AN ABSTRACT OF THE THESIS OF

Ruud Valyasevi   for the degree of Doctor of Philosophy   in

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Title: The Cell Surface Polysaccharides and Membrane Protein required

for Bacteriophage Infection of *Lactococcus lactis*

Signature redacted for privacy.

Abstract approved:_____

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The bacteriophage receptor of lactococci was found on the cell

walls. A carbohydrate analysis of the cell walls from phage-resistant

mutants of *L. lactis* subsp. *cremoris* KH with reductions in phage binding

indicated that a loss of galactose correlated with a loss in binding

and infection of all phage tested: kh, 643, 1, c2 and ml3. In addition,

a loss of rhamnose correlated with a reduction in binding of phages kh

and ml3. Inhibition studies of phage binding by lectins specific to

galactose suggested that phage kh does not bind directly to galactose.

Incubation of any of the five phages with 0.5M rhamnose, but not

galactose, completely inactivated the phage. Addition of rhamnose to

a growing liquid culture infected with all five phages or with phage kh

inhibited the infection. This suggested that the receptor of phage for

L. lactis subsp. *cremoris* KH is the rhamnose of the extracellular

polysaccharide.

In a similar analysis, phage skl was found to require rhamnose and

glucose of the extracellular polysaccharide of *L. lactis* subsp. *lactis*

C2 for binding. The lectin studies suggests that phage sk1 does not bind directly to glucose. The partial inhibition of phage sk1 infection when rhamnose, but not glucose, was added to a liquid culture suggests that phage sk1 binds directly to the rhamnose.

The phage-resistant mutants isolated from superinfections of *L. lactis* subsp. *lactis* C2 with phage c2 did not form plaques, but bound normally to phage c2. The sensitivity of these mutants to phage sk1 was also reduced significantly. In another analysis of mutants isolated from superinfections with phage sk1, none formed plaques with either phage c2 or sk1, but bound normally to both phages. The mutations affected the cell membrane, as the membrane from wild type, but not from phage-resistant cells, inactivated phage c2. The phage-inactivation activity was eliminated by treatment with a protease, but not a glycosidase. The partially purified phage-inactivating protein was found to have an apparent molecular weight of 350,000 under non-denaturing conditions and an apparent subunit size of 32 kDa. It is proposed that a multimeric complex of the 32 kDa protein is required for phage c2 and sk1 infection of strain C2.

The Cell Surface Polysaccharides and Membrane Protein required for

Bacteriophage Infection of *Lactococcus lactis*

by

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Type by the author, _____ Ruud Valyasevi _____

DEDICATION

To my parents,

for their love, sacrifice, patience, understanding and support.

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CONTRIBUTION OF AUTHORS

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INTRODUCTION

Lactococci are members of the lactic acid bacteria and are used widely as starter cultures in the fermentation of milk. During the fermentation, lactose is converted to lactic acid, causing a reduction in pH and coagulation of milk. The ability to increase acidity, break down milk protein and produce flavor and aroma compounds make the lactic acid bacteria indispensable in the manufacturing of cheese. The reduction in pH is also of public health significance, as it discourages the growth of undesirable spoilage and pathogenic bacteria (37).

In almost all dairy fermentations, the lactococcus bacteria of either *Lactococcus lactis* subsp. *lactis* or subsp. *cremoris* are used to increase the acidity while subsp. *lactis* biovar diacetylactis is often used to produce an aroma compound of diacetyl. In certain cases such as Swiss cheese production, the lactococcus bacteria are used in addition to other lactic acid bacteria, particularly Leuconostoc or thermophilic starters to assist in acid production. The lactococci usually make up 90% of the dairy starter population. A good starter culture can increase the titratable acidity to 0.8 - 1.0% calculated as lactic acid and decrease the pH to 4.3 - 4.5 (6).

The greatest threat to fast acid production by lactococci during milk fermentation is bacteriophage infection (37,38). The non-aseptic nature of milk fermentation makes the elimination of bacteriophage not

possible, and bacteriophage infections remain a persistent problem in the dairy industries (30). The bacteriophage can enter through a variety of sources such as air, unsanitized utensils and vats, the media used to grow the starter cultures and perhaps, ironically the most important source of phage is from the starter cultures themselves (36,9,42).

The two most important sources of bacteriophages from the starter cultures are the induction of lysogenic strains (19,27,43,11) and the replication of phages in pseudolysogenic cultures (4). The lysogeny of the commercial lactococci starter by the temperate phage is well established and is inducible by a variety of means such as ultraviolet exposure or stress (27,43). The temperate phages are released and this results in the sudden appearance of phages virulent to the starter strains. The virulence may be due to the mutations or modifications of the temperate phages.

The second source of bacteriophage is from the phage-carrier state or the pseudolysogeny of the culture, resulting in a continual presence of phage. Though much less is understood than for the lysogenized state, the pseudolysogeny of the culture involves lytic phages (4). The phage carrier state occurs particularly in mixed-strain starter cultures of lactococci, as they harbor a variety of mechanisms of phage resistance on their plasmids (16,17,30,41). The inherent instability of the plasmid results in heterogeneous populations of phage-sensitive and phage-resistant cells as some phage resistant cells lose a plasmid-coded resistance upon growth. The virulent phage particles can

propagate on the sensitive cells and start an infectious lytic cycle. The repeated infection of phage in the bacterial culture establishes the phage-carrier state (4).

Once the phages attack sensitive cells during the commercial cheese-making process the bacteria lyse and release large numbers of progeny phages. The subsequent cycles of infection result in a drastic increase in phage particles, near-total lysis of bacterial cells and ultimately slow or inactive fermentation. The attack by phage results in substantial economic losses to the dairy industries. Much effort has been focused on the elimination of phage infection during the manufacturing of cheese. One of the approaches used is to actually encourage the contamination of phage in mother or bulk starter cultures (45). The presence of phage kills sensitive cells and through the selection pressure allows the outgrowth of resistant cells (35). The addition of phage-laden whey to the culture was found to extend the useful life of the culture to over one month. Cultures grown without added whey produced acid satisfactorily for only 10 days, after which the acid production appeared to slow down (44).

The strategy widely used in cheese plants to minimize the attack of phage is to use undefined blends of strains of L. lactis subsp. lactis and subsp. cremoris (46,47). Presumably each strain of the undefined blend is not sensitive to the same species of phage. Therefore, an attack by one species of phage would not affect all strains. By alternating the use of cultures from one fermentation batch to the next, the risk of phage proliferation is further minimized.

However, the multiple strain cultures apparently pose as many problems in cheese making as they solve. The undefined strains tend to vary not only in phage sensitivities, but in acid production and flavor characteristics as well. These strains may introduce more temperate phages, which can then be induced to release more phage particles into the fermentation system (19). New phage produced by the undefined blends are often mutants which are capable of infecting lysogenic strains (21). However, the proliferation of the mutated phage can be effectively controlled by removing the indicator strain supporting the phage growth. This requires an accurate monitor of phage infections during the fermentation. Unfortunately, this is practically impossible when the starter blends are unknown (46,47,28). Because of these problems with mixtures of unknown strains, a program has been developed which uses defined strains (38).

A defined multiple strain culture usually consists of 3 to 6 different strains of *L. lactis* subsp. *cremoris* which are not related in phage sensitivities and are insensitive to phages in the plant. The strains are selected according to their insensitivities to phage by testing each strain with phage-containing whey samples. Furthermore, these strains are also selected by their abilities to produce acid, survive at 37° C, hydrolyze milk protein and produce aroma and flavor compounds. The appearance of phage during the cheese-making is monitored by testing phage-containing whey against each individual strain. If the presence of phage is detected, a phage-sensitive strain is identified and removed from the starter cultures. The isolated

phage-sensitive strain is made into a resistant mutant by superinfecting cells with phage-laden whey (12,44,20,22). The mutant cells are tested to be certain that the mechanism of resistance to phage is not lysogenic immunity. For mutant cells of *L. lactis* subsp. *cremoris*, they are tested for their inabilities to adsorb phage. The mutant cells are also tested for fast acid production, off flavors when grown in milk and production of good flavors. In the meantime, the use of the multiple strain culture is continued in the fermentation without the phage-sensitive strain. Once the resistant strain with desired cheese-making characteristics is isolated, it is reintroduced into the cheese plant.

The successful use of phage-resistant mutants, coupled with the program of phage monitoring, have been reported in New Zealand cheese factories (20), England (22), Australia (12,3) and the Netherlands (7). The defined strain program has recently been used successfully in certain cheddar cheese plants in Ireland and Germany. In the U.S., the use of defined strains and their resistant mutants has enjoyed considerable success in cheddar and monterey jack operations as well as in cottage cheese and buttermilk manufacture (46,47,28).

Despite the popularity of the multiple strain program worldwide, the molecular mechanism of cellular resistance to phage is not well understood. In order to understand this mechanism, the molecular details of the interaction of phage to the host cell surface need further elucidation. With the application of recently developed and innovative molecular biological techniques, most research efforts on phage resistance have been focused on plasmid-mediated phage defense

systems. These mechanisms include deoxyribonucleic acids (DNA) restriction and modification (2,31,8,10) abortive infection (33,18,13,8,23,41) and prevention of adsorption (39,40,5,32). Recently plasmid-directed phage defense mechanisms have been genetically engineered to construct phage-insensitive strains (29,34).

Comparatively little attention has focused on chromosomally encoded genes which are associated with phage resistance. The most prevalent mutations of chromosomally encoded genes which leads to phage resistance in *L. lactis* subsp. *cremoris* cause a loss of phage adsorption to the host (30). These mutants can be isolated relatively easily with frequencies ranging from 8 X 10^{-4} to 6 X 10^{-7} (15). The high frequencies suggest that the mutants are resulted from simple mutations such as frameshifts or base substitutions. A low reversion rate contributes to the length of time - as long as 9 months in commercial cheese manufacturing - that the culture can be used without the appearance of phage (20).

The ability of phage to constantly evolve and adapt to the new environment has made the complete elimination of phage infection during the manufacturing of cheese a formidable task. In order to appreciate this dynamic of evolution, the reproductive cycle of phage must be better understood. In general, a phage infection cycle involves seven steps (1):

    1. Adsorption of phage to a susceptible host cell

    2. Release of phage DNA or RNA into the cell

    3. Early steps of phage-specific enzyme synthesis for replication

of nucleic acid

4. Replication of phage nucleic acid

5. Synthesis of phage structural proteins

6. Assembly of nucleic acid and structural proteins into new phage

7. Release of phage particles

The adsorption of phage involves at least three sequential steps; initial contact, reversible binding, and irreversible binding. The initial contact occurs when the freely moving cells come into contact with the phage by Brownian or random movement. This leads to a reversible binding, which is an association of any phage structure with the cell surface. The reversible interaction allows phage to probe the cell surface for a site of irreversible attachment. The irreversible attachment is closely associated with the partial release of phage DNA or eclipse (24).

