Structural Studies of the Capsular Polysaccharide Produced by *Leuconostoc mesenteroides* ssp. *cremoris* PIA2†

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ABSTRACT: The structure of the capsular polysaccharide (CPS) produced by *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 has been determined using component analysis and NMR spectroscopy. 1H and 13C resonances were assigned using 2D NMR experiments, and sequential information was obtained by 1H-1H-NOESY and 1H,13C-HMBC experiments. The CPS consists of linear pentasaccharide repeating units with the following structure: \( -3)\-\beta-D-Galp\-(1\-6)\-\beta-D-Galp\-(1\-2)\-\beta-D-Galp\-(1\-6)\-\beta-D-Galp\-(1\-3)\-\beta-D-Galp\-(1\-\)\), in which four out of the five sugar residues have the furanoid ring form, a structural entity found in bacteria but not in mammals. The analysis of the magnitude of the homonuclear three-bond coupling constants of the anomeric protons for the five-membered sugar rings indicates that the sugar residues substituted at a primary carbon atom show one kind of conformational preferences, whereas those substituted at a secondary carbon atom show another kind of conformational preferences.

**INTRODUCTION**

Lactic acid bacteria (LAB), including *Leuconostoc*, play an important role in the fermentation of various food products, and the importance of *Leuconostoc* strains in dairy technology is widely recognized. They are often present in dairy starter cultures and also in the dairy environment and thus could be considered as nonstarter LAB. Many LAB species produce extracellular polysaccharides (EPS). EPS is a general term that refers to two types of secreted polysaccharides. The first type of EPS is attached to the cell wall as a capsule (capsular polysaccharides or CPSs), whereas the other is produced as loosely unattached material (further referred to as EPS). Some strains produce both types of EPS, whereas others produce only one type. EPS produced by LAB can be subdivided into two groups, namely, homopolysaccharides (HoPSs) and heteropolysaccharides (HePSs). HoPSs contain only one type of monosaccharide, glucose or fructose, in the glucans and the fructans, respectively. An unusual type of HoPSs containing galactose, the galactan, was also reported. Many different types of HePSs are secreted by several LAB strains with respect to monosaccharide composition. *Leuconostoc mesenteroides* strains produce HoPS consisting of \( \alpha\-\)glucans such as dextrans mainly composed of \( \alpha\-1,6\)-linked residues with variable (strain specific) degrees of branching, alternans composed of \( \alpha\-1,3\)- and \( \alpha\-1,6\)-linkages, and \( \beta\-\)fructans such as levans composed of \( \beta\-2,6\)-linkages.

Limited information is available on CPS production by food-grade LAB and the role of CPS-producing LAB in fermented milk. The CPS-producing *Streptococcus thermophilus* strains can be used advantageously as starters in fat and low-fat mozzarella cheese production to increase moisture retention and improve melt properties without adversely affecting whey viscosity. In mixed yogurt cultures, CPS-producing *S. thermophilus* strains were found to contribute high viscosity, mouth thickness, and creaminess. CPSs of LAB do not cause ropiness nor does production of EPS ensure ropiness. It has been suggested that CPSs play a role in the protection of microbial cells against desiccation, phagocytosis, bacteriophage attack, antibiotics and toxic compounds and provide the cell with the capacity to adhere to solid surfaces. Krinos et al. showed that Gram negative human colonic microorganism, *Bacteroides fragilis*, is able to modulate its surface antigenicity by producing at least eight distinct CPSs. So far, some studies on the EPS of the LAB have been carried out to characterize the physiological effects of these biopolymers, but the number of studies on the CPS of the LAB is limited. The CPS may act as both a physical barrier to phage infection and as an attachment site for specialized bacteriophages. The CPS produced by *Lactococcus lactis* MZ4010 protects cells against bacteriophages. The presence of the CPS of *Streptococcus thermophilus* could play a role in the absorption of specific phages to the cells. The study of Khalil et al. indicated that it is possible to reduce phage infection of CPS-forming *S. thermophilus* by blocking or modifying phage absorption sites. One of the main criteria for the selection of probiotics is the ability to adhere to the intestinal mucosa, allowing a transitory colonization of the intestinal tract, which could modify the adhesion of other microorganisms. Sun et al. reported that the mannose-specific adhesion of *Lactobacillus plantarum* Lp6 to the rat mucus was important for competition with pathogen-binding sites in the gut. This may mean that it can be used to resist the colonization of the pathogens. The study suggested that the CPS is also involved in the adhesion. The results of Yasuda et al. showed that the high-molecular-weight component of the cell wall polysaccharide on cells of *Lactobacillus casei* Shirota, one of

