Isolation and Characterization of Lipoteichoic Acid, a Cell Envelope Component Involved in Preventing Phage Adsorption, from *Lactococcus lactis* subsp. *cremoris* SK110

LOLKE SIJTSMA,† JAN T. M. WOUTERS,‡ AND KLAAS J. HELLINGWERF

Biotechnology Centre, Laboratory of Microbiology, University of Amsterdam, 1018 WS Amsterdam, The Netherlands

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The cell envelope of the phage-resistant *Lactococcus lactis* subsp. *cremoris* SK110 differed from its phage-sensitive variant by the presence of a galactosyl-containing component. This component was present in material obtained from SK110 by a mild alkali treatment. In a similar fraction extracted from SK112, no galactosyl-containing components were detected. With respect to gel permeation chromatography and electrophoretic mobility, identical characteristics of the alkali-extracted material and purified lipoteichoic acid (LTA) were measured. Chemical analysis of the latter component showed the absence of galactose in LTA isolated from SK112, whereas it was present in LTA obtained from SK110. In this paper, we propose that galactosyl-containing LTA is involved in preventing phage adsorption to *L. lactis* subsp. *cremoris* SK110.

In recent years, much attention has been paid to phage resistance in lactic acid bacteria (for a review, see reference 14). Strategies which supply bacteria with resistance towards bacteriophage infection are (i) inhibition of phage adsorption, which may be accomplished by the absence of the phage receptor or by blocking of the receptor, (ii) prevention of DNA injection, and (iii) degradation of phage DNA by bacterial restriction enzymes.

*Lactococcus lactis* subsp. *cremoris* SK110 is resistant to phage SK11G. This is due to poor phage adsorption to this strain as compared with adsorption of the phage to a phage-sensitive derivative, SK112 (6, 27). Previously (27), we demonstrated that phage resistance of SK110 is caused by blocking of the receptor site and not by the absence of phage receptor material.

With respect to cell surface characteristics, the two strains showed large differences (26). Cells of SK110 aggregated with the *Ricinus communis* lectin, while cells of SK112 did not. As this lectin binds galactosyl groups (20), galactose appeared to be present at the surface of the resistant strain. Furthermore, ethanol precipitates obtained from extracts of cells of SK110 contained a high level of galactose compared with the level in an identical fraction from SK112 (27). A mild alkali treatment of the phage-resistant strain enhanced phage adsorption and concomitantly decreased the high galactose level. On the basis of these results, we suggested the involvement of a galactose-containing surface component in preventing phage adsorption to SK110 (27).

Sugar polymers present at the cell surface may be phosphate-containing polysaccharides like teichoic acids, which are covalently bound to the peptidoglycan. In *L. lactis* subsp. *cremoris* strains, however, no teichoic acids have been detected so far (13, 23). Galactose-containing polymers that have been described as cell surface components in lactococci are lipoteichoic acids (LTAs) (9, 24, 34, 35). These phosphate-containing polymers appear to be present throughout the gram-positive bacteria (34) and contribute to several cell surface characteristics, such as hydrophobicity (18) and surface electrostatic charge (19). The LTA molecule characteristically consists of a substituted or unsubstituted poly-(glycerol phosphate) chain, covalently linked to a lipid moiety. It is assumed that this latter part of the molecule serves as an anchor in the cytoplasmic membrane, while the former part protrudes into the cell wall and can become exposed at the outer surface of the wall (9, 34).

Although a vital role of LTAs in bacterial physiology has not been proven yet (for a review, see reference 7), several physiological functions, such as binding of divalent cations and inhibition of the autolytic activity, have been assigned to them (8, 11, 16).

Results of this study indicate yet another function for the galactosyl-containing LTA: it is involved in preventing phage adsorption to *L. lactis* subsp. *cremoris* SK110.

MATERIALS AND METHODS

Organism and growth conditions. *L. lactis* subsp. *cremoris* SK110 and SK112 were obtained from the Netherlands Institute of Dairy Research (Ede, The Netherlands). Cells were grown in M17 broth (30) at 28°C.

Alkaline extraction. Cells from a 1-liter culture of *L. lactis* subsp. *cremoris* were harvested in the late exponential phase of growth, washed in 10 mM phosphate buffer (pH 6.8), and suspended in 10 ml of NaOH (50 mM, 30 min, 25°C). During this treatment, no cell lysis was observed microscopically. The suspension was centrifuged (15 min, 5,000 × g), and the supernatant was removed and dialyzed twice against 300 volumes of distilled water (18 h, 4°C). The nondiffusible material was freeze-dried.