The specificity of phage adsorption and the location of phage receptors for *L. lactis* subsp. *lactis* and subsp. *cremoris* were first reported by Oram and Reiter (26). All the phages, with the exception of phage ml3 and d9, were found to bind irreversibly to the trypsin-treated cell walls from both subspecies. Phages ml3 and d9 were found to adsorb reversibly to the trypsin-treated cell wall of *L. lactis* subsp. *lactis* ML3. If the crude cell walls (not treated with trypsin) were incubated with phage ml3, the binding was irreversible. Treatment of receptor substance with n-butanol or alcohol significantly reduced the adsorption of phage ml3. This suggested that the receptor substance is lipoprotein. The molecular weight of the receptor analyzed by gel

filtration chromatography was reported to be in the range of 200,000 to 400,000 as shown by analysis of supernatant from trypsin treated cell wall (26) and 200,000 to 800,000 as shown by analysis of deoxycholate solublized membranes (25).   Attempts to further purify the receptor substance by precipitation with ammonium sulfate or by DEAE-cellulose resulted in a significant loss in phage inactivation activity (25).   The receptor substance was analyzed by analytical utracentrifuge and found to sediment as a single diffuse boundary of 2.21S at 26° C (S_{20w} 1.91). The ratios of the mole percentages of hydrophilic to apolar amino acids isolated from trypsin treatment and deoxycholate extracts were 1.68 and 2.20 respectively, and the mole percentages of charged amino acids were 37.0 and 34.3 respectively.

Keogh and Pettingill (14) reported that treatment of cell wall from *L. lactis* subsp. *cremoris* EB7 with trypsin and pepsin did not significantly reduce phage eb7 binding.   Preincubation of phage with D-galactosamine and D-glucosamine prevented the phage from infecting the cells.   L-rhamnose preincubated with phage had no inactivating activity, but the activity was observed when used in combination with D-galactosamine.   The study concluded that the major determinants of serological variant specificity of group N streptococci (L-rhamnose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine) are involved in phage binding determinants.   It is not known from these studies if these saccharides are components of wall polysaccharide of *L. lactis* subsp. *cremoris* EB7 or whether these components inactivate the phage by binding specifically to the phage.

These previous studies showed that the phage receptor substances for *L. lactis* subsp. *lactis* and *cremoris* are located either on the cell walls or the cell membranes. None of these studies have yet identified the wall or membrane components serving as phage receptor at the molecular level. In order to fully understand the interaction of phage with the cell, a direct correlation must be established between the presence of specific saccharides and/or proteins on the cell surface and phage attachment to the cell. Ultimately it is believed that these results will be used to construct a phage-resistant strain by genetically altering the genes which control the expression of the cell surface components required for phage infection.

The results of my studies are organized in three chapters as follows:

Chapter 1 describes the binding of phage to the cell wall of lactococci. The receptor for phage kh was found to be the rhamnose and galactose of the exopolysaccharide of *L. lactis* subsp. *cremoris* KH. This was the first report which defined a phage receptor at the molecular level for *L. lactis*.

Chapter 2 describes the receptor for phage skl, which was found to be the rhamnose and glucose of the exopolysaccharide of *L. lactis* subsp. *lactis* C2. This is the first description of the phage receptor at the molecular level in *L. lactis* subsp. *lactis*.

Chapter 3 describes the identification and purification of a membrane protein required for phage c2 and skl infection of *L. lactis* subsp. *lactis* C2. This is the first report which identifies a membrane

protein required for phage infection in lactococci.

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Chapter 1

The Bacteriophage kh Receptor of *Lactococcus lactis* subspecies *cremoris* KH is the Rhamnose of the extracellular wall polysaccharide

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ABSTRACT

A receptor for bacteriophages of lactic acid bacteria, including *Lactococcus lactis* subsp. *cremoris* KH, was found on the cell wall and not on the cell membrane, as determined by a phage binding assay of sodium dodecyl sulfate- and mutanolysin-treated cell walls. The cell wall carbohydrates of *L. lactis* subsp. *cremoris* KH were analyzed by gas chromatography and mass spectrometry and found to contain rhamnose, galactose, glucose and N-acetylglucosamine. Similar analysis of mutants that were reduced in the ability to bind phages kh, 643, c2, ml3 and 1 indicated that galactose was essential for binding all phages. In addition, rhamnose was required for binding phages kh and ml3. Inhibition studies of phage binding using two different lectins with a specificity for galactose indicated that phage kh may not bind directly to galactose. Rather, galactose may be an essential structural component located in the vicinity of the receptor. Incubation of any of the five phages with rhamnose, or phage kh with purified cell walls, inactivated the phages. Inactivation required divalent cations, and was irreversible. Inactivation of phage was stereo-specific for rhamnose, as neither L (+) nor D (-) fucose (the stereoisomers of rhamnose) inhibited the phage. Furthermore, phage infection of a culture was completely inhibited by the addition of rhamnose to the medium. Therefore, the receptor for phage kh appears to be a rhamnose component of the extracellular wall polysaccharide.

INTRODUCTION

Bacteriophage infection initially requires a specific recognition between the phage and the host cell. The specificity of phage adsorption to cell surface receptors has been well studied in *Escherichia coli* and other gram negative bacteria. In these organisms, the phages recognize either the lipopolysaccharide (LPS) or specific proteins of the outer membrane. For example, coliphage ϕX174 adsorbs to the carbohydrate moiety of the LPS (20, 24), whereas lambda phage adsorbs to an outer membrane protein involved in maltose transport (12).

In gram positive bacteria, phage adsorption almost always involves the cell surface carbohydrates. Such is the case for phages of *Bacillus subtilis* (33), *Streptococcus pyogenes* (4, 8), and various species of lactobacillus (7, 18, 31, 32). In lactococcus species, phage receptors have been detected on the cell wall material, and presumedbly are part of the peptidoglycan or group-specific carbohydrate (21, 26, 28). One notable exception is the report of a membrane protein receptor for phage ml3 (25).

Lactococci are important starter cultures in cheese manufacturing. One common cause of failed fermentation is bacteriophage infection, which lyses the starter culture. In a step toward understanding and preventing phage infection in this organism, we have partially characterized the molecular determinants of phage receptors in *L. lactis* subspecies *cremoris* KH. We now report that a rhamnose component of the extracellular polysaccharide may be the primary binding site for two of

five phages tested, including phages kh and m13. Conditions for prevention of phage infection in culture are demonstrated.

MATERIALS AND METHODS

Bacterial strains, phages and media. Phages and their bacterial hosts are listed in Table 1.1. The lactococcus bacteria were grown and maintained on M17 medium at 30° C (29). Strain sensitivities to the various phages were determined by spotting the phages on lawns of the host cells. Single plaques were isolated from sensitive strains and used to prepare phage stocks (29).

Preparation of cell walls. Cell walls were isolation by a modified procedure of Chatterjee (2). Cells were harvested at A_{600} = 1.8, resuspended in 10 mM KH_2PO_4, pH 6.8 to a final concentration of 150 mg/ml, and mechanically disrupted with glass beads (0.1 mm diam.) in a Bead Beater (Biospec Products, Bartlesville, OK), according to the manufacturer's instructions, at 4° C for 7 min. In the preparations used for digestion with mutanolysin (Sigma Chemical Co., St. Louis, Mo) or extraction with sodium dodecyl sulfate (SDS), 0.2 mM phenylmethylsulfonyl fluoride (PMSF) was included during the mechanical disruption. The glass beads were removed by filtration through paper (Whatman Ltd., Maidstone, England) and whole cells and cell debris were removed by centrifugation at 1400 X g for 5 min. Cell walls were sedimented by centrifugation at 15,000 X g for 10 min. Resuspended cell walls were treated with DNase (40 μg/ml), RNase (40 μg/ml) and PMSF (0.2 mM) for 1 hr at 37° C. With the exceptions of cell walls prepared for SDS and/or mutanolysin treatments, cell walls were further purified by extraction with hot SDS (10 mg/ml) at 70° C for 20 min. SDS was removed

by three successive centrifugations (15,000 X g, 25° C for 10 min) using 500 volumes of H_2O per wash. The cell walls were collected by centrifugation as above. After the third wash, the pellet was resuspended in 100 volumes of H_2O and centrifuged at 1,400 X g for 5 min to remove debris. The supernatant was removed and twice again centrifuged at 1,400 X g for 5 min. The cell walls were sedimented by centrifugation as above, resuspended in sterile water (100 mg/ml wet weight) and stored at -70°C.

Phage binding assay. Preliminary experiments were conducted to determine the concentration of cell walls used and the assay incubation time required for phage-cell wall binding to reach the completion point. The concentration of cell walls required was determined by mixing increasing amounts of cell wall with a fixed concentration of phage. The concentrations of cell walls used in the assay depended on the phage and its host. In the case of phage c2, for example, a range of 2-100mg of cell wall was used with a phage concentration of 10^8 PFU/ml. It was found that the amount of phage bound was directly proportional to the concentration of cell walls up to 90% phage bound. Additional amounts of cell walls above the concentration used to bind 90% of phage did not significantly increase the percentage of phage binding. This suggests that the phage receptors were saturated at cell wall concentrations required for 90% binding at $1X10^8$ PFU/ml. Therefore, the concentration of cell walls used in subsequent assays was the minimal amount necessary to bind 90% of the phage. In all cases, this was about 10 mg/ml cell

wall (as determined by the mass of cellular wet weight) for phage c2.

In order to determine incubation time that allows the binding of all phage to the cell wall, phage binding was assayed at different time intervals. It was found that at 5 min incubation, about 90% of the phage bound to the cell wall. Further incubation up to 60 min increased the amount phage bound by only 1%. This suggests that at incubation times greater than or equal to 5 min, binding was complete. Routinely, a 10 min incubation time was used in the assay.

Cell walls at a concentration that was found to bind 90% of the phage were mixed and shaken with phage (1×10^8 PFU/ml) in 50 mM Tris-HCl, pH 6.8 and 15 mM $CaCl_2$ at 25° C for 10 min.The mixtures were centrifuged (12,000 X g, 4 min), and the plaque-forming titers of the supernatants were determined by standard procedures (29). The percentage of phage bound to the cell walls was calculated by dividing the difference in the titers of added phage and unbound phage by the titer of added phage and multiplying by 100.

Treatments with mutanolysin, SDS and proteinase K. Cell wall suspensions in 50 mM Tris-HCl, pH 6.8, at concentrations that had been found to bind 90% of a specific amount of a phage were treated with either SDS (1%) at 37℃ for 30 min, mutanolysin (40 U/ml) at 37℃ for 30 min, or proteinase K (0.1 mg/ml) at 37°C for 30 min followed by PMSF (0.2 mM).

