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Research Article

Characterisation of an Exopolysaccharide Preventing Phage Adsorption in *Lactococcus lactis* subsp. *cremoris* MA39

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Abstract: *Lactococcus lactis* subsp. *cremoris* strain MA39 produces an extracellular polysaccharide containing rhamnose, glucose and galactose. A 16.5 kb plasmid encoding exopolysaccharide production in MA39 was determined by plasmid curing experiments. Phage adsorption assays showed that four different lactococcal phages were adsorbed to MA39-40; only the 16.5 kb plasmid cured an exopolysaccharide non-producing mutant of strain MA39, with high efficiency (93.2-98.5%) while adsorption of these phages was completely inhibited in the wild type strain MA39. These results suggest that exopolysaccharide prevents phage adsorption by masking phage receptor sites.

Key Words: Exopolycaccharide, phage resistance, *Lactococcus lactis* subsp. *cremoris*

Lactococcus lactis subsp. *cremoris* MA39 Suşunda Faj Adsorbsiyonunu Engelleyen Bir Ekzopolisakkarit Yapının Tanısı

Özet: *Lactococcus lactis* subsp. *cremoris* MA39 suşu; ramnoz, glukoz ve galaktoz içeren bir ekzopolisakkarit üretmektedir. Plazmid giderme çalışmaları sonucu, MA39 suşunda ekzopolisakkarit üretiminin 16.5 kb büyüklükte bir palzmid tarafından kodlandığı belirlendi. Faj adsorbsiyon deneyleri; doğal tip MA39 suşunda denenen 4 fajın adsorbsiyonunun engellenmiş olmasına rağmen, bu suşun yalnız 16.5 kb plazmidi giderilmiş MA39-40 mutantında, söz konusu fajların adsorbsiyonunun % 93.2-98.5 gibi yüksek oranlarda gerçekleştiğini gösterdi. Bu sonuçlar, ekzopolisakkarit yapının faj reseptör bölgeleri maskelemek suretiyle faj adsorbsiyonunu engellediğine işaret etmektedir.

Anahtar Sözcükler: Ekzopolisakkarit, faj direnç, *Lactococcus lactis* subsp. *cremoris*

Introduction

Lactococcus lactis is an important organism for the dairy industry, where it is used for the production of many cheese varieties as well as cultured milk products such as buttermilk (1). Lactococci, can, however, succumb to phage infection during the production of fermented products thus compromising the effectiveness of the fermentation process. Improved resistance to phage is an on-going challenge and is an extensively researched area in the lactococcal dairy starter industry (2,3). The defensive strategies employed by *L. lactis* against phage attack have generated much interest and include restriction and modification (4), abortive infection (5,6), blocking of phage adsorption (7, 8), loss of cell surface receptor (9) and alterations of the plasma membrane components required for phage infection (10,11).

Phage infection initially requires a specific recognition between the phage and the host cell (12). Despite many studies, inhibition of phage adsorption is still a poorly understood phenomenon at the molecular level (13). Fortunately, recent work has begun to reveal some details about the cell surface components that play a role in or interfere with phage adsorption (14).

The present study reports the chemical characterisation and genetic nature of an extracellular polysaccharide (EPS), preventing phage adsorption in producer strain *L. lactis* subsp. *cremoris* MA39.

Materials and Methods

Bacterial strains and phages

The bacterial strains and phages used in this study are listed in Table 1. Lactococcal strains were grown at 30 °C

Table 1. Bacterial strains and phages used in this study.

Strain/phage	Relevant characteristics	Source/reference
Strain		
L. lactis subsp. *cremoris*		
MA39	Lac^+, Eps^+, Str^s, Kms, ϕ^r wild type strain	Raw milk/this study
MA39-40	Lac^+, Eps^-, ϕ^s mutant, of MA39	This study
L. lactis subsp. lactis		
P81-1	Lac^-, Eps^-, Str^r, Km^r, ϕ^s plasmid free mutant of P81	1
Phages		
ϕlc12	small isometric-headed	3
ϕld3	large isometric-headed	3
ϕll88	large isometric-headed	3
ϕll203	large isometric-headed	3

Lac^+, Metabolises lactose; Lac^-, does not metabolise lactose; Eps^+, produces exopolysaccharide; Eps^-, does not produce exopolysaccharide; Str^r, resistant to streptomycin (200 µg/ml); Str^s, sensitive to streptomycin; Km^r, resistant to kanamycin, (70 µg/ml); Km^s, sensitive to kanamycin.

in M17 medium (15) supplemented with 0.5% glucose when necessary. Phages and culture stocks were stored in M17 broth containing 40% glycerol at –80 °C.

