Characterization of Loosely Associated Material from the Cell Surface of Lactococcus lactis subsp. cremoris E8 and Its Phage-Resistant Variant Strain 398

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Loosely associated material (LAM) was isolated by gentle extraction procedures from the cell surface of Lactococcus lactis subsp. cremoris E8 and its phage-resistant variant strain 398. LAM from both strains was chemically characterized, and its role in the adsorption of three small isometric bacteriophages, Φ 618, Φ 833, and Φ 852, to the cell surface of the two strains was investigated. The phage-resistant strain (strain 398) produced LAM which differed significantly from the material produced by the parent strain. The total yield of LAM from strain 398 was two- to threefold higher than that from strain E8, and the material contained fivefold more rhamnose and twofold more galactose. Polyclarlamide gel electrophoretic analysis showed that LAM from strain 398 lacked a 21-kDa protein which was present in LAM from the parent strain. Inhibition studies of phage binding by using isolated LAM from two strains showed that although LAM from strain E8 reduced the titer of Φ 618 and Φ 852 by 53 and 82% respectively, LAM from strain 398 had no effect on the plaque-forming ability of any of the three phages tested. Treatment of LAM from strain E8 with sodium metaperiodate destroyed its ability to bind with Φ 618 and Φ 852. Phenotypically, strain 398 differed from its parent strain E8 in that it was more prone to cell lysis and required an osmotically adjusted buffer system for the extraction of LAM.

Lactococci are an important group of microorganisms for the dairy manufacturing industry, where they are used as starter cultures to initiate milk fermentation. One of the major causes of failed fermentation and consequential loss of product in cheese manufacture is bacteriophage infection of the starter cultures. Traditionally, the industry has routinely countered this problem by physical exclusion of phages with strict hygiene and sanitation procedures (7) and by the development of commercial starter culture practices which decrease phage-related problems (9). However, these practices minimize rather than eliminate the risk of phage infection. More effective and sustainable solutions to this problem require a basic understanding of the process of phage infection.

The prerequisite to any successful phage infection of a bacterial cell is the specific recognition by the phage of a receptor molecule on the host cell. Molecular interactions between bacteriophages and gram-negative bacteria, particularly Escherichia coli, have been extensively studied (for a review, see reference 10). The phage receptors in these organisms include cell wall lipopolysaccharides and specific outer membrane proteins. However, in gram-positive bacteria, with one notable exception (19), it is invariably the cell surface carbohydrates that are involved in phage adsorption, such as in Bacillus subtilis (33), Streptococcus pyogenes (3, 8), and Lactobacillus casei (11, 30, 32). One of the earliest reports of the nature of phage receptors in lactic streptococci appeared more than 20 years ago when Oram (19) demonstrated the involvement of membrane protein in phage adsorption in Streptococcus lactis ML3. Recently, much attention has been paid to the phage resistance mechanisms of lactic acid bacteria (for reviews, see references 14 and 20), but data on the biochemical characterization of phage receptors in lactococci remain rudimentary.

In the last few years there has been a renewed interest in defining the mechanism(s) of phage adsorption in starter lactococci (17, 21–24, 28, 29). Most of these studies, however, have used either isolated cell walls or membrane fractions to identify cell surface components involved in phage adsorption. This approach ignores the role of noncovalently bound extracellular material in phage recognition, since most of this material is lost during the procedures used to isolate the cell walls. The present study reports the isolation and characterization of a material which is loosely associated with the cell surface of two strains of Lactococcus lactis subsp. cremoris (strain E8 and its phage-resistant variant strain 398). This material may represent the outermost layer of the cell envelope, and we show that it actively interacts with the phage, which suggests that it may be involved in phage adsorption.