Extraction with trichloroacetic acid and assay for phosphate. Cell walls were extracted with hot trichloroacetic acid for the removal of teichoic acids (7) and assayed for total phosphate (1) as described.

Sample preparation for gas chromatography and mass spectrometry. Dried cell walls (10 mg) with ribose (80 μg/ml) added as an internal standard were resuspended in 2 N HCl (5 mg/ml). The mixtures were flushed with nitrogen gas and hydrolyzed at 100° C for 5 h. The hydrolysates were neutralized with 15 N NH_4OH using phenolphthalein indicator and adjusted to equal volumes. Hydrolysates (300 μl) with added xylose (50 μg/ml) were derivatized to alditol acetates essentially as described (14). Prior to mass spectral analysis, the alditol acetate-derivatized components were flushed with ammonia gas.

Gas chromatography and mass spectrophotometry. The alditol acetate derivatives (3 μl) were separated on a 3% SP-2340 glass column (3 ft length X 12 mm ID) (Supelco Inc., Bellefont, PA) connected to a Hewlett-Packard model 5710A gas chromatography equipped with a flame ionization detector. The temperature was kept at 180° C for 6 min following sample injection and then raised at 2° C/min to 240° C. The oven was kept at the final temperature for 10 min. Chromatographic conditions were according to the manufacturer specifications (GLC/HPLC Bulletin 774B, Supelco, Inc., Bellefonte, PA). Peak areas and retention times were reported by using a Hewlett-Packard Model HW 3390A electronic integrator connected to the gas chromatograph. For identification of components

by molecular weight, ammonium derivatized alditol acetate samples were separated on a J&W DB 5 column (30 m length X 0.32 mm ID) (J&W Scientific, Folsom, CA) by using a Finnigan model 4000 mass spectrometer.

Identification and quantitation of alditol acetates. The derivatized cell wall polymer components separated by gas chromatography were identified by comparison of their retention times with those of reference standards. The identities were confirmed by determination of their molecular weights using mass spectroscopy. Each saccharide was quantified as described (9), with the following modifications: The peak area ratios of reference saccharides/xylose were used for construction of standard calibration curves. A correction for variations in loss of saccharides during acid hydrolysis was made by normalizing each saccharide/xylose ratio to the ribose/xylose ratio in the sample from the parental strain. The final concentration of each component was calculated by correlating the corrected area ratio to the standard calibration curve.

Isolation of resistant mutant cells. *L. lactis* subsp. *cremoris* KH (2 X 10^8 CFU) was superinfected with phage kh (3 X 10^8 PFU) and mixed in soft M17 agar (0.4 %) that also included 15 mM $CaCl_2$. Superinfection is defined as phage and cell were mixed at the multiplicity of infection (MOI) greater than 1. After incubation at 30° C for 36-48 hours, cells were collected by rinsing the top agar layer. Single colonies were

isolated on M17 agar that contained 2 x 10^8 PFU/ml of phage kh (resistant mutants 2 and 3) and on M17 agar without phage (resistant mutant 1). Overnight cultures (0.2 ml) from single colonies were prepared, mixed with phage (1 X 10^2 PFU) and 15 mM $CaCl_2$, and spread on M17 agar plates. Phage-resistant mutants that did not form plaques were selected. Cell walls of selected mutants were isolated and tested for losses in binding to phages kh, 643, c2, ml3 and l. The amount of phage bound to cell walls prepared from each mutant is expressed as a percentage of the amount bound by the same weight of cell walls from the parental strain KH.

Effects of lectins on phage adsorption. Cell walls at concentrations that bind 90 % of the phage were mixed with lectins (50-500 μg/ml; Sigma Chemical Co., St. Louis, MO) from *Vicia faba* (broad bean), *Momordica charantia* (bitter pear melon) or *Maclura pomifera* (seeds of Osage orange tree) in 50 mM Tris-HCl, pH 6.8. For competitive cell wall binding by lectins from *M. charantia* and *M. pomifera*, both lectins were added together to the cell walls. The mixtures were agitated at 25° C for 25 min. Either galactose or glucose was added, and again incubated as above. Phage was added and binding was assayed as described above.

Phage inactivation by saccharides. The ability of various saccharides to inactivate phage kh was determined as follows: Different concentrations of the saccharides (0-0.5 M) were mixed and agitated with phage (1 X 10^8 PFU/ml) in 50 mM Tris-HCl, pH 6.8 and 15 mM $CaCl_2$ for 60

min at 25°C. The mixtures were diluted to 1 X 10^3 PFU/ml, mixed with cells (1 X 10^{10} CFU/ml), and assayed for plaques as described above.

Prevention of infection in culture. Rhamnose (0, 0.1, 0.4, or 0.6 M, as indicated in the fig. legends) or galactose (0.6 M) and $CaCl_2$ (15 mM) were added to mid-exponential phase cultures (A_{600} = 0.2), which were immediately inoculated with phage (2 X 10^6 PFU/ml, Fig. 1.4; 2 X 10^4 PFU/ml. Fig.1.5). A control culture received neither rhamnose, phage nor calcium. The A_{600} was measured every 30 min for 4 hr, and then at 8 and 19 hr (Fig. 1.4), or every hr for 6 hr and then at 15 hr (Fig. 1.5). Cells were recovered by centrifugation, washed free of rhamnose and resuspended in M17 broth to about A_{600} = 0.02. Phage (2 X 10^6 PFU/ml) and 15 mM $CaCl_2$ were added, and growth was again measured as above every hour for 7 hr.

RESULTS

Locating the phage receptor sites. Two strains of *L. lactis* subsp. *lactis*, one strain of *L. lactis* subsp. *lactis* biovar. *diactylactis* and two strains of *L. lactis* subsp. *cremoris* were analyzed for phage binding sites. The cell envelopes were treated with either SDS to remove membrane bound proteins or mutanolysin (N-acetylmuramidase) to hydrolyze the peptidoglycan. After treatment with SDS, there was a total loss of proteins, as measured by the lack of detectable material on Coomassie blue-stained SDS polyacrylamide gel electrophoresis. Treatment with SDS did not reduce significantly phage binding (Table 1.1). However, binding was reduced by 36-99 % for all phages tested, except phage 5, when the cell envelopes were treated with mutanolysin. This suggested that nearly all phages tested bound to a carbohydrate component of the cell wall, which includes the peptidoglycan and associated polysaccharides, such as the group N-specific carbohydrate.

The small changes in binding after SDS treatment are within the approximately 20% variation that we measure with the plaque assay. This suggests that protein components of the plasma membrane and any other protein associated with the peptidoglycan or extracellular material are not necessary for binding between these phages and hosts. Additional suggestive evidence that the cell surface phage receptors are not proteinaceous is supported by the following: cell walls from *L. lactis* subsp. *lactis* C2 and ML3 were treated with proteinase K, and the binding of phage 5 to the former, and ml3 to the latter, was 92 and 91

%, respectively.

Isolation and composition of the cell wall polymer from *L. lactis* subsp. *cremoris* KH. Cell envelopes from the culture of *L. lactis* subsp. *cremoris* KH were isolated and the cell walls purified by differential centrifugations and treatments with DNase, RNase and hot SDS. Analysis of the cell wall exopolysaccharide components by gas chromatography identified rhamnose, galactose, glucose and N-acetylglucosamine. The identities of all except N-acetylglucosamine were confirmed by mass spectrometry. The derivatized rhamnose had a molecular weight of 394, which closely agreed with the calculated value of 393.34. The molecular weights of derivatized glucose and galactose were 452, in close agreement with the calculated value of 452.43 (Table 1.2). Because the gas chromatography analyses were done on alditol acetate derivatives of the cell wall components, it is possible that the cell wall polymer consisted of glucosamine rather than N-acetylglucosamine. However, neither of these could be isolated in sufficient quantity for molecular weight identification by mass spectrometry, and the identity of this one component remains uncertain. The mass ratio of rhamnose:galactose:glucose per milligram of dry weight of cell wall was 6.6:1:1.6.

Teichoic acids were not detected. Hot trichloroacetic acid extraction of the cell wall (peptidoglycan and the associated extracellular polysaccharides) yielded no change in the total phosphorus content (50.3 ng phosphate/mg cell wall dry weight). Additionally,

there was a complete absence of the acetate derivatives of ribitol and glycerol in the gas chromatography analysis.

Isolation of resistant mutants, loss of sensitivity to infection and binding to cell walls, and changes in cell wall composition. Spontaneously occurring phage-resistant mutants of *L. lactis* subsp. *cremoris* KH were selected by superinfection with phage kh. Single colonies of mutants were screened for loss of the ability to adsorb phage kh. The resistant mutants were then assayed for their sensitivities to phage infection and abilities of the cell walls to bind the five different phages, each infectious to the parental strain. The results (Table 1.3) show that for resistant mutant 1 (RM 1), a reduction in binding of the five phages to the cell wall ranged from 7-44%, while the sensitivity to infection was 0-23 %. The resistant mutant 2 (RM 2) cell walls exhibited a 92% loss of binding to phage kh and 99% to phage ml3. The binding of phage 643 and 1 to RM 2 was reduced 12 and 1 %, respectively, while that of phage c2 was increased 12%. RM 2 sensitivity to infection was 98 and 99 % for phages kh and ml3, respectively, and 147, 354 and 181 % for phages 643, 354, and 1, respectively. In the resistant mutant 3 (RM 3), there was a large loss (71-99%) of binding to all five phages, as well as a near-total loss (95-100 %) of sensitivity to infection.

The cell walls of the resistant mutant cells were then analyzed for changes in the sugar composition by gas chromatography (Table 1.3). RM 1 showed a 38% reduction of galactose, a 13% decrease of rhamnose and

an 8% increase of glucose. The partial losses in binding to all five phages correlated with a reduction in the amount of galactose on the RM 1 cell wall. In RM 2, there was a 65% loss of rhamnose, 46% loss of galactose and a 10% reduction of glucose. A reduction of rhamnose, in addition to the partial loss of galactose, correlated with a total loss in binding to phages kh and ml3 and an increase in binding to phage c2. The loss in rhamnose, however, correlated with a slight (8 and 1 %) reduction in the binding of phages 643 and 1 to the RM 2 cell wall. The reductions of galactose in the cell walls of RM 1 and RM 2 were more or less of the same magnitude. Therefore, the partial loss in binding of phage 643 correlated with the reduction in galactose. The RM 3 exhibited an 86% reduction of galactose, a 32% increase of glucose, and a 17% increase of rhamnose. The significant reduction in galactose on the cell wall correlated with the near-total loss of binding to all five phages. This suggests that galactose is required for the binding of all phages. Furthermore, the rhamnose units missing from RM 2 were important for binding phages kh and ml3. The reduction in binding generally correlated with a loss in sensitivity to infection for phages kh, ml3, c2 and 1, but not for 643.

