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Structural studies of the cell wall polysaccharide from *Lactococcus lactis* UC509.9

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ABSTRACT

Lactococcus lactis is the most widely utilised starter bacterial species in dairy fermentations. The *L. lactis* cell envelope contains polysaccharides, which, among other known functions, serve as bacteriophage receptors. Our previous studies have highlighted the structural diversity of these so-called cell wall polysaccharides (CWPSs) among *L. lactis* strains that could account for the narrow host range of most lactococcal bacteriophages. In the present work, we studied the CWPS of *L. lactis* strain UC509.9, an Irish dairy starter strain that is host to the temperate and well-characterized P335-type phage Tuc2009. The UC509.9 CWPS structure was analyzed by methylation, deacetylation/deamination, Smith degradation and 2D NMR spectroscopy. The CWPS consists of a linear backbone composed of a tetrasaccharide repeat unit, partially substituted with a branched phosphorylated oligosaccharide having a common trisaccharide and three non-stoichiometric substitutions.

1. Introduction

Lactococcus lactis is one of the most widely utilised starter bacterial species in dairy fermentations globally. Owing to its economic importance, this species has been the subject of intense research scrutiny in recent decades, and its potential as an alternative to yeasts and *Escherichia coli* as a cell factory for the production of metabolites or other bioactive compounds, in addition to its traditional starter culture function, has been explored [1–3]. The cell envelopes of lactococci have recently been shown to contain a rhamnose-rich or rhamnan component, which is embedded within the cell wall [4], and, in some cases, a thin outer layer, termed the polysaccharide "pellicle" (PSP), which has been implicated in bacteriophage recognition and attachment [5]. To date, the PSP structures of three lactococcal strains are known, being composed of repeating phospho-penta or -hexasaccharides [5–7].

In 2004, the variable gene cluster responsible for the biosynthesis of a cell wall polysaccharide (CWPS) was identified [8]. This gene cluster (termed the *cwps* gene cluster) is typically 25–30 kb in length with a conserved region proposed to encode the biosynthetic machinery for the rhamnan component and a more divergent region that encodes the enzymes for PSP biosynthesis [4,9]. Based on focused sequence analysis of the *cwps* gene cluster, it was possible to define three genotypes (A, B and C), while additional genotypes were also predicted [9]. Furthermore, *cwps* C-(geno)type strains were further grouped into five subtypes, highlighting the genetic diversity and likely structural complexity of lactococcal CWPS [6]. The three lactococcal strains for which the CWPS structure has been defined all belong to the C-type, while little is currently known about the CWPS structure of the remaining genotypic groups (i.e. A and B).

L. lactis UC509.9 is an Irish dairy starter strain that is host to the well characterised temperate P335-type phage Tuc2009 [10]. Tuc2009 has been the subject of several recent studies aimed at elucidating the intricate mechanisms by which tailed phages

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recognise and attach to their hosts [11–17]. Since many lactococcal phage groups, including the P335 phages, recognise saccharidic receptors on the host cell surface, it is essential that detailed characterisation of host CWPS structures are resolved in order to derive meaningful conclusions regarding the specific nature of the receptors of tailed phages. Tuc2009 and its host have become a model system for understanding phage-host interactions thus the analysis of the CWPS of UC509.9 is highly relevant. In the current study, the CWPS of *L. lactis* strain UC509.9, genotypically classified as a *cwps* A-type strain, was determined to be a complex rhamnose-rich structure that is distinctly different from those of the C-type strains.

2. Results

2.1. Extraction and purification of the CWPS

The standard method of extraction of CWPS with cold TCA, used previously for other *L. lactis* strains [5], led to a very poor yield of carbohydrate material. Cell debris was therefore re-extracted with 0.01 M and 0.1 M HCl (100 °C, 20 min), and the obtained extract was purified as described previously [4,18]. Polysaccharide material extracted with TCA and HCl had identical monosaccharide composition and methylation profiles. The sample obtained following hot diluted HCl extraction and re-purification (see Experimental section) provided a satisfactory yield of CWPS (approximately 30–35 mg per liter of culture), which was sufficient for further use in NMR and chemical analyses, including those that involved different chemical modifications: dephosphorylation, Smith degradation and deamination treatments.

2.2. Structural elucidation of the CWPS

The CWPS preparation contained L-Rha, D-Glc, D-Gal, and D-GlcN in an approximate ratio of 3.7:1:0.5:0.5 as revealed by monosaccharide compositional analysis. The NMR spectra for this CWPS were highly heterogeneous, containing signals of different intensities (Fig. 1). However, it was not possible to separate the CWPS preparation into more defined compounds by size exclusion and anion-exchange chromatography. This polymer and other compounds described below were analyzed by 2D NMR. Monosaccharides were identified by COSY, TOCSY and NOESY cross peak patterns and ^{13}C NMR chemical shifts. Connections between monosaccharides were determined from transglycosidic NOE and HMBC correlations. Anomeric configuration of Rha residues was determined from TOCSY and NOESY patterns, and the C-5 signal position at ~70 ppm (expected ~73 ppm for β-Rha), being characteristic for α-Rha (Fig. S2).