MATERIALS AND METHODS

Organisms and culture conditions. All strains of bacteria and bacteriophages were from the culture collection held at the New Zealand Dairy Research Institute. L. lactis subsp. cremoris 398 was derived previously from L. lactis subsp. cremoris E8 by phage challenge as described by Limswotin and Terzaghi (16). Static batch cultures were grown at 30°C in autoclaved reconstituted skim milk (RSM) buffered with β-glycerophosphate solution (autoclaved and added separately at 23 g/60 ml of H2O in 1 liter of sterilized RSM [27]). Φ 852 was isometric, with a head of ca. 58 nm in diameter and a tail ca. 140 nm long (16). The two other phages used in this study, namely Φ 618 and Φ 833, also had similar morphology (the sizes of the head and tail were not measured).

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Extraction of loosely associated material (LAM). To 1 liter of RSM culture was added 60 ml of 25% (wt/vol) sodium citrate, and the pH was adjusted to 7.0 with 4 M NaOH. This treatment dissolves the caseins of RSM, and the culture becomes clear. Cells were harvested from the clarified RSM by centrifugation (12,000 x g for 10 min). Harvested cells were thoroughly washed with cold (4°C) 50 mM sodium acetate buffer (pH 6.4) to remove the remnants of the medium and any caseins from the surface of harvested cells. Cell pellets were resuspended in 50 ml of prewarmed (30°C) 0.35 M glycylglycine buffer (pH 7.5) containing 10 mM MgCl₂. The suspension was incubated at 30°C for 30 min in a water bath and gently shaken at 50 rpm. Solubilized material was removed by centrifugation (12000 x g for 10 min) and dialyzed against deionized water for 24 h with three changes of water. The dialyzed material was freeze-dried.

Identification and quantitation of monosaccharides. Component monosaccharides of the LAM were identified and quantitated after total acid hydrolysis by gas-liquid chromatography and by high-performance anion-exchange chromatography on a carbohydrate analyzer equipped with a pulsed electrochemical detector and an advanced computer interface (Dionex Corp., Sunnyvale, Calif.) linked to a 386 Unicon computer.

A 10-ml sample of material was suspended in 2 ml of 3 M HCl in a hydrolysis tube, evacuated, and heated for 5 h at 100°C to hydrolyze the polysaccharides. In some cases the hydrolysis time was increased to 18 h to release glycerol from teichoic acids. For the gas-liquid chromatography analysis, the hydrolysates were neutralized with ammonium hydroxide and converted to alditol acetates as described by Blakeney et al. (2). Alditol acetates were separated on a DB225 capillary column (30-m column with internal diameter of 0.25 mm and film thickness of 0.25 μm; J & W Scientific, Folsom, Calif.) connected to a Shimadzu 16 A gas chromatograph equipped with a flame ionization detector. Chromatographic conditions were those recommended by the column manufacturer. Peak areas and retention times were determined with a Shimadzu CR6A Chromatopac electronic integrator. Peaks were identified by comparing the retention times of standard compounds derivatized in a similar fashion.

For analysis on the Dionex system, the hydrolysates were taken to dryness on a SpeedVac evaporator (Savant Instrument Inc., Farmingdale, N.Y.) to remove the acid and then reconstituted in deionized water. Insoluble material was removed from the samples by centrifuging in a microcentrifuge at 12,000 x g, and then a fixed volume (25 μl) of hydrolyzed material was injected directly into the system. The analysis was carried out on a CarboPac PA-1 (4 by 250 mm) pellicular anion-exchange column (Dionex Corp.) with appropriate guard. The column was equilibrated with 16 mM NaOH, and the neutral and basic monosaccharides were separated by eluting the column isocratically with 16 mM NaOH at a flow rate of 1.0 ml min⁻¹. The column was washed with 100 mM NaOH for 10 min at the end of each run and reequilibrated in 16 mM NaOH. The pulsed electrochemical detector was used in integrated amperometry mode at carbohydrate settings to detect and quantify various monosaccharides.