Inhibition of phage binding by lectins. Preincubation of cell wall from *L. lactis* subsp. *cremoris* KH with a lectin from *Vicia faba* that specifically binds glucose and N-acetylglucosamine, did not inhibit the binding of phage kh (Fig. 1.1A). This suggests that glucose and N-acetyl glucosamine are not part of the phage binding site.

In contrast to the above results, a significant reduction in phage binding occurred when the cell wall was preincubated with a lectin from *Mormodica charantia* that specifically recognizes galactose (Fig. 1.1A). Moreover, when free galactose, but not glucose, was added to compete with the cell wall for binding to the lectin, the inhibition of phage binding was completely reversed (Fig. 1.1B). This suggests that galactose may be part of the phage binding site.

However, another lectin with a specificity for galactose, from *Maclura pomifera*, did not inhibit phage binding (Fig. 1.2, upper curve). As a control to show that the *M. pomifera* lectin bound to the cell wall and had the same specificity as the lectin from *M. charantia*, cell walls were preincubated with 50 μg of lectin from *M. charantia* and 50 to 500 μg of the lectin from *M. pomifera*. The results show that phage binding increased with increasing concentrations of the lectin from *M. pomifera* (Fig. 1.2, lower curve). Therefore, both lectins bound to the cell wall with the same specificity.

The apparent discrepancy in the results from the galactose-specific lectins can be explained as follows: The lectin from *M. pomifera* has a molecular weight of ~40 X 10^3, about one-third the size of the lectin from *M. charantia* (mol wt 110 X 10^3). The smaller lectin will occupy a lesser volume, and when bound to the cell wall may interfere less with neighboring components of the phage receptor sites. The results of these experiments are interpreted to mean that galactose is located near the phage binding site, but does not actually bind to the phage itself.

Inactivation of phage by rhamnose. Incubation of phage kh with 0.5 M L(+) rhamnose resulted in 99% inactivation (Fig. 1.3). The effect was specific for rhamnose, as shown by the lack of inactivation by glucose and galactose (Fig. 1.3), as well as glucosamine and N-acetylglucosamine (data not shown). Moreover, neither L(-) fucose nor D(+) fucose (the stereoisomers of rhamnose) inactivated the phage (data not shown). This indicates that the phage binds to rhamnose. The other four phages were tested in an identical manner, and found also to be inhibited by rhamnose, but not by galactose (Table 1.4). These results suggest that rhamnose may inactivate many phages of *L. lactis* subsp. *cremoris*, and that there may be more than one environment or conformation for the rhamnose units in the exopolysaccharide.

These effects of rhamnose were found to require divalent cations such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+} and Zn^{2+} (Figs. 1.3 and 1.6B). Monovalent cations such as Na^+ could not substitute.

Rhamnose also prevented or delayed phage kh infection in liquid culture (Fig. 1.4A). When 0.1 and 0.4 M rhamnose was included in the growth medium, lysis of cultures inoculated with phage kh was delayed for either 2 hr (0.1 M) or indefinitely (0.4 M). Not only did 0.4 M rhamnose prevent lysis by the phage, but the cells continued to grow like the uninoculated culture. This suggests that rhamnose protected the cells by interfering at an early step in phage infection, such as the binding of the phage to the cell wall. In a similar experiment, a culture was inoculated with all five phages tested above. As with phage kh alone, rhamnose added to the culture prevented lysis (Fig. 1.5). As

a control, *L. lactis* subsp. *lactis* biovar. *diacetylactis* 18-16 and phage 18-16 were also tested, and found to be unaffected by rhamnose (Fig. 1.5).

To eliminate the possibility that the resistance to phage infection shown in Fig. 1.4A was a result of a selection for a spontaneous phage-resistant mutant, the following was done: The cells from the experiments shown in Fig. 1.4A were harvested and washed free of rhamnose, and re-inoculated with phage. No phage resistant mutants were found in either of the cultures previously grown in the presence of rhamnose (Fig. 1.4B). A phage resistant mutant was found in the culture previously inoculated with phage in the absence of rhamnose (Fig. 1.4B). Thus, rhamnose delayed or prevented phage infection of a growing culture by a mechanism other than acting as a selection pressure for a phage resistant mutant. An identical control was done on the cultures used for the experiment shown in Fig. 1.5, and no phage resistant mutants were found (data not shown).

DISCUSSION

Treatment of cell envelopes from three subspecies of *L. lactis* with mutanolysin and SDS indicated that the binding sites for 13 of the 14 phages tested are located on the carbohydrate components of the bacterial cell walls. No evidence for a membrane protein receptor was found.

The cell wall carbohydrate polymer from one of the hosts, *L. lactis* subspecies *cremoris* KH was analyzed, and found to contain rhamnose, galactose, glucose and N-acetylglucosamine. We have also found similar compositions in *L. lactis* subsp. *lactis* C2 and *L. lactis* subsp. *lactis* biovar. *diacetylactis* 18-16, although the latter lacks galactose and may contain some teichoic acids (data not shown). This carbohydrate composition is similar to that of some streptococci (6, 22, 23) and various species of lactobacillus (7, 32). We were unable to determine if any of the N-acetylglucosamine was derived from glucosamine.

Chemical analyses of the cell wall carbohydrates from three phage-resistant mutants of *L. lactis* subsp. *cremoris* KH showed that a near total loss in galactose correlates with the loss of phage binding. Therefore, galactose may be essential for the binding of all five phages kh, 643, ml3, c2, and 1. In addition to galactose, reduction in the amount of rhamnose also correlates with a total loss of binding of phages kh and ml3. Therefore, rhamnose is important for the binding of phages kh and ml3. For the other three phages tested, a loss of rhamnose had almost no effect (phage 643 and 1), or even seemed to increase binding (phage c2). An increase in phage binding as the result

of the loss of cell wall components has also been demonstrated to be a mechanism of unmasking cryptic phage binding sites (28). It is likely that phages kh and ml3 recognize different determinants than do phages 643, c2, and 1, and may be similar in binding specificity. It is interesting that phages kh and ml3 have different morphological types; small isometric and prolate heads, respectively (5, 19). Apparently the receptor site is independent of phage morphology.

The general pattern of sensitivity to infection of the resistant mutants correlates with the binding of phages kh, ml3, c2 and 1 (Table 1.3). However, phage 643 shows a large difference in sensitivity between RM 1 and 2, with little difference in binding. Based on differences in sensitivity to mutants selected for resistance to phage kh, there appear to be two groups: (1) phages kh and ml3, and (2) phages 643, c2, and 1. Sensitivities greater than 100% (Table 1.3, RM 2, phages 643, c2, and 1) may be attributable to faster growth rates of RM 2 as compared to KH. Other explanations are possible, such as changes that affect the kinetics of binding and eclipse, without affecting the number of binding sites.

Studies of the inhibition of phage kh binding by lectins indicated that a large lectin prevented phage binding, whereas a small lectin with the same specificity for galactose did not inhibit the phage. This suggests that the larger lectin blocks phage adsorption by physically hindering the phage receptor site. We interpret this to mean that the phage does not bind directly to galactose, although galactose is probably located in the vicinity of the receptor. Alternatively, the

binding of the two lectins at the same site may have had different effects on the structure of the cell wall, such as changes in the conformation of the carbohydrate polymer. Considering these results and those from the compositional analyses of the mutants discussed above, we conclude that galactose probably plays an essential, but indirect role in phage kh binding, perhaps by maintaining a required structure of the polymer or acting as a secondary binding site to stabilize the phage as it binds to the receptor.

If galactose is not the primary receptor, then the analysis of the mutants implicates rhamnose. Rhamnose was reported to be a determinant of a phage receptor in *Lactobacillus casei* (18, 32). Results in this report demonstrate that phage kh was inactivated by treatment with free rhamnose. Additionally, phage kh infection of a culture was prevented by including rhamnose in the growth medium. More recent experiments have demonstrated that antiserum against the rhamnose of the group G streptococcus inhibited phage kh binding to the cell wall by about 50%. From this and the results from the analysis of the mutants, we conclude that the phage kh receptor on the cell wall is rhamnose.

Treatment with rhamnose also inactivated the other four phages tested, and prevented lysis in liquid culture. These results were surprising, especially for phages 643, c2, and 1, because these phages appeared relatively unaffected in binding to the resistant mutants that were reduced in rhamnose content. However, it must be noted that all the resistant mutants still contained substantial amounts of rhamnose. In fact, we have been unsuccessful in finding a phage kh-resistant

mutant that completely lacks rhamnose. One explanation is that rhamnose exists in more than one environment or conformation within the polymeric cell wall carbohydrate. If this is true, then the partial loss of one type or group of rhamnose might differentially affect many phages that bind directly to the rhamnose components of the polysaccharide.

Earlier reports by Oram and Reiter (26) and Oram (25) demonstrated that phage ml3 binds to a membrane lipoprotein of *L. lactis* subsp. *lactis* ML3. Our results are in apparent conflict with these. In our experiments, cell envelopes from *L. lactis* subspecies *lactis* ML3 contained both cell membrane and wall (peptidoglycan and extracellular polysaccharides, presumably including the group N specific carbohydrate). These envelopes lost none of their ability to specifically bind phage ml3 after treatments with proteinase K and SDS, even though there was a total loss of detectable protein. The binding of phage ml3 was 91 and 96% to proteinase K- and SDS-treated cell envelopes, respectively. On the other hand, treatment with the glycosidase mutanolysin reduced the phage binding capacity by 83%, and this was not further reduced by subsequent treatment with proteinase K and SDS. This clearly shows that this phage receptor is on the cell wall and is not proteinaceous. One explanation for the apparent contradiction in results is the possibility that the phage or host is actually not identical in both laboratories. Alternatively, a membrane protein could be involved in the steps of the infectious process concurrently with, or subsequent to the binding. In this case, a membrane protein might have a specific affinity for the phage that could

lead to its misidentification as a receptor. There is precedence for such a mechanism of binding in the case of coliphage T4, which apparently has two separate types of receptors: the lipopolysaccharide and the cell surface protein OmpC (34).

An important characteristic of the phage kh binding to *L. lactis* subspecies *cremoris* is the apparent irreversibility. A mixture of phage and cell walls (sufficient to bind 90 % of the phage) added directly to cells produced the same number of plaques as the supernatant from the same mixture which had been centrifuged to remove the phage-bound walls (data not shown). If binding were reversible, more plaques would have formed in the mixture that had not been centrifuged. Further evidence is shown in Fig. 1.6A. After phage was bound to the cell wall complex, isolated by centrifugation and resuspended in buffer, no infectious particles were released into the supernatant.

