCAGCTGGTACACTTGAAGC</td>
<td>Priming glycosyltransferase</td>
<td>1,150</td>
<td>35 cycles of 94°C (45 s), 46°C (1 min), 72°C (1 min)</td>
<td>11, 2</td>
</tr>
<tr>
<td>φpsC rev</td>
<td>GTAGTCAGTGACACTTGAAC</td>
<td>Priming glycosyltransferase</td>
<td>1,150</td>
<td>35 cycles of 94°C (45 s), 46°C (1 min), 72°C (1 min)</td>
<td>11, 2</td>
</tr>
<tr>
<td>φpsD fw</td>
<td>TGCAGCTGGTACACTTGAAGC</td>
<td>Priming glycosyltransferase</td>
<td>1,150</td>
<td>35 cycles of 94°C (45 s), 46°C (1 min), 72°C (1 min)</td>
<td>11, 2</td>
</tr>
<tr>
<td>φpsD rev</td>
<td>GTAGTCAGTGACACTTGAAC</td>
<td>Priming glycosyltransferase</td>
<td>1,150</td>
<td>35 cycles of 94°C (45 s), 46°C (1 min), 72°C (1 min)</td>
<td>11, 2</td>
</tr>
<tr>
<td>φpsE fw</td>
<td>TGCAGCTGGTACACTTGAAC</td>
<td>Priming glycosyltransferase</td>
<td>1,150</td>
<td>35 cycles of 94°C (45 s), 46°C (1 min), 72°C (1 min)</td>
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<td>φpsE rev</td>
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<td>Priming glycosyltransferase</td>
<td>1,150</td>
<td>35 cycles of 94°C (45 s), 46°C (1 min), 72°C (1 min)</td>
<td>11, 2</td>
</tr>
</tbody>
</table>

The presence and sequence analysis of the corresponding genes were confirmed by DNA sequencing.
viscoelastic properties of yoghurt has been shown before (15, 16). Moreover, the large CPS production by *S. thermophilus* MR-1C was responsible for the improved melting properties of low-fat mozzarella cheese (20).

The structural characteristics of the HePS analyzed in this study revealed a large diversity. A broad MM range, from 8 to >5,000 kDa, was found (Tables 2 and 3). LMM-EPS were more likely to occur than HMM-EPS. Only six LAB strains

### TABLE 2. HePS production by thermophilic LAB strains in milk and characterization of their polymers

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Cell count (CFU ml⁻¹)</th>
<th>Final pH</th>
<th>PDM (mg liter⁻¹)</th>
<th>R</th>
<th>CPS</th>
<th>MM (kDa)</th>
<th>Monomer ratioᵃ</th>
<th>Groupᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. delbrueckii</em> subsp. <em>bulgaricus</em> CRL142</td>
<td>Yoghurt</td>
<td>1.0 × 10⁷</td>
<td>3.94</td>
<td>21</td>
<td>−</td>
<td>+</td>
<td>None</td>
<td>946</td>
<td>1.0</td>
</tr>
<tr>
<td>CRL406</td>
<td>Yoghurt</td>
<td>3.8 × 10⁸</td>
<td>3.95</td>
<td>83</td>
<td>−</td>
<td>−</td>
<td>None</td>
<td>642</td>
<td>1.0</td>
</tr>
<tr>
<td>CRL553</td>
<td>Yoghurt</td>
<td>4.1 × 10⁸</td>
<td>3.83</td>
<td>47</td>
<td>−</td>
<td>−</td>
<td>None</td>
<td>324</td>
<td>1.0</td>
</tr>
<tr>
<td>CRL563</td>
<td>Yoghurt</td>
<td>1.1 × 10⁹</td>
<td>4.02</td>
<td>105</td>
<td>−</td>
<td>+</td>
<td>None</td>
<td>270</td>
<td>1.0</td>
</tr>
<tr>
<td>CRL852</td>
<td>Yoghurt</td>
<td>1.6 × 10⁷</td>
<td>3.90</td>
<td>150</td>
<td>−</td>
<td>−</td>
<td>None</td>
<td>10</td>
<td>1.5</td>
</tr>
<tr>
<td>CRL861</td>
<td>Yoghurt</td>
<td>1.4 × 10⁶</td>
<td>4.56</td>
<td>&lt;10</td>
<td>+</td>
<td>+</td>
<td>None</td>
<td>203</td>
<td>1.0</td>
</tr>
<tr>
<td>CRL863</td>
<td>Yoghurt</td>
<td>3.4 × 10⁸</td>
<td>4.63</td>
<td>10</td>
<td>+</td>
<td>−</td>
<td>2,203</td>
<td>None</td>
<td>1.0</td>
</tr>
<tr>
<td>CRL865</td>
<td>Yoghurt</td>
<td>5.0 × 10⁶</td>
<td>3.85</td>
<td>120</td>
<td>−</td>
<td>−</td>
<td>None</td>
<td>15</td>
<td>1.5</td>
</tr>
<tr>
<td>CRL870</td>
<td>Yoghurt</td>
<td>4.0 × 10⁷</td>
<td>4.19</td>
<td>126</td>
<td>−</td>
<td>−</td>
<td>&gt;5,000</td>
<td>None</td>
<td>1.0</td>
</tr>
<tr>
<td>CRL874</td>
<td>Yoghurt</td>
<td>8.7 × 10⁶</td>
<td>3.97</td>
<td>24</td>
<td>−</td>
<td>−</td>
<td>None</td>
<td>8</td>
<td>1.5</td>
</tr>
</tbody>
</table>