Standards were run under similar conditions to determine the retention times and the response factors. Standard curves were generated for individual monosaccharides, and controls were run to correct for losses of material that occurred during the hydrolysis and further processing of samples.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by the method of Laemmli (15) with the Protean II minigel system (Bio-Rad Laboratories, Richmond, Calif.). A stacking gel containing 5% acrylamide (acrylamide/bisacrylamide ratio, 30:0.8) and a separating gel containing either 12.5 or 17.5% acrylamide were routinely used. Separated proteins were fixed and stained with 0.1% Coomassie brilliant blue R-250 dissolved in 40% (vol/vol) methanol–10% (vol/vol) acetic acid and destained with the same solvent mixture. When required, gels were also developed by the silver-staining method with the kit provided by Bio-Rad Laboratories. Gels were also stained for glycoprotein bands with Schiff's periodate stain (34).

Phage adsorption assays. Each of the phages tested (10⁶ to 10⁷ PFU/ml) was preincubated at 30°C for 15 min with a predetermined concentration of LAM (either 1 to 1.2 or 10 to 12 mg/ml) in 1 ml of adsorption buffer (50 mM Tris-HCl [pH 7.2] containing 0.1 M NaCl, 2 mM MgCl₂, and 10 mM CaCl₂). The preparation was centrifuged at 12,000 x g for 5 min, the supernatant was assayed for residual phages by standard plaque assay on the sensitive host as described by Terzaghi and Sandine (26), and the percentage of phages adsorbed was calculated.

Other procedures. Total carbohydrates were determined by the phenol-sulfuric acid method of Dubois et al. (6). Protein was estimated by using the Bio-Rad DC protein assay kit (based on the Lowry method), and total phosphate was estimated by the method of Ames and Dubin (1). LAM was treated with sodium metaperiodate as described by Schafer et al. (21). Fructose-1,6-bisphosphate aldolase activity was measured by the method of Crow and Thomas (5).

RESULTS

Extraction of LAM. The phage-resistant variant of L. lactis subsp. cremoris (strain 398) produced a soft and slimy pellet on centrifugation at 12,000 x g, whereas the parent strain (strain E8) produced the normal hard pellet. Strain 398 was a preexisting mutant of strain E8, and its properties have been characterized previously (16). Part of the reason for this mucoid character of strain 398 was an increased tendency of the cells to lyse. Initially, the method and conditions described by Coolbear et al. (4) were used to extract the LAM from the two strains of L. lactis subsp. cremoris, but this method produced an average of 15 to 20% cell lysis in strain 398. The degree of cell lysis was estimated by measuring the relative release of intracellular fructose-1,6-bisphosphate aldolase into the extraction buffer. To minimize the cell lysis and avoid the contamination of the extracellular material with the cytoplasmic contents, we tested a number of extraction buffer systems with different ionic strengths and pHs (Table 1). We found that osmotically stabilized buffer systems (between 0.3 and 0.4 M glycylglycine in the presence of 10 mM MgCl₂ [pH 7.5]) gave the best results. Therefore a final buffer system comprising 0.35 M glycylglycine and 10 mM MgCl₂ (pH 7.5) was used routinely to extract the LAM from the two strains of L. lactis subsp. cremoris. However, even under these conditions some cell lysis of strain 398 was observed (usually less than 5%), but this was comparable to the lysis observed in strain E8.

Age of the culture and cell lysis. We harvested cells at different stages of growth to examine the relationship between cell lysis during the extraction of LAM and the age of the culture. Younger cultures (early log to mid-log phase of growth; harvest pH 6.2) of strain 398 were less prone to lysis
TABLE 1. Optimization of the conditions for the extraction of LAM from L. lactis subsp. cremoris 398