Extraction of LTA. Cells from 10 liters of a culture in the late exponential growth phase were harvested by centrifugation (3,000 × g, 10 min), washed, and stirred overnight in 0.1 M sodium acetate (pH 5.0) with chloroform-methanol (2:1, vol/vol). After centrifugation, the defatted cells were suspended in 0.1 M sodium acetate (pH 5.0, 6.5 g [wet weight] of cells per 13 ml) and an equal volume of 80% phenol preheated at 65°C was added. The suspension was

* Corresponding author.
† Present address: ATO Agrotechnological Research Institute, P.O. Box 17, 6700 AA Wageningen, The Netherlands.
‡ Present address: The Netherlands Institute for Dairy Research, 6710 BA, Ede, The Netherlands.
stirred at 65°C for 45 min. The mixture was then centrifuged at 4°C for 30 min at 5,000 × g to obtain phase separation, and the upper layer was carefully removed. Phenol was removed from this supernatant by dialysis against 150 volumes of sodium acetate (0.1 M, pH 5.0) at 4°C. Nucleic acids were degraded by incubation of the dialyzed material with nucleases (RNase [20 μg · ml−1], DNase [5 μg · ml−1], and 1 mM MgCl₂). Toluene (1 ml) was added to prevent microbial contamination, and the solution was incubated at 20°C for 24 h. The enzymes were removed by a second phenol treatment as described above. After centrifugation, phenol was removed from the water phase by dialysis as mentioned before. The nondialyzable material was freeze-dried and finally taken up in a small volume of sodium acetate (50 mM, pH 4.0). Further purification was achieved by gel permeation chromatography with a Sepharose 6B column (50 by 2.5 cm; Pharmacia) equilibrated with 50 mM sodium acetate (pH 4.5) at 4°C. Upward elution took place at a flow rate of 15 ml · h−1, and fractions (3 ml) were collected. Every second fraction was assayed for phosphorus as described below, and the extinction at 260 nm (A₂₆₀) was measured. Appropriate fractions were pooled, dialyzed against 150 volumes of distilled water (18 h) to remove buffer salts, freeze-dried, and weighed.

**Analytical methods.** The level of phosphorus was determined according to the method of Chen et al. (4) after oxidation of the dried sample with 70% HClO₄ as described by Kruyssen et al. (15). Total hexose was measured with anthrone reagent with glucose as a standard as detailed by Ashwell (2). Fatty acid esters were assayed by the procedure of Snyder and Stephens (28) with methylstearate as the standard. Protein was assayed according to the method of Bradford (3) with bovine serum albumin as a standard. Levels of sugars and glucosamine were determined by gas chromatography. LTA (300 μg) was subjected to methanolysis and subsequent trimethyl silylation as described by Gerwig et al. (10). Gas-liquid chromatography was carried out according to the method of Schuring et al. (25). The level of alanine was determined by high-pressure liquid chromatography after hydrolysis of the LTA (4 M HCl, 16 h, 100°C) and dansylation of the hydrolyzed extract. LTA suspension (1 volume) was mixed with 1 volume of sodium borate (0.4 M, pH 10), and the mixture was warmed to 65°C to dissolve in ultrapure, water-free acetonitrile. The mixture was incubated in the dark at 37°C. When the yellow color had disappeared, the reaction was stopped by the addition of 1 volume of acetic acid (0.3 M). The dansylated amino acids were separated by high-pressure liquid chromatography with an LKB instrument (Bromma, Sweden) with a Hypersil 5 ODS column (250 by 4.6 mm; Chrompack International B.V., Middelburg, The Netherlands), a UV detector (LKB 2158 uvicord SD) at 206 nm, an SP4270 integrator (Spectra Physics, San Jose, Calif.), and a mixture of sodium phosphate (20 mM, pH 6.25) and acetonitrile (Merck, Darmstadt, Federal Republic of Germany) as eluants, at room temperature. During elution, the percentage of acetonitrile increased from 15 to 70% in 25 min and remained at 70% for 10 min. The level of glycerol was determined, after acid hydrolysis of LTA (6 M HCl, 16 h, 100°C), by high-pressure liquid chromatography (LKB) with an Aminex HPX 87H organic acid analysis column (Bio-Rad, Richmond, Calif.) with a 2142 refractive index detector (LKB), an SP4270 integrator (Spectra Physics), and 5 mM H₂SO₄ (Merck) as an eluant at 55°C.

**Precipitation assay.** For the precipitation tests, 20 μl (2 mg [dry weight] · ml⁻¹) of cell envelope material, obtained by one of the procedures described above, was used. Precipitation tests were carried out in 1% agarose gels in a sodium diethyl barbiturate buffer (8.25 g · liter⁻¹, pH 8.6) by the double-diffusion method (22). Immunoelectrophoresis was carried out in the same buffer at 300 V for 1.5 to 2.5 h. The amount of R. comminis lectin (RCA 120) used in the experiments was 20 μl (300 μg · ml⁻¹) for the Ouchterlony experiments (22) or 50 μl (300 μg · ml⁻¹) in the electrophoresis experiments.