EPS purification and characterization

L. lactis subsp. *cremoris* was grown in 1 L of reconstituted milk for 24 h at 20 °C, trichloroacetic acid was added to a final concentration of 12%, and bacterial cells and precipitated proteins were removed by centrifugation (30,000 x g, 20 min, 4 °C). The supernatant was adjusted to neutral pH using 10 N NaOH, concentrated by ultrafiltration, dialysed against running tap water (48 h), and lyophilised. The lyophilised EPS was dissolved in double-distilled water and the contaminating protein was removed by gel filtration on a sephacryl S-500 (Pharmacia, Kalamazoo, MI, USA) column (75 x 2.6 cm) by elution with 50 mM NH_4HCO_3 at 0.75 ml/min, monitoring the refractive index and the A280 (16).

For quantitative analysis, monosaccharide units were hydrolysed completely by the treatment of 4 N HCl and the EPS hydrolysates were analysed by HPLC [column CHO 682 Interchim Pb. (Montlucon, France), 85 °C, 0.4 ml/min deinonized water] and detected by a refractive index detector. Extracellular polysaccharides were also analysed by gas liquid chromatography (GLC) [column type macrobore SP-2380 phase (CPG, Sigma, Paris, France), 225 °C, nitrogen 3 ml/min].

Total carbohydrates were determined by the phenol-sulphuric acid method of Dubois et al. (17), and total phosphate was estimated by the method of Ames and Dublin (18).

Phage assays

The preparation of phage lysates and determination of titres (pfu/ml) were conducted as described by Terzaghi and Sandine (15).

Phage adsorption to the host cell was determined by the method of Lucey et al. (8). Phage adsorption was calculated as follows:

$$\text{Percentage adsorption} = \frac{(\text{Control titre} - \text{residual titre})}{\text{Control titre}} \times 100$$

Curing trials and isolation of plasmid DNA

Plasmid cured derivatives were obtained by using the protoplast induced curing method described by Gasson (19). The lysis procedure of Anderson and McKay (20) was used to isolate plasmid DNA. Purification of plasmids in caesium chloride-ethidium bromide density gradients and analysis on agarose gels were performed as described previously (21).

Conjugal matings

For conjugation experiments, filter matings were conducted as described by McKay et al. (22). Transconjugants were selected on lactose indicator agar (23) supplemented with streptomycin (200 µg/ml) and kanamycin (70 µg/ml) to select for Lac^+, Str^r and Km^r recombinants. Transconjugants were tested for EPS production.

Electron microscopy of phages

Phage suspensions were negatively stained with uranyl acetate and examined with a JEOL S-100 transmission electron microscope (7).

Results and Discussion

A pure EPS isolate, free of proteins and other contaminating compounds, was used to study the nature of EPS from the slime-forming strain *L. lactis* subsp. *cremoris* MA39. The chemical analysis showed that the EPS contained 92% carbohydrate and 4.0% phosphate (Table 2). These results pointed out that the EPS produced by MA39 was a phosphopolysaccharide. Total acid hydrolysates of the EPS were analysed both quantitatively and qualitatively for the component monosaccharides by gas-liquid chromatography and high performance liquid chromatography. The two methods indicated that the only three sugars were present in EPS. Data from high performance liquid chromatographic analysis only are represented in Table 3. The levels of rhamnose, glucose and galactose were 287.6, 112.7 and 90.3 µg/100 µg dry wt, respectively. The molar ratios of rhamnose/glucose/galactose were 2.55/1/0.80. The results presented here show similarities with research stating that lactococcal EPSs typically contain rhamnose, glucose, galactose and phosphate (8,12,14,16).