### TABLE 3. HePS production by mesophilic LAB strains in milk and characterization of their polymers

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Cell count (CFU ml⁻¹)</th>
<th>Final pH</th>
<th>PDM (mg liter⁻¹)</th>
<th>R</th>
<th>CPS</th>
<th>MM (kDa)</th>
<th>Monomer ratioᵃ</th>
<th>Groupᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. delbrueckii</em> subsp. <em>lactis</em> CRL564</td>
<td>Yoghurt</td>
<td>9.6 × 10⁸</td>
<td>3.95</td>
<td>55</td>
<td>+</td>
<td>−</td>
<td>None</td>
<td>314</td>
<td>1.0</td>
</tr>
<tr>
<td><em>L. helveticus</em> CRL1176</td>
<td>Cheese</td>
<td>3.9 × 10⁹</td>
<td>4.38</td>
<td>41</td>
<td>+</td>
<td>−</td>
<td>1,800</td>
<td>None</td>
<td>2.0</td>
</tr>
</tbody>
</table>

### a Relative to other monomers in compound (on a molar basis).

### b Classified according to the monomers present in the HePS.

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**Viscoelastic Properties of Yoghurt**: The viscoelastic properties of yoghurt have been previously described in the literature. For instance, *S. thermophilus* MR-1C was shown to produce a large amount of CPS, which contributed to the improved melting properties of low-fat mozzarella cheese. The structural characteristics of the HePS analyzed in this study revealed a large diversity, with a broad MM range from 8 to >5,000 kDa. LMM-EPS were more likely to occur than HMM-EPS. Only six LAB strains showed significant HePS production in milk, with various MM ranges and monomer ratios. The detailed data for each strain is provided in Table 2, which includes information on cell count, final pH, and polymer characteristics. The table categorizes the strains into groups based on their monomer ratios. The structural diversity of the HePS produced by these strains is consistent with the observed properties of yoghurt and mozzarella cheese.

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**HePS Production by Mesophilic LAB Strains**: Table 3 presents the HePS production by mesophilic LAB strains in milk. These strains include *E. faecalis*, *E. faecium*, *L. casei*, *L. paracasei*, and *L. rhamnosus*. Each strain is characterized by its cell count, final pH, PDM, and polymer characteristics, including MM and monomer ratios. The table also categorizes the strains into groups based on their monomer ratios. The details for each strain are provided, highlighting the diversity in HePS production and polymer characteristics.

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**Conclusion**: The study demonstrates the role of LAB strains in the production of heteropolysaccharides (HePS) and their impact on the viscoelastic properties of yoghurt and mozzarella cheese. The structural diversity of the HePS, characterized by their MM and monomer ratios, correlates with the observed properties of these dairy products. Understanding the specific contributions of LAB strains to HePS production is crucial for optimizing dairy product quality and functionality.
produced HMM-EPS, including E. faecium CRL210. In contrast, Vanigelgem et al. (34) showed that HePS-producing S. thermophilus strains harbored HMM-EPS or both HMM-EPS and LMM-EPS. L. delbrueckii subsp. bulgaricus CRL870 produced an HMM-EPS of >5,000 kDa, which could be of interest to improve the rheology of fermented milks, as the MM positively influences the rheological properties of a polysaccharide solution (9, 30). Five out of six HMM-EPS-producing strains were R ̶; hence, ropiness is correlated with the size (HMM) of the HePS. However, the rheology of fermented milks with EPS ̶ strains is affected not only by polymer structure, MM, and yields but also by EPS-protein interactions (6, 29).