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The uniformity of the CPS was further checked by size exclusion chromatography using a column (75 × 1.5 cm) of Bio-Gel P-10 polycrylamide gel (exclusion limit 20 000 Da, 200–400 mesh, Bio-Rad Laboratories, Richmond, CA). A sample (1.0 mg) was loaded onto the column and eluted with 0.05 M NH₄OAc (Riedel-de Haën AG, Seelze, Germany) with UV monitoring at 280 nm (Econo UV monitor, Model EM1, Richmond, CA) to exclude the presence of proteinaceous material. The presence of sugar in the fractions was tested qualitatively with a Molish test.²⁷

**Component Analyses.** The CPS was hydrolyzed with 2 M trifluoroacetic acid at 120 °C for 2 h. The sample was then reduced with NaBH₄ and acetylated, after which it was analyzed with GLC. The absolute configuration of the sugar components was determined by GLC analysis of their acetylated (+)-2-butyl glycoside derivatives (prehydrolysis 15 min, (+)-2-butanol, AcCl, 80 °C, overnight) essentially as described, using also racemic 2-butanol.²⁸

**GLC Analyses.** The alditol acetates were separated using a temperature program of 190 °C for 2 min, 4 °C min⁻¹ up to 220 °C, and then 10 min at 220 °C. The injector and detector temperatures were set to 200 and 250 °C, respectively. Acetylated butyl glycosides were separated using a temperature program of 170 °C for 12 min, 10 °C min⁻¹ up to 220 °C, and then 2 min at 220 °C. The injector and detector temperatures were set to 180 and 250 °C, respectively. Separations were carried out on a PerkinElmer Elite-5 column with hydrogen as carrier gas (25 psi). The column was fitted to a PerkinElmer Clarus 400 gas chromatograph equipped with a flame ionization detector.

**NMR Spectroscopy.** NMR spectra of the CPS (5 mg) in D₂O solution (0.55 mL) were recorded at 20 °C on a Bruker AVANCE III 700 MHz spectrometer equipped with a 5 mm TCI Z-Gradient Cryo-probe. Data processing was performed using vendor-supplied software. Chemical shifts are reported in ppm using internal sodium 3-trimethylsilyl-(2,2,3,3-2H₄)-propanoate (TSP, δ_H 0.00) or external 1,4-dioxane in D₂O (δ_C 67.40) as references.

The assignments of the ¹H and ¹³C resonances of the CPS were obtained by analysis of 1D ¹H and ¹³C NMR spectra together with 2D NMR spectra from a ¹³C-HETCOR experiment,²⁹ ¹H-¹³C-HSQC experiments, including a multiplicity-edited version,³⁰ ¹H-¹H-TOCSY experiments with mixing times of 10, 30, 60, 90, and 120 ms, ¹H-¹H-NOESY experiments with mixing times of 50 and 100 ms, and a ¹H,¹³C-H–H2BC experiment with a constant time delay of 22 ms for the ¹H,¹H evolution. The inter-residue correlations were assigned using ¹H-¹H-NOESY experiments and a ¹H,¹³C-H–HMB experiment with a 6 ms delay for evolution of long-range couplings. The chemical shifts were compared with those of the corresponding monosaccharides.³⁶,³⁷

**RESULTS AND DISCUSSION**

*Leuconostoc mesenteroides* ssp. *cremoris* PIA2, grown in skim milk supplemented by tryptone, produced only CPS but not loosely unattached EPS. The CPS was isolated and purified from the culture by extraction first with saline solution and then with 5% phenol solution, filtered, and dialyzed. The uniformity of the CPS sample was confirmed by size exclusion chromatography. The ¹H NMR spectrum of the CPS (Figure 1) revealed five signals in the region for anomeric protons, indicating pentasaccharide repeating units. Sugar analysis of the CPS showed that all hexasaccharide repeating units. Sugar analysis of the CPS showed that all hexasaccharide repeating units.