<table>
<thead>
<tr>
<th>Composition of extraction buffer*</th>
<th>% Lysis*&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris-HCl</td>
<td>11.3</td>
</tr>
<tr>
<td>50 mM Tris-HCl*</td>
<td>12.2</td>
</tr>
<tr>
<td>100 mM Tris-HCl</td>
<td>10.6</td>
</tr>
<tr>
<td>100 mM glycylglycine</td>
<td>14.2</td>
</tr>
<tr>
<td>200 mM glycylglycine</td>
<td>13.4</td>
</tr>
<tr>
<td>300 mM glycylglycine</td>
<td>4.2</td>
</tr>
<tr>
<td>350 mM glycylglycine</td>
<td>3.5</td>
</tr>
<tr>
<td>400 mM glycylglycine</td>
<td>5.6</td>
</tr>
<tr>
<td>600 mM glycylglycine</td>
<td>13.4</td>
</tr>
<tr>
<td>Cell extract (total activity)</td>
<td>100</td>
</tr>
</tbody>
</table>

* Extraction buffers were adjusted to pH 7.5 and, with the exception of those indicated *, contained 10 mM MgCl₂.
<sup>c</sup> Lysis is expressed as the percentage of aldolase activity found in the cell extract preparation of cultures used in the extraction experiments.
<sup>c</sup> Data are from a typical experiment and are representative of the trend.

(typically ca. 5%). As the culture aged, cells became more prone to lysis; up to 8% lysis was observed with cells in the late log phase of growth (harvest pH ca. 5.4), and if the culture was allowed to reach the stationary phase of growth (harvest pH ca. 4.8), cell lysis was as high as 18%. A similar relationship between culture age and cell lysis was found for the parent strain E8, but the levels of lysis observed for this strain during different phases of growth were significantly lower than for strain 398. Typically, less than 2% cell lysis was observed in cultures of strain E8 in the late mid-log phase of growth (harvest pH ca. 5.8); this increased to ca. 5% lysis by the late log phase of growth (harvest pH ca. 5.2) but never exceeded 8% in the stationary phase of growth.

Composition of LAM. There was a significant difference in the yield of LAM from the two strains; strain 398 produced between two and three times more material than did the parent strain E8. In strain 398 LAM typically accounted for between 6 and 10% of the total dry weight of the cells, whereas in strain E8 the value was between 2 and 3% (Table 2). Of the three components measured, protein was a major component of LAM in both strains, accounting for between 40 and 54% of LAM in strain 398 and between 40 and 42% in strain E8. Carbohydrate accounted for only 8 to 11% of LAM in strain 398 and 6 to 8% in strain E8. The other component measured was phosphate, which made up 10 to 12% of LAM in strain 398 and up to 13% in strain E8.

Analysis of the proteins of LAM. From the protein profile of LAM on SDS-polyacrylamide gels, there was one distinct protein band present in LAM from E8 that was absent from the LAM extracted from the phase-resistant strain 398 (Fig. 1). The relative molecular mass of this band was 21 kDa on an SDS–17.5% polyacrylamide gel. Both strains otherwise showed qualitatively similar banding patterns, with the major protein species having a relative molecular mass of 19 kDa. Other prominent common bands gave relative molecular masses of 22, 24, and 30 kDa. A high-molecular-mass band was detected in both strains when the gels were stained for glycoproteins with Schiff’s periodate stain (gel not shown). It was difficult to assess the apparent molecular mass of this band because it was highly diffused and migrated minimally into the separating gel.

Monosaccharide composition of LAM. Total acid hydrolysates of LAM were analyzed both quantitatively and qualitatively for the component monosaccharides by gas-liquid chromatography and liquid chromatography. The two methods indicated that only five sugars were present in both strains, with glucosamine being the major monosaccharide. Other component sugars detected were rhamnose, galactosamine, galactose, and glucose. Data from liquid-chromatographic analysis only are presented in Table 3 and show significant differences in the molar ratio of component sugars. The levels of rhamnose and galactose were particularly high in strain 398, representing 173.8 and 79.9 μg/100 mg (dry weight) of cells, respectively, compared with 37.6 and 44.7 μg/100 mg (dry weight) of cells, respectively, in strain E8. The molar ratios of rhamnose/galactosamine/glucosamine/galactose/glucose for the two strains were 2.2:0.3:1.2:0.9:1 for strain 398 and 0.6:0.08:1.0:0.65:1 for strain E8.