In contrast to this, when an equivalent amount of cell wall was digested with mutanolysin, all the cell wall components became soluble, but were not able to inactivate the phage by preventing plaque formation (Table 1.1). Similar results were noted with rhamnose at concentrations estimated to be similar to those in the intact cell wall, although increased amounts of either the mutanolysin-solubilized wall (data not shown) or rhamnose (Fig. 1.3) did prevent plaque formation. This suggests that binding to soluble receptor determinants does occur, but probably with a lesser affinity than to the intact cell wall. It seems likely that the conformation of the cell wall carbohydrate polymer and the linkage between sugars would be important aspects of the phage

recognition site.

The significance of the apparent irreversibility of binding phage kh is that it suggests a mechanism for the early steps of the infectious cycle. Coliphage ϕX174 also binds irreversibly to the cell wall lipopolysaccharide of its host (17). However, the step that makes it irreversible is the process of eclipse, where part of the ϕX174 DNA is ejected from the phage capsid (10). The actual binding step can be a typical, thermodynamically reversible reaction, as has been shown with coliphages T4 (11, 13), T5 (15) and T1 (3), and for two phages of *Lactobacillus casei* (30, 31). Experiments are currently in progress to determine if eclipse occurs with purified cell walls from lactococcus, and what role membrane components may have in this process.

Another notable result was that both the inactivation of phage by soluble rhamnose (Fig. 1.3) and adsorption of phage to the cell wall require divalent cations (Fig. 1.6B). This not only suggests that inactivation occurs because of adsorption, but identifies an important role for divalent cations in the interaction of the phage with the cell surface. This has important implications for the mechanism of the early steps of phage infection. Divalent cations are involved in at least two functions of ϕX174 infection: (1) neutralizing the repulsive forces between the negative charges on the phage and the *E. coli* cell surface (27), and (2) releasing phage DNA from the capsid (16). Ongoing investigations should help to understand the role of calcium in phage infections of lactococcus.

Lactococcus bacteria are important starter cultures in milk

fermentations, and bacteriophage infection during fermentation is a major economic concern to the dairy industry. A deeper understanding of all phases of bacteriophage infections in the lactococcus bacteria will provide the details necessary for testing new and creative ways of eliminating the problem. One approach suggested by this report is the use of "mimic" phage receptors that would compete with growing cells for the phage. Perhaps cells could be programmed to secrete rhamnose or other decoys. Additional studies will be needed to focus on practical solutions.

ACKNOWLEDGMENTS

TABLE 1.1. Phage binding to cell walls
treated with SDS and mutanolysin

			% Phage Binding[a] to walls treated with:		
Host[b]		Phage	None	SDS	Mut[c]
L1	C2	c2	78	68	42
		sk1	99	97	18
		5	91	89	85
		13	76	66	24
		16	84	89	17
		102	78	68	8
		108	84	84	40
		1	88	88	48
		h	87	85	10
		ml3	82	94	26
		kh	87	70	0
L1	ML3	ml3	90	96	7
Lc	KH	kh	99	95	0
		643	76	80	0
		1	80	82	23
		ml3	99	96	0
		c2	84	79	0
Lc	205	1	90	69	2
		h	89	78	8
		205	99	90	0
		16	76	70	0
Ld	18-16	18-16	95	92	18

[a]Average values of two experiments.
[b]Ll, *L. lactis* subsp. *lactis*; Lc, *L. lactis* subsp. *cremoris*; Ld, *L. lactis* subsp. *lactis* biovar. *diacetylactis*.
[c]Mut, mutanolysin.

TABLE 1.2. Gas chromatography and mass spectrometry
analysis of saccharides from cell wall carbohydrates

| saccharide[a] | cell wall | molecular weight | |
		stds[b]	calc[c]
rhamnose	394	394	393.34
galactose	452	452	452.43
glucose	452	452	452.43

[a]ammonium alditol acetate derivatives
[b]reference standards
[c]calculated molecular weight

TABLE 1.3. Phage binding, sensitivity to infection, and saccharide analysis of resistant mutants

Strain[a]	% of phage binding					% sensitivity to phage					Carbohydrate[b] (μg/mg cell wall)		
	kh	m13	643	c2	1	kh	m13	643	c2	1	Rhm ± SD	Gal ± SD	Glu ± SD
KH	99	99	82	84	91	100	100	100	100	100	177 ± 10	27 ± 0	42 ± 1
RM 1	66	70	66	40	84	6	11	23	0	14	153 ± 7	17 ± 0	46 ± 3
RM 2	7	0	70	96	90	2	1	147	354	181	61 ± 4	14 ± 0	38 ± 3
RM 3	0	17	5	13	20	9	0	9	5	5	207 ± 11	3 ± 0.3	56 ± 4

[a] KH, wild type strain KH; RM, phage-resistant mutant.
[b] Rhm, rhamnose; Gal, galactose; Glu, glucose.

TABLE 1.4. Inhibition of plaque formation
by treating phage with monosaccharides

	% Inhibition							
	[Rhamnose][a]				[Galactose][a]			
phage	0	0.1	0.3	0.5	0	0.1	0.3	0.5
kh	0	13	84	99	0	22	15	26
m13	0	20	87	99	0	34	54	41
643	0	21	98	100	0	0	0	18
c2	0	32	98	100	0	30	32	27
1	0	92	99	100	0	0	0	0

[a]saccharide concentrations in mol/l.

FIG. 1.1. (A) Effects of different concentrations of lectins from *V. faba* (□) and *M. charantia* (O) on phage kh binding to the cell walls of *L. lactis* subsp *cremoris* KH. Cell walls (200 μg) were preincubated with various concentrations of lectins prior to addition of phage kh (10^8 PFU). The mixtures were centrifuged and the supernatants were titered for phage. (B) Galactose reverses lectin inhibition of phage binding. Cell walls (200 μg) were preincubated with lectin from *M. charantia* (50 μg). The indicated concentrations of galactose (▪) or glucose (•) were added, followed by phage (10^8 PFU). The mixtures were assayed as in (A).
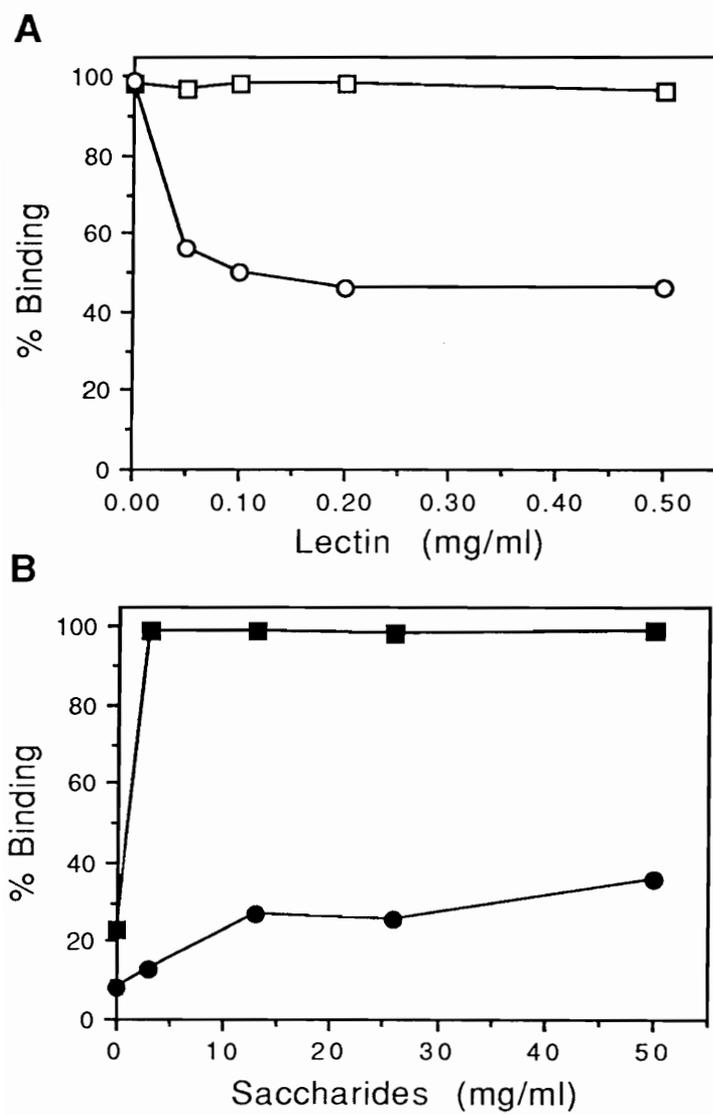
FIGURE 1.1

FIG. 1.2. Effects of lectins specific for galactose on phage kh binding to the cell walls of *L. lactis* subsp *cremoris* KH. Cell walls (200 μg) were incubated with the indicated concentrations of lectin from *M. pomifera* with (▲) and without (Δ) a constant amount of lectin from *M. charantia*. After phage kh was added (10^8 PFU), the mixtures were centrifuged and the supernatants titered for phage.
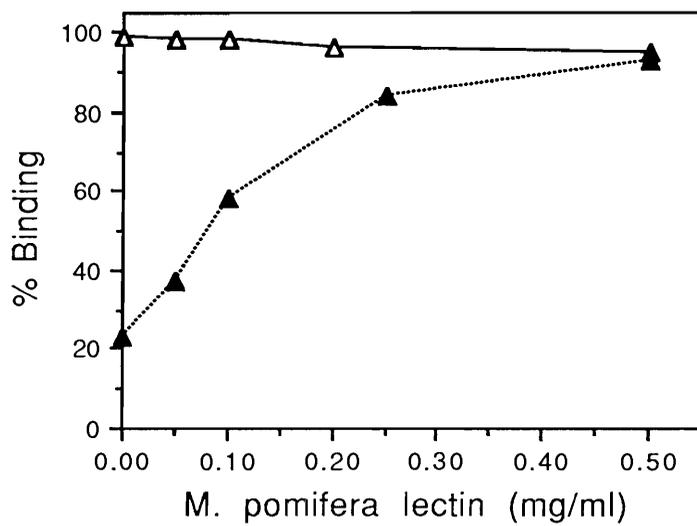
FIGURE 1.2

FIG. 1.3. Effects of different concentrations of saccharides on infectivity of phage kh. Phage kh was preincubated with $CaCl_2$ (10 mM) and the indicated concentrations of saccharides. The mixtures were diluted, mixed with cells, and assayed for the number of plaques. The amount of inhibition is expressed as % of a control that was not preincubated with a saccharide. Saccharides: ▲ , L(+) rhamnose; ● , D(+) glucose; and ■ , D(+) galactose. Δ , L(+) rhamnose without $CaCl_2$.
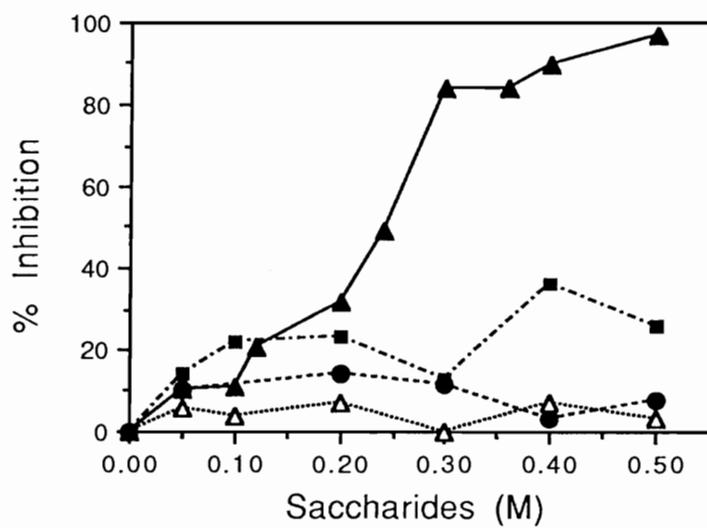
FIGURE 1.3