Concerning the HePS monomer composition, galactose, glucose, galactosamine, glucosamine, and rhamnose were present but in different ratios (Tables 2 and 3). The isolated HePS were classified into nine groups, the number of different monomers varying from one to four, and each group harboring strains of different LAB species. Four of these groups were previously described for HePS from S. thermophilus strains (34). In our work, rhamnose was the least common monomer found, in contrast to other reports (7, 34). The presence of rhamnose could play a role in the bacteriophage sensitivity of the strains (12, 26). Galactose was the only monosaccharide present in all of the HePS. Also, galactose was present in all of the HePS produced by 26 S. thermophilus strains (34). Galactose incorporation could be due to poor expression of genes for galactose breakdown (4). The HMM-EPS produced by E. faecium CRL210 belonged to group VII, and the LMM-EPS produced by E. faecalis CRL316 and E. faecalis CRL434 belonged to groups VI and V, respectively. This is the first report on two strains of L. delbrueckii subsp. bulgaricus (CRL406 and CRL142) secreting polysaccharides composed solely of galactose. These unusual EPS were synthesized as HePS as no sucrose was added to the medium (5). To date, L. lactis strains have been shown to synthesize HePS with the same chemical composition (14, 35). In general, the biodiversity of HePS may be ascribed to the sugar nucleotide biosynthesis routes and the genetic potential of the strains (7). A correlation between the HePS monomers and the activity of glycosyltransferases (17, 18, 35) and enzymes for sugar nucleotide biosynthesis has been reported (2, 21, 22, 32).

The sequences of the eps PCR products of the genomic DNA of the control strains showed identities (61 to 97%) with the corresponding eps genes (GenBank database). With the epsEFG primers, a 300-bp PCR product was obtained with the DNA of L. helveticus CRL1176, while a 1,600-bp band was obtained with the DNA of L. delbrueckii subsp. bulgaricus CRL870. These differences were attributed to the absence of the epsF gene in the eps cluster of L. helveticus (18, 19, and GenBank accession no DQ222973). Most of the EPS ̶ LAB strains possessed eps genes, as verified with the primers used. DNA of nine L. delbrueckii subsp. bulgaricus strains gave 1,600-bp PCR products with the epsEFG primers, while a 300-bp amplicon was obtained with the DNA of L. delbrueckii subsp. bulgaricus CRL861. All S. thermophilus strains exhibited 800-bp PCR products with the epsA primers. With the epsDE primers, all of S. thermophilus strains displayed positive results, except for two strains (CRL815 and CRL638). Regarding the epsB primers, only DNA of S. thermophilus CRL815 yielded a PCR product. PCR products of 200 bp were amplified with the epsDE primers for the L. casei group strains. No PCR products were obtained with the DNA from L. delbrueckii subsp. lactis CRL564 and from the enterococci tested with any of the primers used; only DNA of E. faecalis CRL434 gave an amplicon with the epsB primers. A good correlation between the presence of eps genes with the specific primers described for HePS and the HePS phenotype was observed. Specific primers to detect eps genes in the DNA of L. delbrueckii subsp. lactis CRL564 and enterococcal strains remain to be designed. Screening with different sets of eps primers could be a rapid alternative way to seek HePS-producing strains, although the HePS phenotype has to be confirmed afterwards.

For the first time, it was shown that enterococcal strains are able to produce HePS. Although enterococci are not widespread in the dairy industry, they are usually present in artisinal Argentinian cheeses (Tafi) and are often part of the dominant microbiota of ripened Mediterranean cheeses (13, 31). Safe HePS-producing enterococcal strains could improve the textural characteristics of cheeses, as long as they are not involved in virulence (13).

This extensive and systematic study showed a wide biodiversity of HePS from LAB. Furthermore, novel polysaccharides and new HePS-producing LAB species were found which will be of further interest for technological exploitation.

**Nucleotide sequence accession numbers.** The sequences obtained in this study are available under GenBank accession no. DQ222973, DQ249312 to DQ249314, DQ249316, DQ249317, and DQ249319.

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