The structure of the CPS was subsequently determined using NMR spectroscopy. The ¹H,¹³C-HSQC spectrum (Figure 2)
corroborated that the polymer had pentasaccharide repeating units, and information from the multiplicity-edited version of the experiment identified the chemical shifts from the five methylene groups. The sugar residues are denoted A–E based on decreasing \(^{1}H\) chemical shifts of their respective anomeric protons. The assignments of \(^{1}H\) and \(^{13}C\) resonances were carried out by \(^{1}H\), \(^{1}H\)- and \(^{1}H\),\(^{13}C\)-correlated 2D NMR experiments, and the \(^{1}H\) and \(^{13}C\) NMR chemical shifts are compiled in Table 1. Notably, the \(^{13}C\) chemical shifts of the anomic carbons of residues A–D reside between 107 and 110 ppm, which indicates that these sugar residues have the furanoid ring form. This was confirmed by three-bond correlations observed in the \(^{1}H\),\(^{13}C\)-HMBC spectrum between H1 and C4 in the four residues. The \(^{13}C\) chemical shifts of residue E are consistent with the pyranoid ring form. Thus, four of the sugar residues have the furanoid ring form, whereas one sugar residue has the pyranoid ring form. The anomic configuration of furanosides may be determined from \(^{13}C\) chemical shifts and homonuclear coupling constants for the anomeric protons.

Using the \(^{13}C\) chemical shifts of the anomic carbons of these residues and the small coupling constants of their anomeric protons, viz., \(J_{H1,H2} \approx 1\) Hz for C and D, it is concluded that residues A–D have the \(\beta\)-configuration at their anomeric center. Residue E also has the \(\beta\)-configuration because \(J_{H1,H2} \approx 7.8\) Hz and \(J_{H1,C1} = 160\) Hz.

The substitution positions of the five sugar residues were determined from \(^{13}C\) NMR glycosylation shifts, that is, the difference in chemical shifts to those of the corresponding monosaccharide. These changes in chemical shift are highly indicative of the substitution position(s) in an oligo- or polysaccharide, and they usually have a magnitude of 5 to 10 ppm. The chemical shift displacement is toward a higher chemical shift. Residues A and B are both six-substituted because \(\Delta \delta_{C} = 6.1\) and 6.3, respectively. Residue C is two-substituted because \(\Delta \delta_{C} = 5.9\) and residues D and E are both three-substituted because \(\Delta \delta_{C} = 8.1\) and 7.1, respectively. Therefore, the sugar components and their absolute configuration, ring form, and substitution pattern have all been determined.

The sequence of the sugar residues in the repeating unit of the CPS was determined from the \(^{1}H\),\(^{13}C\)-HMBC experiment (Table 1). At each glycosidic linkage, two or three three-bond heteronuclear correlations were observed. Furthermore, the results from the \(^{1}H\),\(^{1}H\)-NOESY experiment (Figure 3) are in agreement with those from the \(^{1}H\),\(^{13}C\)-HMBC experiment. Therefore, the structure of the repeating unit of the CPS produced by \textit{Leuconostoc mesenteroides} ssp. \textit{cremoris} PIA2 is

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\text{Figure 1.} \quad \text{\(^{1}H\) NMR spectrum of the CPS from \textit{Leuconostoc mesenteroides} ssp. \textit{cremoris} PIA2.}
\]

\[
\rightarrow 3\)-\(\beta\)-\(D\)-\textit{Galp}(1 \rightarrow 6\)-\(\beta\)-\(D\)-\textit{Galp}(1 \rightarrow 2\)-\(\beta\)-\(D\)-\textit{Galp}(1
\]

\[
\rightarrow 6\)-\(\beta\)-\(D\)-\textit{Galp}(1 \rightarrow 3\)-\(\beta\)-\(D\)-\textit{Galp}(1
\]

\[
\rightarrow 3\)-\(\beta\)-\(D\)-\textit{Galp}(1 \rightarrow 6\)-\(\beta\)-\(D\)-\textit{Galp}(1 \rightarrow 2\)-\(\beta\)-\(D\)-\textit{Galp}(1
\]

The homonuclear coupling constants of the anomic protons of the furanosidic residues show for residues A and B, which are six-substituted, larger values of 1.7 Hz, whereas for residues C and D, which are two- and three-substituted, respectively, the anomic resonances were not resolved and the corresponding coupling constants are smaller, estimated to be on the order of 1 Hz. These results indicate different conformational preferences between the two types of furanosic residues present in the CPS.