The EPS producing (Eps^+) *L. lactis* subsp. *cremoris* MA39 is capable of fermenting lactose (Lac^+) and harbour plasmids of 62.4, 55.2, 25.1, 16.5 and 4.2 kb (Figure 1). After protoplast induced plasmid-curing of strain MA39, Lac^+ and Lac^- (lactose non-fermenting) colonies were selected on lactose indicator agar. Then the colonies were tested for EPS production. A Lac^+ Eps^- (EPS non-producing) mutant was identified and designated as MA39-40. Plasmid profiles showed that loss of the 16.5 kb plasmid alone produced the Lac^+ Eps^- mutant (Figure 1). This result provides strong correlative evidence that the ability of EPS production in strain MA39 is linked with the 16.5 kb plasmid. Various reports describe the involvement of specific plasmids possessing conjugal ability in *L. lactis* (16,24-27). The conjugal ability of these plasmids is important for the identification and manipulation of the relevant genes for EPS production. In order to determine the conjugal ability of the 16.5 kb plasmid, conjugal matings were performed between *L. lactis* subsp. *cremoris* MA39 and the non-mucoid, plasmid free strain *L. lactis* subsp. lactis P81-1 (7). None of the conjugants became Eps^+ indicating that the EPS plasmid could not be transferred to recipient strain P81-1. Conjugal matings produced only one type of conjugant which contained 62.4 kb plasmid, and all conjugants were found to be Lac^+ (data not shown). These results indicate that lactose fermenting ability is encoded by the 62.4 kb plasmid in *L. lactis* subsp. *cremoris* MA39.

To test whether EPS production is correlated with phage insensitivity in MA39, the adsorption and plaque forming abilities of four different lactococcal phages to MA39 and MA39-40 were monitored (Table 4). Lactococcal phages were isolated from different host strains and characterised by electron microscopy to confirm their distinctions (Figure 2). Phage ϕlc12 had a small isometric head 60 nm in diameter with a 247 nm

Table 2. Chemical composition of EPS produced by *L. lactis* subsp. *cremoris* MA39.

Component	Strain MA39[a]	
	EPS concentration (µg/100 mg [dry wt] of cells)	% of EPS extracted
Total EPS extracted	6.75	100
Carbohydrate	6.21	92
Phosphate	0.27	4.0

[a] Results are means of three independent experiments

Table 3. Monosaccharide composition of EPS produced by *L. lactis* subsp. *cremoris* MA39[a].

Component	Strain MA39[a]	
	concentration (µg/100 mg [dry wt] of cells)	Molar ratio
Rhamnose	287.6	2.55
Galactose	90.3	0.80
Glucose	112.7	1

[a] Data from HPLC analysis

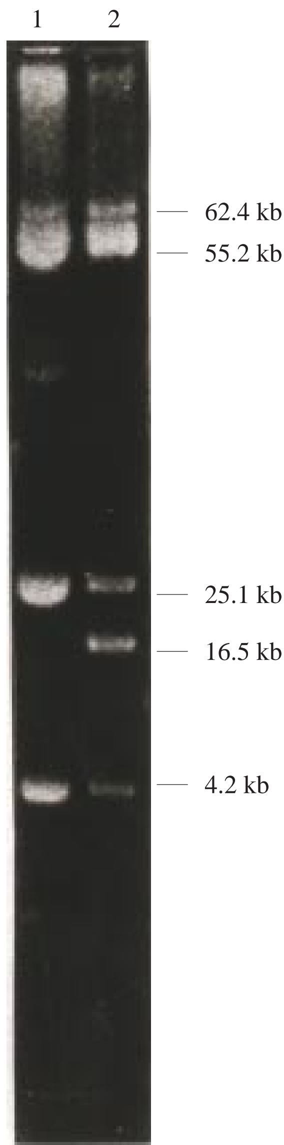

Figure 1. Plasmid profiles of *L. lactis* subsp. *cremoris* MA39 (Lane 2) and MA39-40 (Lane 1).