Fig. 1.4. (A) The effects of L(+)rhamnose on the growth of phage-infected liquid cultures of *L. lactis* subsp. *cremoris* KH. Growing cultures containing 0.1 M (Δ), 0.4 M (▪) and no (O , ▲) rhamnose were infected with phage kh at a multiplicity of 0.01, except the control (O) that was not infected. Growth was measured by A_{600}. (B) Growth curves of washed cells from the cultures described in (A) that were re-inoculated with phage kh.
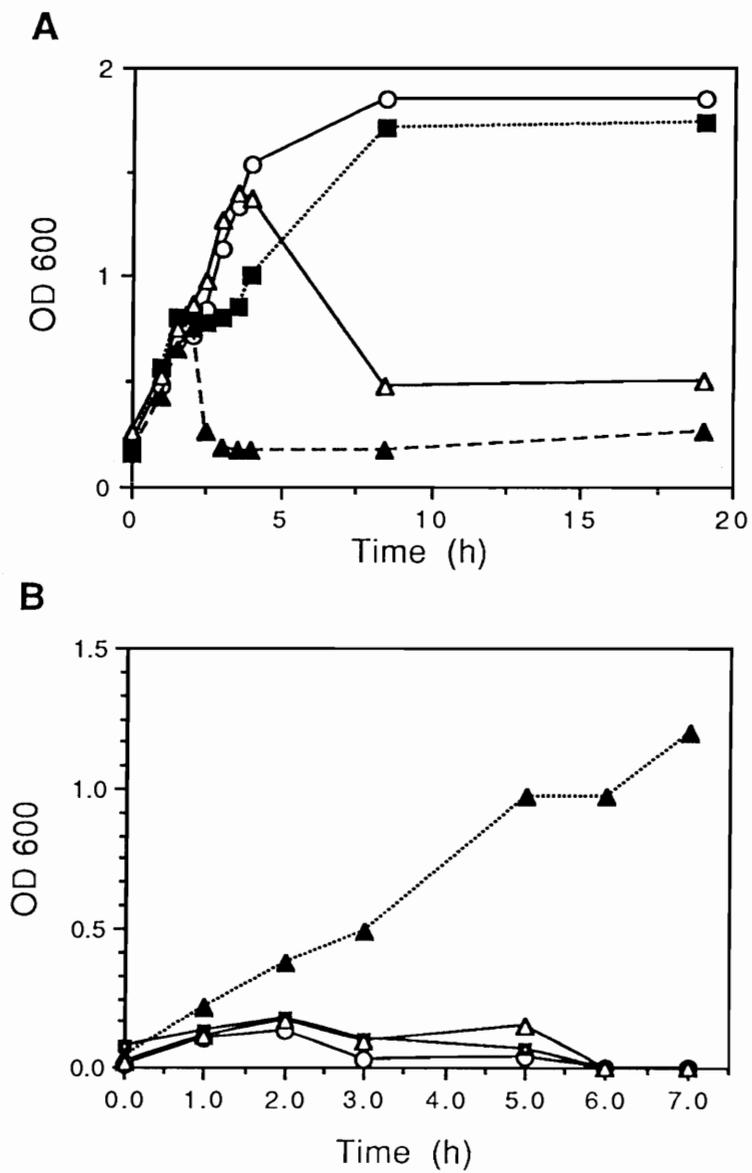
FIGURE 1.4

Fig. 1.5. The effects of L(+)rhamnose on the growth of phage-infected liquid cultures. Mid-exponential phase cultures of *L. lactis* ssp. *cremoris* KH (▲,■,●,O) were mixed with $CaCl_2$ (10 mM) and 0.6 M rhamnose (■), no rhamnose (▲), or 0.6 M galactose (●), and immediately inoculated with all five phages: kh, ml3, 643, c3, and 1 (2 X 10^4 PFU/ml, each). A control culture received no rhamnose, galactose nor phages (O). A culture of *L. lactis* ssp. *lactis* biovar. diacetylactis 18-16 was mixed with $CaCl_2$ (10 mM) and 0.6 M rhamnose, and immediately inoculated with 2 X 10^4 PFU/ml of phage 18-16 (□). Growth was measured by A_{600} during the following 15 hr.
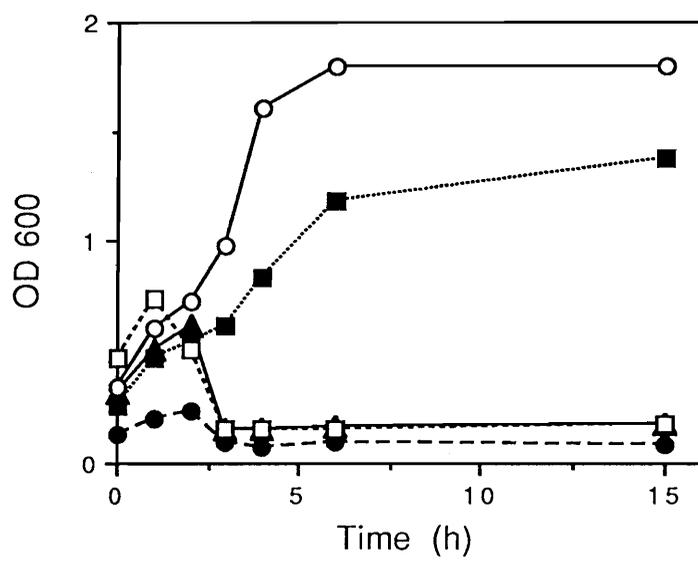
FIGURE 1.5

Fig. 1.6. (A). The irreversibility of phage binding to the cell wall. Cell walls (400 μg/ml) mixed with phage kh (1 X 10^8 PFU/ml) and 15 mM $CaCl_2$ were incubated for 15 min and centrifuged. The supernatants were removed and the pellets were resuspended in 1 ml of 50 mM Tris-HCl, pH 6.8. The amount of infectious phage was assayed at the indicated times after resuspension. (B) Effects of cations and trimagnesium diphosphate on phage binding to the cell wall. Cell walls (200 μg/ml) were incubated with phage kh (1 X 10^8 PFU/ml) in the absence or presence of 15 mM Ca^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, $Mg_3(PO_4)_2$, or Na+. The mixtures were centrifuged and assayed for the titer of phage in the supernatant.

A

B

FIGURE 1.6

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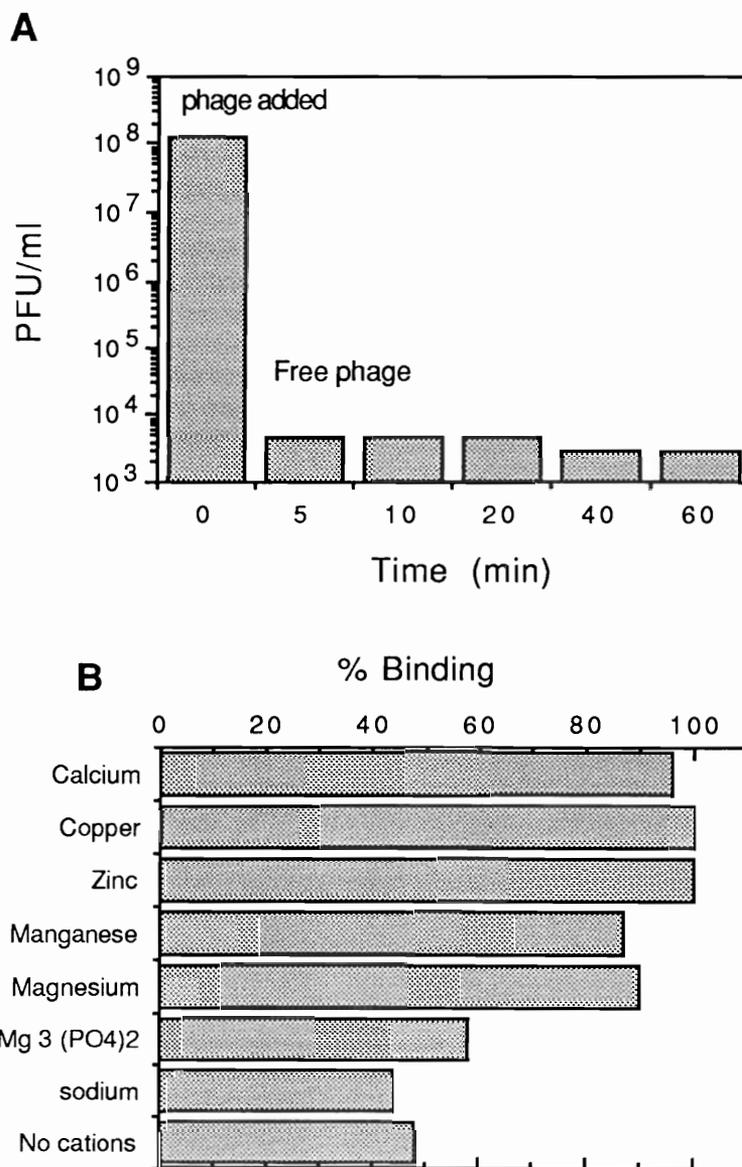
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Chapter 2

The Bacteriophage skl Receptor is the Rhamnose and Glucose of the

Exopolysaccharide of *Lactococcus lactis* subspecies *lactis* C2

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ABSTRACT

The bacteriophage skl bound to the exopolysaccharide of *Lactococcus lactis* subsp. *lactis* C2. Chemical analysis by gas chromatography of the cell walls from mutants which failed to bind the phage revealed changes in rhamnose, glucose and possibly N-acetylglucosamine. Lectins specific for N-acetylglucosamine and galactose did not prevent phage binding, suggesting that these saccharides do not bind directly to the phage. However, three lectins specific for glucose, but different in size, reduced phage binding to variable degrees, from 0-50%. Rhamnose, but not glucose, partially inhibited phage infection when added to a growing culture. We propose that phage skl directly binds to a rhamnose of the exopolysaccharide, and that glucose is near to, or is an important conformational determinant of, the binding site.