Analysis of the glycome of mammals and bacteria reveals several characteristcs. When carried out on the level of detail where the sugar, the absolute configuration, the anomic configuration, and the ring form are used as criteria, \(\beta\)-\(D\)-\textit{Galp} is found among the 10 most abundant monosaccharides in bacteria. Notably, it is absent in mammals. \(\beta\)-\(D\)-\textit{Galp} is mostly found in Gram positive \textit{Actinobacteria} and \textit{Bacilli} and to a small extent in Gram negative \textit{Enterobacteria}. In the bacterial taxonomy, \textit{Leuconostoc mesenteroides} ssp. \textit{cremoris} PIA2 belongs to the Gram positive \textit{Leuconostoc} genera. The disaccharide element A–E in the above structure has been previously observed, for example, as part of the cell surface polysaccharide of \textit{Streptococcus oralis} C104, an acidic glycan from the reference strain for \textit{Serratia marcescens}. 

\[
\text{Figure 2.} \quad \text{Multiplicity-edited \(^{1}H\),\(^{13}C\)-HSQC NMR spectrum of the CPS from \textit{Leuconostoc mesenteroides} ssp. \textit{cremoris} PIA2 showing (a) the anomeric region and (b) the region for ring and hydroxymethyl atoms. Correlations for methine groups are shown in black and those for methylene groups are shown in red.}
\]
Table 1. $^1$H and $^{13}$C NMR Chemical Shifts (ppm) of the Resonances from the CPS Produced by Leuconostoc mesenteroides ssp. cremoris PIA2 and Inter-Residue Correlations from $^1$H,$^1$H-NOESY and $^1$H,$^{13}$C-HMBC Spectra$^a$.

<table>
<thead>
<tr>
<th>sugar residue</th>
<th>$^1$H/$^1^3$C</th>
<th>correlation to atom (from anomeric atom)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\rightarrow$-6)$\beta$-D-Galp(1$\rightarrow$) A</td>
<td>5.24 [1.7]</td>
<td>H3, E</td>
</tr>
<tr>
<td></td>
<td>(0.02)</td>
<td>C3, E</td>
</tr>
<tr>
<td>109.98 [175]</td>
<td>82.21</td>
<td>H3, E</td>
</tr>
<tr>
<td></td>
<td>(7.96)</td>
<td>C2, C</td>
</tr>
<tr>
<td>$\rightarrow$-6)$\beta$-D-Galp(1$\rightarrow$) B</td>
<td>5.19 [1.7]</td>
<td>H2, C</td>
</tr>
<tr>
<td></td>
<td>(0.03)</td>
<td>C2, C</td>
</tr>
<tr>
<td>107.94 [174]</td>
<td>82.08</td>
<td>H3, C</td>
</tr>
<tr>
<td></td>
<td>(5.92)</td>
<td>H2, C</td>
</tr>
<tr>
<td>$\rightarrow$-2)$\beta$-D-Galp(1$\rightarrow$) C</td>
<td>5.16 [2.8]$^b$</td>
<td>H6a, A</td>
</tr>
<tr>
<td></td>
<td>(0.14)</td>
<td>C6, A</td>
</tr>
<tr>
<td>107.46 [176]</td>
<td>88.22</td>
<td>H6b, A</td>
</tr>
<tr>
<td></td>
<td>(5.44)</td>
<td>H6a, A</td>
</tr>
<tr>
<td>$\rightarrow$-3)$\beta$-D-Galp(1$\rightarrow$) D</td>
<td>5.09 [2.7]$^b$</td>
<td>H6a, B</td>
</tr>
<tr>
<td></td>
<td>(0.30)</td>
<td>C6, B</td>
</tr>
<tr>
<td>108.92 [175]</td>
<td>80.49</td>
<td>H6b, B</td>
</tr>
<tr>
<td></td>
<td>(6.90)</td>
<td>H6a, B</td>
</tr>
<tr>
<td>$\rightarrow$-3)$\beta$-D-Galp(1$\rightarrow$) E</td>
<td>4.63 [7.8]$^b$</td>
<td>H3, D</td>
</tr>
<tr>
<td></td>
<td>(0.10)</td>
<td>C3, D</td>
</tr>
<tr>
<td>103.12 [160]</td>
<td>70.66</td>
<td>H3, D</td>
</tr>
<tr>
<td></td>
<td>(5.75)</td>
<td>(0.03)</td>
</tr>
</tbody>
</table>

$^a$ $J_{\text{H}_1,\text{H}_2}$ and $J_{\text{C}_1,\text{H}_1}$ Values are given in Hertz in square brackets and in braces, respectively. Chemical shift displacements $\Delta \delta$ are reported in parentheses, compared to the corresponding galactose residue. $^b$ Full width at half-maximum. $^c$ May be interchanged.