**RESULTS**

Previously (27), we showed that material extracted from whole cells of the phage-resistant L. lactis subsp. cremoris SK110 and precipitated with ethanol contained a high level of galactose compared with the level in a similar fraction obtained from SK112. After extraction of the cells with mild alkali, however, the high level of galactose in the cell envelope of SK110 was reduced significantly. Material isolated from the cell envelope of SK110 by mild alkali treatment precipitated with the R. comminis lectin in a double-diffusion test in a single precipitation line (Fig. 1, well d). This indicates the presence of one galactosyl-containing polymer in this extract. No precipitation line, however, was observed between the alkali-extracted material isolated from the phage-sensitive SK112 and the R. comminis lectin (Fig. 1, well c). Chemical analyses (protein assay) and staining of the gel with Coomassie blue showed the presence of proteinaceous material in the alkali extracts of SK110 and SK112 (Fig. 1, wells c and d).

A galactose-containing component which is known to be present at the cell surfaces of lactococci is LTA (9, 34, 35). To investigate whether the phage-resistant SK110 and its phage-sensitive variant SK112 possess different LTAs, this cell envelope constituent was isolated by a hot-aqueous-phenol treatment (33). Figure 2 shows the elution profile of nuclease-treated hot-phenol extracts of whole cells of L. lactis subsp. cremoris SK110 and SK112 on a Sepharose 6B column. Two phosphorus-containing peaks were observed. The fractions containing the component with the lowest apparent molecular weight (fractions 52 to 80) probably contained mostly digested nucleic acids, as judged by their high A₂₆₀, and were not studied further. Since it is known that LTA forms micelles (33) because of its amphipathic nature and thus shows an apparent high molecular weight in gel permeation chromatography in aqueous buffers without detergent, the phosphorus-containing fractions eluting directly after the void volume of the column most likely contained LTA. These fractions were pooled, dialyzed,
freeze-dried, and weighed. The yield of LTA was about 0.2% of the dry weight of the cells. However, since no corrections have been made for losses during the isolation procedure, the actual amount of LTA in intact cells may be larger. No difference in elution patterns of LTAs isolated from SK110 (Fig. 2A) and SK112 (Fig. 2B) was observed. However, LTA extracted from SK110 formed a precipitate in the double-diffusion test with a lectin from *R. communis* (Fig. 1, well b), whereas LTA isolated from SK112 did not (Fig. 1, well a). The inability of the latter LTA to react with this lectin indicated a lack of galactosyl groups in this cell envelope component. The present of galactose in LTA extracted from SK112 was further confirmed by chemical analyses (Table 1). The molar ratio of phosphorus to total carbohydrate was about 1:0.25 for SK110 and 1:0.15 for SK112, indicating a low degree of glycosidic substitution in LTA. Alanine, which has been observed in LTAs from several different procaryotic species, was detected in both strains. The ratios of alanine to phosphorus did not show significant differences between the two strains investigated, being 0.33 and 0.30 for SK110 and SK112, respectively. Unlike the amount of alanine, the amount of esters in LTA differed between the two strains: it was relatively large in SK112 as compared with SK110 (Table 1). In order to check whether the material isolated from *L. lactis* subsp. *cremoris* SK110 by the mild alkali treatment was LTA or a part thereof, this polymer was subjected to gel permeation chromatography over a Sepharose 6B column. No significant peaks were observed upon spectral analysis of the fractions at 260 nm (data not shown), indicating the absence of significant amounts of contaminating nucleic acid in this sample. Since the amount of material in the fractions obtained was too small for an accurate phosphate determination, the fractions were tested for the ability to form precipitates with the *R. communis* lectin in a double-diffusion test. Only samples from fractions 31 to 49 formed precipitation lines with this lectin (Fig. 3).

These fractions correspond to the LTA-containing fractions obtained from hot-phenol-treated SK110 cells (Fig. 2). That the galactosylated component in the alkali extract obtained from SK110 was equivalent to LTA was further substantiated by precipitation with the *R. communis* lectin after electrophoresis. The component in the alkali extract, which reacted with the *R. communis* lectin, did not migrate under the electrophoresis conditions used (Fig. 4). The same electrophoresis characteristic was observed for purified LTA.

**DISCUSSION**

From previous results (27) and the data presented in this paper, it can be concluded that the cell envelope of the phase-resistant SK110 differs from its phase-sensitive derivative SK112 by the presence of a galactose-containing component in the former strain. This component was present in material isolated by mild alkali extraction. With precipitation

![Figure 3](http://jb.asm.org/)

FIG. 3. Precipitation of *R. communis* lectin with material isolated from *L. lactis* subsp. *cremoris* SK110 by a mild alkali treatment, after fractionation over Sepharose 6B. Wells a to l contain odd-numbered fractions 29 to 51, respectively.