Inhibition of phage binding by LAM from the two strains.

TABLE 2. Composition of LAM from L. lactis subsp. cremoris E8 and 398

<table>
<thead>
<tr>
<th>Component</th>
<th>Strain E8&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Strain 398&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amt (mg/100 mg [dry wt] of cells)</td>
<td>% of LAM extracted</td>
</tr>
<tr>
<td>Total LAM extracted</td>
<td>2.5</td>
<td>100</td>
</tr>
<tr>
<td>Protein</td>
<td>1.0 ± 0.17</td>
<td>41.4</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>0.20 ± 0.03</td>
<td>8.0</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.32 ± 0.06</td>
<td>12.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are means from three independent experiments.
TABLE 3. Monosaccharide composition of the LAM extracted from L. lactis subsp. cremoris E8 and 398*

<table>
<thead>
<tr>
<th>Component</th>
<th>Strain E8</th>
<th>Strain 398</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amt (μg/100 mg [dry wt] of cell)</td>
<td>Molar ratio</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>37.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>5.6</td>
<td>0.08</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>73.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Galactose</td>
<td>44.7</td>
<td>0.65</td>
</tr>
<tr>
<td>Glucose</td>
<td>68.9</td>
<td>1</td>
</tr>
</tbody>
</table>

* Data from liquid-chromatographic analysis; for details see Materials and Methods.

LAM from the phage-resistant strain 398 and the phage-sensitive strain E8 was tested for its ability to inhibit the binding of Φ 618, Φ 833, and Φ 852 to the sensitive E8 cells. Results of these experiments are summarized in Table 4. LAM from strain 398 did not inhibit the binding ability of the three phages tested, whereas LAM from the parent strain showed different results with different phages. At the higher concentration tested, LAM from strain E8 inactivated 81% of Φ 852 and 53% of Φ 618 but did not reduce the titer of Φ 833. Furthermore, LAM treated with sodium metaperiodate did not inhibit the plaque formation on strain E8 by Φ 618 and Φ 852. This indicates that the phage-binding activity of LAM from strain E8 is related to its carbohydrate components, since treatment with sodium metaperiodate destroys the carbohydrate structure.

**DISCUSSION**

*L. lactis subsp. cremoris* 398 was isolated from *L. lactis* subsp. cremoris E8 by Limsworth and Terzaghi (16) after the parent strain was challenged by the small isometric phage Φ 852 in milk cultures. Since its isolation, strain 398 has retained its resistance against Φ 852, as well as a number of other phages with similar morphology, including Φ 618 and Φ 833. The parent strain E8 remains sensitive to these three phages. Further work with these strains has shown that the resistance was due to the inability of the phages to bind to the variant strain (8a). This result has suggested that the cell surface of strain 398 is different from that of its parent strain E8.

One notable phenotypic change observed in strain 398 compared with the parent strain is an inability to form a firm pellet when centrifuged at 12,000 × g. Recently Lucey et al. (17) reported a similar phenotypic change in *L. lactis* subsp. cremoris 503 and 505. These strains contained plasmid pC1528, which conferred adsorption-related phage resistance. However, part of the reason for the production of a looser and slimier pellet in strain 398 was a susceptibility to cell lysis when resuspended in buffer of low ionic strength. In buffers commonly used for washing the cells after harvesting from liquid RSM medium, up to 20% of the cell population of strain 398 lysed (Table 1), whereas under similar conditions the lysis of the parent strain was less than 5%. The up-to-10-fold increase in susceptibility to cell lysis found in strain 398 compared with strain E8 suggests a change in the integrity of the cell wall in the resistant strain. Interestingly, the cells of strain 398 harvested in the early log to mid-log phase of growth behaved much like the parent strain, and cell lysis during the extraction of LAM was 5 to 8%. This result is not surprising since it has been shown by controlled pulse-chase sequence experiments that in other gram-positive bacteria such as *B. subtilis* (13), newly synthesized walls are most resistant to solubilization by autolysins whereas older cell walls are more susceptible. However, the observation that the degree of cell lysis in strain 398 could be controlled by using buffer of higher ionic strength (Table 1) suggests that the stabilization effect may have been due to the osmolarity of the buffer and that a certain population of cells may have an incomplete or weaker wall structure. Recently Coolbear et al. (4) used 0.6 M glycyglycine buffer to stabilize spheroplasts from a number of strains of *L. lactis* subsp. lactis and *L. lactis* subsp. cremoris.