INTRODUCTION

Lactococci are used widely as starter cultures in the manufacturing of cheese (12). A significant problem occurs when these bacteria are attacked by bacteriophages which contaminate the milk or the cheese manufacturing equipment. Infection results in lysis of the cells, an impaired fermentation, and a greatly inferior product (11). As a strategy to prevent phage infection of cheese starter cultures, we have begun to characterize, at the molecular level, some of the cellular components required to initiate the phage infection cycle.

Bacteriophage infection is initiated by attachment of the phage to the cell surface at specific recognition sites called receptors. In lactococci, various receptors have been found on the cell wall polysaccharides (5,17; chapter 1). One notable exception located a phage receptor for *Lactococcus lactis* subsp. *lactis* ML3 in the membrane protein fraction of the cell (10,9). In addition to differences in the reported locations of the receptors, attachment of phage can be either reversible or irreversible, depending on the phage and host (3,2).

Previously, we identified the rhamnose of the exopolysaccharide of *L. lactis* subsp. *cremoris* KH as the binding site for phage kh (17; chapter 1). In this report, we present evidence that the phage skl receptor of another subspecies, *Lactococcus lactis* subsp. *lactis* C2, is the rhamnose and glucose of the exopolysaccharide. The binding of phage is apparently an irreversible step in the infection cycle.

MATERIALS AND METHODS

Bacterial strains, phage and medium. Phages sk1 and c2 and *L. lactis* subsp. *lactis* C2 were the generous gifts of Dr. Todd Klaenhammer (Department of Food Science, North Carolina State University, Raleigh, NC). *Lactococcus* bacteria were grown on M17 medium at 30° C and phages were propagated as described (16).

Phage sensitivity and plaque assay. Plaque assays were done by mixing an appropriate dilution of the phage-containing sample with 200 μl of an overnight (stationary) culture, 20 mM CaCl$_2$, and 3 ml of top agar, as described (17; chapter 1). The sensitivity of a strain to a phage is measured as the number of plaques formed divided by the number of plaques formed by the same amount of sample on the wild type, parental strain, times 100.

Preparation of cell wall and phage binding assay. Cell wall of *L. lactis* subsp. *lactis* C2 was prepared by glass bead disruption, differential centrifugation, and hot sodium dodecylsulfate extraction as previously described (17; chapter 1). The binding of phage to the cell wall was assayed as described (17; chapter 1) except the titer of phage sk1 added to the cell wall was 10^6 pfu/ml.

Gas/liquid chromatography and mass spectrometry. The acid hydrolysis and derivatization of cell wall, and identification and quantitation of

the alditol acetate derivatives were done as described (17; chapter 1).

Isolation of phage-resistant mutants. Cells of *L. lactis* subsp. *lactis* C2 ($2X10^8$) were superinfected with phage skl (10^6) in soft M17 agar (0.4%) with 10 mM $CaCl_2$. Superinfection is defined as phage and cell were mixed at the multiplicity of infection (MOI) greater than 1. The phage-resistant mutants were isolated, selected and screened for their abilities to bind to, and form plaques, with phage skl, as described (17; chapter 1).

For N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced mutants, *L. lactis* subsp. *lactis* C2 was grown at 30° C on M17 medium, harvested at the exponential phase ($A_{600} = 1$) and washed in equal volume of 0.1 M citrate buffer, pH 5.5. Cells were resuspended in the same volume of buffer and treated with MNNG (20 μg/ml, 4h at 30° C) as described by Miller (1972; 8). Cells were washed free of MNNG twice by using 0.1 M phosphate buffer, pH 7, transferred to M17 broth and grown overnight. The overnight cells were streaked on M17 plates containing phage skl (10^6 PFU). Single colonies of the selected mutant cells were screened for their abilities to bind to, and form plaques with, phage skl.

Lectin treatment of cell walls. Cell walls, at a concentration determined empirically to bind approximately 90% of phage skl, were mixed with 0.3, 0.1, 0.05, and 0.025 mg lectins (Sigma Chemical Co.) from *Concanavalin A* (Con A; Jack bean), *Vicia faba* (faba bean), *Vicia sativa*, *Momordica charantia* (bitter pear melon) or *Bandeiraea*

simplicifolia, respectively, in a final volume of 1.0 ml 50 mM Tris-HCl, pH 6.8. Either glucose or galactose was added at concentrations of 0, 0.5, 1.0, 5.0, and 10 mg/ml. The inhibition of phage adsorption by lectins was assayed as described (17; chapter 1).

Addition of saccharides to phage-infected cultures. Rhamnose (0.4M or 0.6M) and/or glucose (0.4M) and $CaCl_2$ (15mM) were added to mid-exponential phase cultures (A_{600} = 0.2), which were immediately inoculated with phage sk1 ($4X10^5$ or $4X10^4$ PFU/ml in experiments shown in fig. 2.2A and 2.2B, respectively). A control culture did not receive phage or calcium. Growth of bacteria was measured by absorbance at 600 nm.

RESULTS.

Isolation and characterization of phage-resistant mutants. Spontaneous phage sk1-resistant mutants were selected from superinfected cultures of *L. lactis* subsp. *lactis* C2 at a frequency of 1 X 10^{-6}. Of the approximately 50 mutants that were screened for phage binding to whole cells, a total of three had reductions of greater than 15% in binding phage sk1 (Table 2.1, RMSK1/1, /2, and /3), compared to the wild type strain.

Additional mutants with defects in phage binding were sought, by treating the same wild type strain with the mutagen, MNNG. Phage-resistant mutants were selected at a frequency of 2 X 10^{-5}, and screened for defects in phage binding. Ten of the 50 mutants screened had reductions in phage sk1 binding of greater than 15%. A preliminary carbohydrate analysis of the cell wall from four of the MNNG-induced binding mutants showed that all had similar cell wall compositions. One of these, referred to as RMSK1/4, was analyzed in more detail, and is described in the following sections.

A further analysis of binding, using isolated cell walls from the four mutants, revealed that the cell walls of each mutant were defective in binding to nearly the same extent as the whole cells from which they were derived (Table 2.1). The RMSK1/1 cells had an 82% reduction in phage sk1 binding, and were completely insensitive to the formation of plaques. The RMSK1/2 cells exhibited a reduction in phage sk1 binding of 52% and a sensitivity to infection of 37%. For RMSK1/3, there was

a loss in phage sk1 binding of 27%, and a sensitivity to phage infection of 75%. There was a total loss of both phage sk1 binding and sensitivity to infection in the MNNG-induced RMSK1/4 cells.

Carbohydrate analysis of cell walls. The cell walls of the wild type strain *L. lactis* subsp. *lactis* C2 and the phage-resistant mutants were analyzed for carbohydrates by gas chromatography. The cell walls from the wild type strain contained rhamnose, galactose, glucose and N-acetylglucosamine (Table 1.1). The identity of each of these carbohydrates was confirmed by mass spectrometry. Changes in the cell wall carbohydrate composition of each mutant were found (Table 1.1). The RMSK1/1 cell walls showed a 126% increase in rhamnose, a 13% decrease in galactose, a 45% decrease in glucose and a 47% increase in N-acetylglucosamine. In RMSK1/2, there was a 135% increase in rhamnose, a 2% increase in galactose, a 20% decrease in glucose as well as a 52% increase in N-acetylglucosamine. The RMSK1/3 cell wall showed a 70% increase in rhamnose, a 15% decrease in galactose, a 27% decrease in glucose and a 15% increase in N-acetylglucosamine. The MNNG-induced RMSK1/4 cell walls had no detectable rhamnose, a 13% decrease in galactose, a 78% decrease in glucose and a 63% decrease in N-acetylglucosamine.

Together with the phage binding analysis, these results show a correlation between the loss of phage binding and the loss of glucose. However, this does not eliminate rhamnose and N-acetylglucosamine as potential phage receptor determinants. There is no apparent correlation

between phage binding and the levels of galactose.

Inhibition of phage binding by lectins. Lectins are glycoproteins that can bind to a specific monosaccharide component of polysaccharides, such as those of the cell wall from Lactococci. This may cause steric hindrance and prevent phage binding, if the lectin and phage binding sites are identical or in close proximity. Preincubation of wild type cell walls with lectins from *M. charantia*, which specifically binds galactose, and *B. simplicifolia*, which specifically binds N-acetylglucosamine, did not inhibit the binding of phage skl (Fig. 2.1A). This suggests that both the galactose and N-acetylglucosamine are not the molecular determinants of phage skl binding.

However, a 50% reduction in phage skl binding occurred when the cell walls were preincubated with the glucose-binding lectin from *Concanavalin A* (Fig. 2.1A). When glucose was added to the pre-incubation mixture, the effect of the *Concanavalin A* lectin was prevented (Fig. 2.1B). Adding galactose to the pre-incubation mixture did not prevent the effects of the lectin on phage binding. This suggests that both the lectin from *Concanavalin A* and phage skl recognize the same glucose component of the cell wall. However, it also suggests that the soluble glucose, which prevented the effects of the lectin, does not inhibit the phage binding. In fact, a control pre-incubation of phage and glucose, but without the lectin and cell wall, did not reduce the phage binding to cell walls added subsequently.

To test further the role of glucose, cell walls were preincubated

with a lectin from *V. sativa*, that also specifically recognizes glucose. Surprisingly, this lectin did not inhibit phage skl binding (Fig. 2.1A). Furthermore, a third glucose-specific lectin from *V. faba* reduced phage binding by only 20% (data not shown).

The different effects of the three glucose-recognizing lectins apparently are not the result of differences in the lectin binding affinities, because equal amounts of lectins added on the basis of affinities for glucose gave results essentially the same as those shown in Fig. 2.1A. Instead, the ability of these three lectins to prevent phage binding correlates with the molecular weights of the lectins. The molecular weights of the lectins from *Concanavalin A, V. faba, and V. sativa* are 102,000, 50,000 and 40,000, respectively (4,7,1). This suggests that phage skl does not bind directly to glucose, but rather that glucose is located in the vicinity of the phage receptor.

Delay of infection by rhamnose. To investigate the role of rhamnose as the phage receptor, phage skl was first mixed with 0.4 M rhamnose for 30 min before adding cells. Rhamnose apparently did not irreversibly bind to the phage, nor compete for the receptor on the cell surface, because there was no detectable difference in the number of plaques formed with or without rhamnose in the preincubation with phage.

