

Analysis and production of two exopolysaccharides from *Lactococcus lactis* subsp. *cremoris* LC330

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SUMMARY. Two polysaccharides produced concurrently by *Lactococcus lactis* subsp. *cremoris* strain LC330 have been identified. One had a high molecular mass ($> 1 \times 10^6$ Da) and was neutral. The second was smaller (~ 10000 Da), charged and had a high phosphorus content. Sugar composition also differed. In chemostat culture the neutral polysaccharide was influenced by temperature and by nitrogen limitation. This polysaccharide was branched with terminal galactose moieties and contained galactose, glucose and glucosamine. The phosphopolysaccharide was more complex with glucose, rhamnose, galactose and glucosamine in an approximate ratio of 6:5:4:1.

A number of authors (Brooker, 1976; Macura & Townsley, 1984; Cerning *et al.* 1986, 1988, 1992; Doco *et al.* 1989, 1990, 1991; Nakajima *et al.* 1990; Toba *et al.* 1990) have investigated the ropy nature of milk fermented with mesophilic and thermophilic dairy lactic acid bacteria. It is generally accepted that the ropiness produced by these bacteria is related to synthesis and secretion of exopolymers (for review, see Cerning, 1990).

Macura & Townsley (1984) suggested that the ropy characteristic of milk-grown cultures was the result of a glycoprotein of which 47% was proteinaceous in nature. Schellhaass (1983), however, found that the exopolymer isolated from cultures grown in milk ultrafiltrate to which casamino acids were added contained 85% carbohydrate, composed of glucose and galactose. There now appears to be a consensus that, unlike the homopolysaccharides produced by mesophilic leuconostoc bacterial species, those from the lactococci are heteropolysaccharides. There is disagreement concerning the composition of the heteropolysaccharide, although the presence of glucose and galactose is always reported. Some differences in composition may be due to a lack of distinction between extracellular, capsular polysaccharide and exopolysaccharide that can be dissociated easily from the cell wall, but such distinction is difficult (Sutherland, 1982). For example, Toba *et al.* (1991) examined capsules removed from cells by sonication of strains of *Lactococcus lactis* subsp. *cremoris* and found that they contained 44% protein and only 22% carbohydrate.

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These authors found that the polysaccharide fraction of the capsular material contained rhamnose, glucose, glycerol and phosphorus and suggested that it was deacylated lipoteichoic acid. Macura & Townsley (1984) found a similar composition in the carbohydrate fraction of the glycoprotein isolated from whey-grown cultures of lactococci. Others (Schellhaass, 1983; Cerning *et al.* 1992; Cowie, 1993) found that the polymer precipitated from a culture supernate with ethanol was predominantly carbohydrate.

Synthesis and secretion of exopolysaccharide occur during different growth phases and may be regulated by proteins located on the cell surface (Forsén & Häivä, 1981; Kontusaari & Forsén, 1988). In some cases the ropy character may be associated with plasmid DNA. This has been reported for the ropy strains of *Lactobacillus casei* NCIB 4114 (Vescovo *et al.* 1989), *Lb. casei* CG11 (Vedamuthu & Neville, 1986; Kojic *et al.* 1992) and for strains of lactococci (Von Wright & Tynkkynen, 1987; Neve *et al.* 1988). Heteropolysaccharides are made by polymerizing precursors formed in the cell cytoplasm. Here sugar nucleotides are formed which play an important role, as does the lipid isoprenoid carrier located within the cytoplasmic membrane. The lipid carrier is also involved in syntheses of cell wall lipopolysaccharide, peptidoglycan and teichoic acid, so there is competition for this facilitating membrane component during different phases of growth. The competition may explain the appearance of exopolymers and capsules during different phases of growth and different growth conditions (Sutherland, 1982).

This paper reports on a ropy strain of *Lc. lactis* subsp. *cremoris* where two different exopolysaccharides, with different composition and molecular mass, could be isolated from the fermentation medium. In addition we report that the production of the two polymers was influenced by different growth conditions.