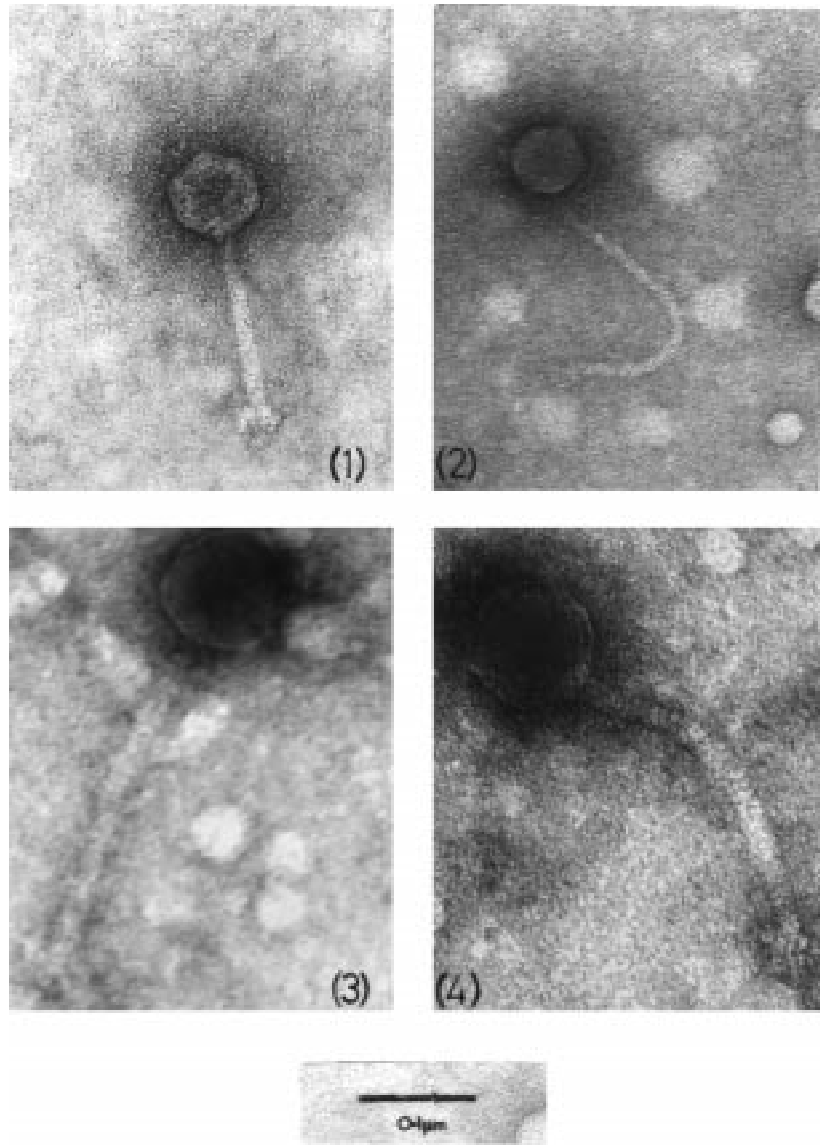

Figure 2. Electron micrographs of lactococcal phages. ϕll203 (1), ϕlc12 (2), ϕld3 (3) and ϕll88 (4).

tail. Phages ϕll88, ϕld3 and ϕll203 had large isometric heads 107, 100 and 80 nm n diameter with 340, 355 and 180 nm tails, respectively. Adsorption experiments showed that ϕlc12, ϕld3, ϕll88 and ϕll203 were adsorbed to the Eps^- mutant strain MA39-40 with high efficiency (93.2-98.5%), whereas adsorption of these phages was completely inhibited in the Eps^+ wild type parent strain MA39. After adsorption to MA39-40, plaque formation was determined in double-layer M17 agar for all phages (data not shown). These results suggest that the EPS produced by *L. lactis* subsp. *cremoris* MA39 is responsible for the complete inhibition of ϕlc12, ϕld3, ϕll88 and ϕll203 adsorption by masking the receptor(s) of these phages.

Bacterial extracellular polysaccharides occur in two basic forms. As a capsule (capsular polysaccharide, [CPS]) the polysaccharide is intimately associated with the cell surface and may be covalently bound. In contrast, slime polysaccharides are only loosely associated with the cell surface. The distinction between CPS and slime is often

Table 4. Adsorption of lactococal phages to *L. lactis* subsp. *cremoris* MA39 and MA39-40.

Component	Phage adsorption (%)[a]			
	ϕll88	ϕll203	ϕlc12	ϕld3
MA39	-	-	-	-
MA39-40	96.4	98.5	94.7	93.2

[a] Results are means of three independent experiments

operationally defined by the degree of cell association following centrifugation (28). Dairy mesophilic starters containing slime polymer-producing *L. lactis* subsp. *cremoris* strains for making Scandinavian ropy sour milk products, such as "viili" and "longfil" , are available in Europe and the United States (29). In response to the continuing need for phage resistant strains in the dairy industry, researchers have focused on the cell surface components required for phage adsorption or inhibition of phage adsorption. Most of these studies, however, have used either isolated cell walls or membrane fractions to identify the cell surface involved in phage adsorption (4,30-32). This approach ignores the role of loosely associated extracellular material, such as the phosphopolysaccharide produced by strain MA39 in phage adsorption, since most of this material is lost during the procedures used to isolate cell walls.

This report shows that the loosely associated cell material is a phosphopolysaccharide, produced by the involvement of the 16.5 kb plasmid DNA in *L. lactis* subsp. *cremoris* MA39, and this component prevents phage adsorption by blocking phage receptor(s) sites. There is need for further work, especially on the chemical composition of the phage receptor(s) in strain MA39 in order to identify the exact nature of receptor(s)-masking material interactions.

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