Two- to three-fold more LAM was extracted from strain 398 than from the parent strain. This suggests that the cell wall metabolism of the phage-resistant variant was altered and resulted in the overproduction of LAM or that the wall architecture of strain 398 was affected in such a way that more material was accessible for extraction. Production of additional extracellular material in phage-resistant derivatives of *L. lactis* subsp. cremoris has been observed in other studies (17).

The chemical analysis of the carbohydrates of LAM from both strains showed the presence of the same monosaccharides. The sugars found, namely rhamnose, galactosamine, glucosamine, glucose, and galactose, are widespread in bacterial exopolysaccharides (25). Cell walls of a number of strains of lactococci (17, 28) and *Lactobacillus casei* (11, 30, 31) also have similar monosaccharide composition. There was a significant difference, however, in the composition of LAM carbohydrate with respect to the amount of rhamnose and galactose in the two strains. The phage-resistant variant strain 398 contained almost fivefold more rhamnose and twofold more galactose than did the parent strain (Table 3). In a study of the cell wall of phage-resistant mutants of *L. lactis* subsp. cremoris KH, Valyasevi et al. (28) showed that galactose and rhamnose were essential for the binding of a number of phages. The loss of galactose and rhamnose from the cell wall polysaccharides of phage-resistant mutants resulted in loss of phage adsorption. Rhamnose has also been implicated as a determinant of phage receptor in other studies of *Lactobacillus casei* (11, 31). It is possible that LAM is a defense mechanism for strain 398. Overproduction of material enriched in monosaccharides believed to be involved in phage adsorption could inactivate the phages before they interact irreversibly with discrete cell surface-binding sites. Lucey et al. (17) reached similar conclusions in their study of strains of *L. lactis* subsp. cremoris containing plasmid pC1528. They found that plasmid-containing phage-
resistant strains produced a cell surface material that had elevated levels of galactose and rhamnose. In the present study, however, this possibility seems unlikely because it has been demonstrated here that the LAM isolated from strain 398 showed a complete lack of ability to bind to any of the three phages tested (Table 4). Material isolated from the parent strain could, however, inactivate 82% of Φ 852 particles and 53% of Φ 618 particles. This result suggests that the changes in strain 398 have most probably resulted in the production of LAM that has a chemical structure different from that of LAM of the parent strain and does not interact directly with the phage particle against which the strain was originally selected.

The ability of LAM from strain E8 to inactivate the phages was completely lost after treatment with sodium metaperiodate. This suggests that the polysaccharide portion of the LAM is critical for phage binding.

The disappearance of a 21-kDa protein band (Fig. 1) from the LAM of strain 398 is interesting, but it is unclear whether this protein plays any role in phage adsorption. There is, however, evidence in the literature which suggests a role for cell surface proteins in determination of the agglutination characteristics and cell surface hydrophobicity of streptococci (12, 18) and a role for membrane proteins in phage adsorption in lactococci (19, 29).

This is the first study in which methods have been developed specifically for the isolation of component(s) of the lactococcal cell surface which constitute the outermost layer of the organism and therefore present a first point of contact for bacteriophages. In this study we investigated the chemical composition of this material and showed that the material may be functionally significant in bacteriophage interaction and in inhibition of phage adsorption by undergoing structural modifications.

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