MATERIALS AND METHODS

Organism and growth conditions

Lc. lactis subsp. *cremoris* LC330 (*Lc. lactis*) was obtained from the Nestlé Culture Collection (CH-1000 Vers-Chez-les-Blanc, Switzerland). It was maintained on glass beads at $-70\text{ }^{\circ}\text{C}$ (Jones *et al.* 1984), resuscitated and routinely grown in M17 (Lab M, Bury BL9 6AU, UK) liquid medium. All experiments were carried out in *cremoris* defined medium (CDM), based on a defined medium described by Otto *et al.* (1983). Glucose, galactose or lactose at 20 g/l was added and the medium filter sterilized ($0.45\text{ }\mu\text{g}$; Millipore Ltd, Watford WD1 8YW, UK) and stored at $4\text{ }^{\circ}\text{C}$. For experiments with limiting lactose, the sugar was replaced by lactose at 2.5 g/l. For nitrogen-limiting growth the concentration of glucose, galactose and lactose remained at 20 g/l, trisodium citrate replaced triammonium citrate and casamino acids were added at 300 mg/l. An inoculum (10 g/l) from an overnight M17 medium at $30\text{ }^{\circ}\text{C}$ was used to start the fermentation. For batch experiments, cultures were grown in 250 ml conical flasks at $30\text{ }^{\circ}\text{C}$ unless otherwise stated.

Chemostat cultures

Cultures (700 ml) were maintained at constant temperature and pH in a Microlab fermenter (LH 500; LH Fermentation Ltd, Reading RG2 0EB, UK). Samples (10 ml) were removed for analysis and steady state re-established by allowing at least three fermenter volumes to flow through the system before further sampling.

Exopolysaccharide separation and determination

Cultures from CDM were centrifuged at 1000 g for 10 min to remove cells and the supernatant fluid (10 ml) was dialysed (molecular mass exclusion, 12000 Da) at 4 °C for 4 d against frequent changes of distilled water. Total carbohydrate was determined with phenol-sulphuric acid, measuring absorbance at 490 nm and using glucose standard solutions (Dubois *et al.* 1956). Neutral sugars were determined by gas chromatography (Carlo-Erba 4160, equipped with on-line injector and flame-ionization detector; Carlo Erba Strumentazione, I-20090 Rodano, Italy) as the *o*-methyloxime derivatives after hydrolysis with 4 M-trifluoroacetic acid (Neeser & Schweizer, 1984). The derivatives were separated on a Carbowax 20M NP fused silica capillary column (0.3 mm × 25 m) with hydrogen (70 kPa) as the carrier gas. Sugar linkages were determined after methylation according to the method of Hakomori (1964). Permethyl D-allose and quebrachitol (Aldrich Chemical Co., Gillingham SP8 4JL, UK) were used as the internal standards. Partly methylated alditol acetates were separated by gas chromatography (Carlo-Erba 5160) on three different column packings: SP-1000 (Sepelco Ltd, Poole BH17 7NH, UK), CPSiL88 and OV-1 (Chrompack UK Ltd, London E14 9TN, UK) using helium as the carrier gas, and identified by their retention times and by mass spectrometry (Lomax *et al.* 1983).

Acidic and neutral polysaccharides were separated using DEAE sepharose. Molecular masses were determined using gel filtration chromatography (Sephacryl CL 4B and Sephacryl S300; Pharmacia Biotechnology, St Albans AL1 3AW, UK) by comparing retention times with known dextran standards.

Phosphorus determination

Phosphorus was determined using the method of Dittmer & Wells (1969).

RESULTS

Lc. lactis grown in CDM as a batch culture without pH control achieved a maximum population of 5.6×10^8 cfu/ml. Exopolysaccharide was produced towards the end of the exponential phase and during stationary phases of growth (Fig. 1). The total exopolysaccharide produced was 25 (SD 2.0) µg/ml from three determinations.

Analysis of polysaccharides

The polysaccharide produced by strain LC330 in CDM was resolved by column chromatography into two components. The neutral polysaccharide was eluted with the void volume and the charged polysaccharide, which bound to the DEAE, was eluted using M-NaCl. The neutral polysaccharide was large with a molecular mass in excess of 2×10^6 Da. The charged polysaccharide had a molecular mass corresponding to 10000 Da.