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**TABLE 1.** Chemical composition of LTA isolated from *L. lactis* subsp. *cremoris* SK110 and SK112

<table>
<thead>
<tr>
<th>Component</th>
<th>SK110</th>
<th>SK112</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amt (μg/mg ± SD)</td>
<td>Molar ratio</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>50.2 ± 5.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Total hexose*</td>
<td>72.5</td>
<td>58.1</td>
</tr>
<tr>
<td>Glycerol</td>
<td>141.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>41.9 ± 3.2</td>
<td>0.143</td>
</tr>
<tr>
<td>Galactose</td>
<td>19.7 ± 3.4</td>
<td>0.067</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>13.5 ± 2.9</td>
<td>0.046</td>
</tr>
<tr>
<td>Fatty acid</td>
<td>76.9 ± 15</td>
<td>0.135</td>
</tr>
<tr>
<td>Alanine</td>
<td>48.1 ± 5.3</td>
<td>0.33</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Protein</td>
<td>12.0 ± 0.5</td>
<td>12.0 ± 0.5</td>
</tr>
</tbody>
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* Measured with the anthrone reagent.

* ND, Not detectable.
L. lactis subsp. cremoris SK11 strains are used in multiple-strain starter cultures, and a proper regulation of physiological functions may be a requirement for survival during competition. Sterkenburg et al. (29) found that neither SK112 nor SK110 had a competitive advantage when these strains were grown in a chemostat culture, in MRS medium (5a), with lactose as the limiting substrate. When cells were grown in milk, in which amino acids are assumed to be growth limiting (31), a lower growth rate was found for the phage-resistant strain (12). These results indicate that possession of plasmid pSK112, which encodes phage resistance in SK110 (6), renders a competitive disadvantage to strains grown in milk. It is not clear whether this disadvantage is due to the differences in LTA composition at the cell surface or whether it is determined by other factors.

The way in which LTA of SK110 can prevent phage adsorption is not clear yet. For several gram-positive and gram-negative bacteria, phage resistance due to changes in the phage receptor has been described (1, 17). In order to understand the role of galactosylated LTA in preventing phage adsorption to L. lactis subsp. cremoris SK110, one would expect that (i) LTA itself acts as the receptor for phage sk11G or (ii) the location of LTA in the cell envelope is so close to the phage receptor that steric hindrance is exerted on binding of the phage to the receptor. Although a plasma membrane component has been proposed as a phage receptor in L. lactis ML3 (21), no evidence that this component was LTA has been presented. Several authors, however, demonstrated the presence of phage receptor material in all walls of gram-positive bacteria (for a review, see reference 1). Previously (27), we reported the presence of phage receptor material in purified cell walls of the two strains investigated in this study. In vitro, phage sk11G does not bind to isolated LTA of L. lactis subsp. cremoris SK110 or SK112 (data not shown). Therefore, we consider resistance caused by steric hindrance most likely to be the mechanism of phage resistance in SK110. Resistance for phages caused by steric shielding of the phage receptor has previously been shown for Salmonella typhimurium. Smooth strains, with lipopolysaccharides containing an average of 5 to 15 repeating units per O chain, adsorbed phage FO at a significantly lower rate than strains with one repeating unit but were still FO sensitive (17). It was proposed that the O chain prevents access of the phage to its receptor.

With respect to phage resistance in Rhizobium mellotili, Ugalde et al. (32) recently reported the presence of an inner-membrane-bound galactosyltransferase. This enzyme transfers galactose from UDP-galactose to a water-insoluble anionic polymer in a phage-resistant strain. The phage-sensitive variant lacks this enzyme as well as galactose in the polymer.

Since phage resistance of L. lactis subsp. cremoris SK110 is encoded by plasmid pSK112 (6), the differences in galactose contents of LTAs isolated from SK110 and SK112 may be due to one or more enzymes present in the phage-resistant strain. Transfer of galactose from UDP-galactose into lipids and an extracellular polymer by membrane-bound enzymes prepared from Streptococcus mutans has recently been reported by Chiu (5). Unfortunately, experiments to demonstrate the presence of such enzymes in SK110 were not successful until now.

In this paper, evidence that galactosylated LTA prevents phage adsorption in L. lactis subsp. cremoris SK110 is presented. It cannot, however, be ruled out that the mild alkali treatment that enhances phage adsorption to this strain
liberates a second polymer which may be involved in preventing phage adsorption. This possibility might be further investigated with chemical analysis and reconstitution experiments. Another approach to obtain more information about the cell surface component(s) that prevents phage adsorption is to characterize the gene(s) that gives rise to phage resistance. However, this approach can be successful only when it is possible to isolate plasmid pSK112 or the relevant part thereof.

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LITERATURE CITED