The polysaccharide contained β-galactopyranose, substituted at O-6 by 1-glycerophosphate, identified in 2D NMR spectra (Table 1). Both H-6 of β-Gal and H-1 of glycerol yielded correlation signals with the same phosphate at 0.5 ppm in the ^{1}H-^{31}P HMQC spectrum, indicating substitution of β-Gal O-6 with 1-glycerophosphate. Methylation of dephosphorylated CWPS led to the identification of 2-substituted Rha and 6-substituted Glc (all sugars in pyranose form) as the main components in a ratio of approx. 3:1. Other methylated monosaccharides were terminal, 3-, 2,3-, and 2,4-substituted Rha, terminal Glc, terminal Gal, 3- and 3,6-substituted GlcN. Attempts to determine the structure of intact original polysaccharide by 2D NMR methods led to the identification of several fragments, but it was difficult to find linkages between them. Therefore, this complex CWPS was chemically modified by deamination and periodate oxidation.

2.3. Deamination of CWPS and structural analysis of products

The CWPS was deacylated and desalted on a Biogel P6 column in an effort to reduce the background noise, but in general appeared highly similar to the original preparation, without an N-Ac signal, and H-2 of GlcN had shifted to high field (2.80 ppm). The deacylated CWPS was deaminated by $NaNO_2$/AcOH and products separated on a Sephadex G15 column resulted in a preparation of a deaminated polysaccharide (**DPS**) and a mixture of oligosaccharides. The DPS had a simple linear structure and was composed of tetrasaccharide repeating units containing one 6-α-Glc and three 2-α-Rha residues (Figs. 1 and 2). The structure was solved using 2D NMR (Table 1, Figs. 3 and 4). In agreement with NMR data, methylation analysis of the **DPS** highlighted the presence of 6-substituted Glc and 2-substituted Rha.

In addition to the **DPS**, four oligosaccharides OS1-4 could be identified (Fig. 2). Their structures were determined by 2D NMR (Table 1). Oligosaccharides exhibited a common trisaccharide fragment **F-E-N**, with a 3-substituted 2, 5-anhydro-mannose **N** (product of the deamination of glucosamine) at the reducing end. Assignment of residue **E** signals was very problematic due to considerable signal overlap. The common trisaccharide was substituted with non-stoichiometric units, β-Gal **M**, α-Rha **G** and α-Glc **X** (**OS 1–4**). Units **G** and **X** were present only in different oligosaccharides, never occurring together in the same structure. Glycerophosphate from β-Gal **M** was completely lost in the deamination products.

Thus, structural data of deamination products of *L. lactis* UC509.9 CWPS indicate that the latter is composed of a linear backbone **DPS**, which carries various oligosaccharide side chains with GlcNAc **N** at a branching point.

2.4. Smith degradation and NMR analysis of the original CWPS

Methylation analysis of the dephosphorylated CWPS showed, among other products, the presence of 2,4-substituted Rha. This indicated the branching of lateral chains at O-4 position of one of Rha residues **B**, **A** or **D**. In order to identify the linkage points of the side chains to the main DPS backbone, the original CWPS material was subjected to Smith degradation. It afforded a mixture of oligosaccharides, which were analyzed by NMR, compositional and methylation analyses. It contained exclusively Rha and GlcNAc in an approximate molar ratio of 1:0.8. Methylation analysis afforded the identification of a terminal Rha, 4-linked Rha and a terminal GlcNAc as the major products, as well as small amounts of 2-, and 3-, and 2,4-linked Rha. The main product OS5 (Fig. 1) identified by NMR analysis was a disaccharide β-GlcNAc-4-α-Rha-2-glyceraldehyde. This structure was in good agreement with the results of monosaccharide composition and methylation analysis. It exhibited 2-substituted glyceraldehyde at the reducing end, corresponding to a product of Smith degradation of a 2-substituted sugar. Accordingly, β-GlcNAc **N** from the OS side chains was assigned to represent an O-4 attachment of the Rha residue **A** or **D** of the **DPS** linear backbone.

Careful examination of the 2D NMR spectra (Fig. 3) of the original (i.e. untreated) CWPS material revealed that each of the components of the **DPS** had a lower intensity duplicate (**A′**, **B′**, **C′**, **D′**) in the spectra of the CWPS. Rha residues **B′** and **D′** were not substituted at O-4, as followed from their C-4 chemical shifts. Their H-4 signals were well visible in TOCSY, and displayed no NOE cross-peaks with H-1 of GlcNAc **N**. However, a NOE correlation was observed from H-1 of GlcNAc **N** to the signal at 3.69 ppm, which pointed to a signal at 81.0 ppm in the HSQC spectrum (Fig. S1). This signal was assigned to H4/C4 of Rha **A'** (Table 1). Long range ^{1}H-^{13}C HMBC correlation from Rha H-6 (Fig. S2) had a correlation to this