Gas chromatography of the *o*-methyloxime acetate derivatives showed that the charged polysaccharide was composed of glucose, rhamnose, galactose and glucosamine in an approximate ratio of 6:5:4:1 with a phosphorus content of 56 g/kg. The larger, neutral polysaccharide had a simpler sugar content of glucose, galactose and glucosamine in an approximate ratio of 6:3:2. Nakajima *et al.* (1990) also found a phosphopolysaccharide from a strain of *Lc. lactis* subsp. *cremoris* composed of a branched repeat unit of rhamnose, galactose, and glucose in a molar ratio of 1:2:2, but no glucosamine was found.

Previous work (J. Cerning, pers. comm.) has indicated a change in polysaccharide composition isolated from *Lb. casei* as a result of growth on different sugars. The

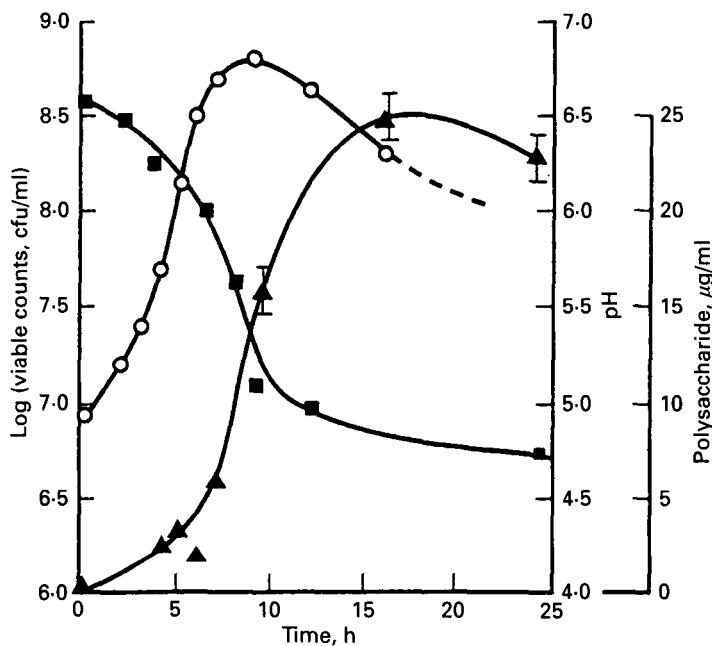


Fig. 1. Growth of, and acid and polysaccharide production by, *Lactococcus lactis* subsp. *cremoris* LC330 in a batch-grown culture at 30 °C without pH control: ○, viable count; ▲, polysaccharide; ■, pH. Cultures were grown in cremoris defined medium with lactose as the carbon source. Values are means for three experiments with SD indicated by vertical bars.

Table 1. *Sugar linkages of neutral exopolysaccharide from Lactococcus lactis strain LC330†*

Linkage	Ratio of linkages with different sugar sources		
	Lactose	Glucose	Galactose
Gal—1—	1·0	1·0	1·0
—4—Gal—1— 3 	1·3	1·2	1·3
—4—Gluc—1—	2·4	2·3	2·4
—6—Gluc—1—	1·1	1·1	1·2

Gal, galactose; gluc, glucose.

† Cultures were grown at 30 °C in cremoris defined medium plus lactose to late stationary phase and the neutral exopolysaccharide was separated using gel chromatography and analysed as methylated alditol acetate derivatives by gas chromatography.

Convention: —4—Gal—1— indicates that carbons 1, 3 and 4 of the galactose moiety are involved in linkages.

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Table 2. Effect of temperature on exopolysaccharide production from *Lactococcus lactis* strain LC330 grown as a batch culture in cremoris defined medium

Temperature, °C	Exopolysaccharide, µg/ml†		
	Total	Neutral	Acidic
20	55 ± 5.5	62	10
25	50 ± 4.5	65	10
30	50 ± 4.0	18	10
35	32 ± 3.0	ND	ND

ND, not determined.

† Values for total exopolysaccharide are means ± SD for four experiments; neutral and acidic values were determined in a separate experiment.

composition of the neutral polysaccharide from *Lc. lactis* grown in CDM with lactose, glucose or galactose as the carbon source was the same. Further analysis of methylated alditol acetates revealed similar linkage patterns in polysaccharides produced with different carbon sources (Table 1). The linkage patterns revealed a branched polysaccharide which had terminal galactose residues.

Growth conditions and polysaccharide production

Strain LC330 grown in continuous culture in CDM under lactose limitation at different temperatures produced more neutral polysaccharide at the lower growth temperatures (Table 2). At 40 °C LC330 produced very little polysaccharide and growth was poor.