Figure 3. Part of a $^1$H,$^1$H-NOESY NMR spectrum ($t_{\text{mix}} = 100$ ms) of the CPS from Leuconostoc mesenteroides ssp. cremoris. Trans-glycosidic correlations from anomeric protons are annotated.

serogroup O22 and the Streptococcus pneumoniae type 29 polysaccharide.44–46 The structural disaccharide element D–B makes up the backbone of the galactose-rich polysaccharide in the cell wall of the Gram-positive bacterium Renibacterium salmoninarum, in which it is repeated twice in forming a repeating unit because every other six-substituted $\beta$-D-Galp residue carries a side-chain consisting of three sugar residues.47 A homogalactan is present in mycobacterial arabinogalactans where the five- and six-substituted $\beta$-D-Galp residues alternate in the polysaccharide.48

Another unusual galactan structure of the EPS from LAB was found to be produced by Lactococcus lactis ssp. cremoris H414, and it was composed of a branched pentasaccharide repeating unit of $\beta$-galactopyranosyl residues.49 A galactan structure of a microbial polysaccharide has also been found as a constituent of the cell wall, viz., as part of a lipopolysaccharide (LPS), that is, in the structure of the O-polysaccharide from the LPS of a Hafnia alvei strain. The O-antigen was composed of different disaccharide repeating units of $\beta$-galactose, where two distinct regions, which are connected, were identified.49

A number of LAB strains have been reported to produce both EPS and CPS. In this study, the strain Leuconostoc mesenteroides ssp. cremoris PIA2 was seen to produce only CPS without loose unattached EPS. LAB strains producing only the capsular type have not been previously confirmed.50 Low et al.51 were the first to report the constituent monosaccharides of capsular EPS from the LAB; that is, the CPS of Streptococcus thermophilus MR-1C was composed of repeating monomers of galactose, rhamnose, and fucose in the molar ratio of 5:2:1. Later, Robitaille et al.52 suggested using fluorescently labeled lectins that the lectin PNA (peanut agglutinin) was specifically bound to galactose-containing CPS located at the cell surface of MR-1C. Binding of the CPS at the cell surface was strong and probably electrostatic in nature. Lactobacillus plantarum EPS6 isolated from corn silage was shown to produce both cell-bound EPS and released EPS composed of glucose, galactose and N-acetylgalactosamine in a molar ratio of approximately 3:1:1 and also phosphate.53 Recently, Yang et al.54 reported on the CPS of Lactobacillus rhamnosus JAAS8 to be composed of $\beta$-galactose and N-acetylglucosamine in a molar ratio of 5:1. The SMFS (single-molecule force spectroscopy) showed that the cell wall of L. rhamnosus GG contains two different types of surface polysaccharides: long galactose-rich polysaccharides and also shorter glucose-rich polysaccharides.55 The occurrence of the former is
fully consistent with our previous structural studies of the EPS isolated from HYLA milk. In addition, TEM studies show that L. rhamnosus GG produces CPS when grown in HYLA milk or in MRS. The CPS is very likely to contain shorter glucose-rich polysaccharides.

Although many Lactobacillus strains used as probiotics are believed to modulate host immune responses, the molecular nature of the components of such probiotic microorganisms directly involved in immune modulation processes are largely unknown. Kekkonen et al. reported some potentially probiotic LAB strains to have immunomodulatory effects, but the role of the EPS/CPS as immunomodulators was not included in the study. Leuconostoc mesenteroides sps. cremoris PIA2 of this study and Streptococcus thermophilus THS were more potent inducers of Th1-type cytokines interleukin-12 (IL-12) and interferon-γ (IFN-γ) than the other probiotic Lactobacillus strains used in that study.

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Notes
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(11) 71 €Dr. Soile Tynkkynen and Mrs. Anja Valjus-Tiensuu of Valio Ltd., Finland (project number 210653), the Swedish Research Council, were more potent inducers of Th1-type cytokines interleukin-12 (IL-12) and interferon-γ (IFN-γ) than the other probiotic Lactobacillus strains used in that study.
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