When cultures were limited by availability of nitrogen a marked effect was noted when the amount of polysaccharide per dry weight of cells was measured. Nitrogen limitation increased the amount of neutral polysaccharide produced (from 58 µg/mg for carbon limited cells to 92 µg/mg cells) but had only a slight effect on the phosphopolysaccharide, reducing the production from 23 µg/mg cells under carbon limitation to 18 µg/mg cells when nitrogen limited. When incubation temperature was varied, different effects were noted for the two polysaccharides: more neutral polysaccharide was produced at the lower temperatures, but there was little temperature effect on production of phosphopolysaccharide.

DISCUSSION

There has been considerable interest in the secretion of polymers by lactic acid bacteria. A recent publication has revealed the structure of an exopolysaccharide from *Lb. delbrueckii* subsp. *bulgaricus* as having galactose, glucose and rhamnose in a molar ratio of 5:1:1 with predominantly 1 → 4 and 1 → 3 linkage patterns (Gruter *et al.* 1993). Similarly, Doco *et al.* (1989, 1990, 1991) have reported that the exopolysaccharide from *Streptococcus thermophilus* was composed of galactose, glucose and *N*-acetylgalactosamine in a molar ratio of 2:1:1 with 1 → 3 and 1 → 6 linkage patterns.

Our results with strain LC330 of *Lc. lactis* subsp. *cremoris* also indicated the presence of branched heteropolysaccharide. However, we showed that two polysaccharides of different composition were produced. The results presented in this paper on the larger neutral polysaccharide are in agreement with other reports (Schellhaass, 1983; Toba *et al.* 1991). However, direct comparisons cannot be made as no mention is made elsewhere of any heterogeneity of the polysaccharide (i.e. the possibility of two types).

There were differences in size and charge between the two polysaccharides, the charged polysaccharide having an association with phosphate. Earlier work with CDM showed that growth and acid production by this strain was similar to that achieved in milk (Cowie, 1993) and total polysaccharide production was also similar. The CDM was designed so that growth and polymer production could be achieved from utilization of small molecular mass compounds, thus allowing good recovery of polysaccharides. Sugar linkage patterns of total polysaccharide from milk were also found to be similar to those for the total polysaccharide from CDM (Cowie, 1993). Gruter *et al.* (1992) also examined polysaccharide from *Lc. lactis* subsp. *cremoris* strain H414 grown in defined medium and in milk. These authors found that the polysaccharides from milk and defined medium had similar composition, but it differed from that of strain LC330 reported in this paper, in that it was composed only of D-galactose. This underlines the importance of the strain.

The large neutral polysaccharide was influenced by growth conditions. Sutherland (1982) would predict that for heteropolysaccharides that are extracellular rather than cell associated, slow growing cells (i.e. cells growing below their optimum growth temperature, in this case 30 °C) would be able to produce more polymer because of increased availability of the isoprenoid carrier. The results presented may, therefore, indicate that the neutral polysaccharide was exocellular and that the phosphopolysaccharide was more closely associated with cell wall material. The results shown in Fig. 1 would indicate that exopolysaccharide synthesis towards the end of exponential phase was likely to begin with synthesis of the neutral polymer and that the phosphopolysaccharide was a minor component of the total polysaccharide. The presence of two polysaccharides produced at different growth phases may also occur in other milk fermentations and in part explain the results of Gancel & Novel (1994), who found different modes of polymer production in response to temperature and sugar availability, with hyperproduction occurring at onset of the stationary phase.

Although a minor component, the phosphopolysaccharide was found consistently. It was smaller and more complex and was not subject to changes in growth conditions. It is proposed, therefore, that this smaller polysaccharide is associated with cell wall material and its synthesis is linked to growth but can become detached. Nakajima *et al.* (1992), in their work with an isolate of lactococci from the Scandinavian fermented milk *långfil*, found a phosphopolysaccharide of similar composition but with a larger molecular mass. Previous workers (Schellhaass, 1983; Doco *et al.* 1991; Cerning *et al.* 1992) have isolated exopolysaccharide by ethanol precipitation. The method used in the work reported in this paper employed exhaustive dialysis to remove small molecular mass compounds. The carbohydrate content of the retentate was then analysed. This permitted good resolution of the two components.

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