In a slightly different approach to phage inactivation, monosaccharides were added to a growing culture just prior to inoculation with phage skl (Fig. 2.2). In the absence of added monosaccharides, phage skl stopped cell growth within 2 hr, and caused

complete cell lysis within about 3 hr (Fig. 2.2A). Adding rhamnose delayed the cessation of growth to about 2 hr. Rhamnose had no effect on the growth of an uninfected culture (Fig. 2.2B). When glucose was added to a culture just prior to inoculation with phage sk1, growth stopped after 1-2 hr, and total cell lysis occurred within 3 hr (Fig. 2.2A) Addition of both rhamnose and glucose had the same effect on infection as did rhamnose alone (Fig 2.2A). When the amount of phage in the inoculum was reduced by a factor of 10, rhamnose delayed the length of time required for lysis from about 3 to 6 hr (Fig. 2.2B). These results demonstrate that rhamnose, but not glucose, delays the ability of phage sk1 to lyse the cells, and suggests that the phage may bind to a rhamnose residue of the cell wall.

Quantitation of the phage titer in the cultures after 20 hr indicated that rhamnose reduced the final number of phage by 3-17 fold, depending on the titer of the initial phage inoculum. Thus, in the culture without added carbohydrates, or with rhamnose, glucose, or rhamnose and glucose (Fig. 2.2A), the final titers were 6×10^9, 2×10^9, 6×10^9, and 2×10^9 PFU/ml, respectively. In the experiment shown in Fig. 2.2B, where less phage was used in the inoculum, the final titers were 1×10^{10} and 6×10^8 PFU/ml for the culture without and with rhamnose, respectively. This indicates that rhamnose lowered the amount of phage produced in liquid cultures, and supports the contention that the phage bind to a rhamnose-containing component on the cell wall.

Does an increase in the rhamnose or N-acetylglucosamine mask the receptor? Mutations that increase the carbohydrate components of the cell surface can occlude or mask a phage receptor (6). In the case of the closely related subspecies *cremoris*, the masking carbohydrates from a phage-resistant mutant could be chemically stripped from the cells. This exposed the receptor and restored phage binding (13,14).

In an attempt to remove any carbohydrates that might be masking phage sk1 binding to the cell walls of the subspecies *lactis* C2, cell walls from the resistant mutants were treated with mild alkali (100mM NaOH, 30 min and 25°C). The amount of phage sk1 bound was the same before as after treatment. Additionally, the carbohydrate analysis by gas/liquid chromatography revealed no changes in the cell wall components after alkali treatment. This suggests that the cause of phage-resistance is not due to a masking of the cell surface receptor.

DISCUSSION

About 50 spontaneous phage-resistant mutants from a phage skl-superinfected culture of *L. lactis* subsp. *lactis* C2 were screened for reduction in binding to the phage. Three were found with significant defects in phage skl binding, and analyzed further. One additional phage-resistant, binding-defective mutant from a MNNG-treated culture was also analyzed.

In general, a decrease in phage binding correlates with a quantitatively similar loss in sensitivity to phage infection. Although RMSK1/3 was totally insensitive to phage skl, a small amount (16%) of phage binding to the cell wall was detected. This could be due to changes in the cell wall that do not completely perturb some of the phage skl binding, but in an unknown way prevent subsequent steps required for infection (such as eclipse, for instance). A similar observation was reported with phage-resistant mutants from the subsp. *cremoris* (17; chapter 1). Future experiments to determine if phage eclipse occurs include analysis of phage washed from the surface of the mutants and wild type cells and determine if the phage DNA had been ejected from the phage head. Eclipsed phage can be separated from intact phage on sucrose gradients which separate phage particles according to their sizes. The partial release of phage DNA can also be assayed by susceptibility of radiolabelled phage DNA to deoxyribonuclease.

The cell wall of *L. lactis* subsp. *lactis* C2 was analyzed by

gas/liquid chromatography and mass spectrometry and found to contain rhamnose, galactose, glucose, and N-acetylglucosamine in a molar ratio of approximately 7:1:2:2, respectively. Although glycerol, a component of teichoic acids, has been reported on the cell walls of several species of lactococci (18,19,15), it was not detected on the cell walls of *L. lactis* subsp. *lactis* C2 by using gas chromatography or mass spectrometry.

Carbohydrate analyses of the cell walls from RMSK1/1, RMSK1/2 and RMSK1/3 showed that there is no correlation between the galactose content and phage binding, suggesting that galactose plays no specific role in phage skl adsorption. The analyses also revealed that a loss in glucose and increases in rhamnose and N-acetylglucosamine correlate with a loss in phage skl binding. However, the lack of an exact, quantitative relationship between the loss or gain of any one of these three monosaccharides and phage binding suggests a number of possibilities: 1) The receptor may be composed of a combination of the monosaccharides, arranged in a specific geometry. 2) The loss of phage binding may not be as simple as the loss of the receptor determinants. For instance, the increase in rhamnose or N-acetylglucosamine might cover or mask the receptor, and physically block the association between cell and phage. Such a mechanism has been proposed previously for phage resistance in *Salmonella typhimurium* (6), and a *cremoris* subspecies of *Lactococcus lactis* (13). In the latter case, phage resistance is associated with a specific plasmid. Our unsuccessful attempts to unmask the receptor by alkali treatment of the cell walls may indicate that

this mechanism of resistance is not applicable in the case of phage skl infection of subspecies *lactis* C2.   3) Both of the first two possibilities may occur simultaneously.

Although receptor masking may be a possible mechanism of phage resistance in the three spontaneous mutants analyzed, a loss of receptor is apparent in the MNNG-induced phage-resistant mutant RMSKl/4. Unfortunately, all four of the MNNG-induced, phage-resistant mutants that were analyzed had nearly identical cell wall carbohydrate compositions to each other. Thus, the loss of phage binding can not be assigned to the loss of any specific cell wall component on the basis of the carbohydrate analysis alone. However, it does suggest that the loss of cell wall carbohydrates correlates with the loss of phage binding. Because glucose, galactose and N-acetylglucosamine are still present, albeit at lesser levels, while rhamnose is undetectable, this suggests that rhamnose is the phage receptor.

Lectins were employed as competitive inhibitors of phage binding. The inability of lectins specific for galactose and N-acetylglucosamine to inhibit binding suggests that neither galactose nor N-acetylglucosamine is directly involved in phage binding. On the other hand, glucose appears to play a role in phage skl binding, because a lectin specific for glucose inhibited phage binding. However, other lectins specific for glucose, but smaller in size, did not compete with the phage as efficiently. This differential effect of the glucose-specific lectins is consistent with the idea that glucose is in close proximity to, and may influence the conformation of, the phage skl

receptor, but does not bind directly to the phage. A similar result was previously reported for the effects of galactose-specific lectins on the binding of bacteriophage kh to *L. lactis* subsp. *cremoris* KH, where the receptor was proposed to be near, but not identical to, a galactose component of the cell wall (17; chapter 1).

Mixing soluble monosaccharides and phage, or adding monosaccharides to culture media, can often prevent phage infection. This occurs if the monosaccharide mimics the determinants of the receptor, and successfully competes with the receptor by binding to the phage. The results of experiments in this report indicate that glucose was ineffective in preventing or delaying phage skl infection. Preincubating rhamnose and phage did not reduce the number of plaques formed upon subsequent incubation with sensitive cells. However, when rhamnose was added to a liquid culture at the time of phage inoculation, infection was delayed, but not prevented. When the ratio of rhamnose to phage in the inoculum was increased by a factor of 10, the onset of infection was further delayed. The final number of phage in the culture containing rhamnose was reduced 3-17-fold compared to phage-infected cultures without rhamnose, demonstrating that the delay caused by adding rhamnose reduced the final number of phage produced. Together, these results may be explained by a reversible, competitive binding between rhamnose and phage skl. This might effectively reduce the number of unbound phage at any given moment, practically slowing the rate of attachment, without reducing the initial number of potentially infective particles. We propose that phage skl binds directly to a rhamnose contained on the

exopolysaccharide. However, the evidence is only suggestive, and we cannot rule out the possibility that other components of the exopolysaccharide bind directly to the phage.

The bacteriophage skl binding to the sodium dodecylsulfate-extracted, purified cell wall was irreversible. This is evident from the fact that when phage and cell walls are incubated, the same number of plaques are formed from the mixture, as from the supernatant following centrifugation to remove the cell wall-bound phage. This suggests that either the apparent thermodynamic binding constant of phage to receptor is extremely high, or an irreversible step subsequent to the binding has taken place. Examples of the latter possibility have been documented for the initial attachment and release of phage DNA from the capsid of coliphage ϕX174 (3). This characteristic is also shared by phage kh of *L. lactis* subsp. *cremoris* KH (17; chapter 1). However, this apparently is <u>not</u> a general feature of interactions between lactococcal cell walls and phages. Recently, we have begun to characterize the interaction of another phage, c2, whose host range includes strain C2. The binding of phage c2 to the sodium dodecylsulfate-extracted, purified cell wall appears to be reversible. Although the differences in the reversibility of binding may merely reflect differences in binding affinities of the phages, it may instead be an indication of major mechanistic differences of the infection cycles of the various phages.

Rhamnose may be a common determinant of many lactococcal phage receptors. In addition to this report, we are aware of only one other

molecular description of a lactococcal, cell wall phage receptor: that of *L. lactis* subsp. *cremoris* KH (17; chapter 1). There also, rhamnose appears to be the primary site of phage attachment. The secondary determinants are apparently different, however, being glucose and galactose in the cases of phages skl and kh, respectively. Additional studies of other phages will be necessary to determine if rhamnose is required for all lactococcal phage binding.

Our results suggest a strategy for constructing a strain which may be resistant to a broad spectrum of phages. Rhamnose on the extracellular polysaccharide can be totally eliminated without any apparent loss of viability. Using molecular genetic techniques, it should be possible to delete or inactivate genes coding for the synthesis of rhamnose or the assembly of rhamnose on the exopolysaccharide. Presumedly these genes are located on the chromosomes, and deletions or insertional inactivations may be more stable than plasmid-encoded phage defense mechanisms. Given the adaptability of phages to accommodate changes in the host, we think it likely that multiple mechanisms of phage-resistance will be required in strains constructed for phage resistance. Chromosomal-based defense mechanisms offer another tool which might be combined with plasmid-encoded mechanisms to best combat the problem.

In summary, the evidence suggests that phage skl binds to a specific part of the cell wall of *Lactococcus lactis* subsp. *lactis* C2 that contains rhamnose. Glucose residues are apparently in close proximity to the binding site, but do not bind directly to the phage.

ACKNOWLEDGMENTS

Table 2.1. Cell Wall binding, sensitivity to infection and saccharide analysis

Strain	% binding of phage skl to cell walls	% sensitivity to phage skl	carbohydrate[a] (μg/mg cell wall±SD)			
			Rha	Gal	Glu	GlcNAc
c2[b]	98	100	234±20	37.1±2.2	66.0±5.3	73.5±6.2
RMSK1/1	16	0	529±18	32.4±0.8	36.5±0.3	108 ±2.7
RMSK1/2	46	37	550±4.6	38.0±0.9	53.0±0.6	112 ±7.4
RMSK1/3	71	75	398±20	31.6±0.5	47.9±1.6	84.8±1.5
RMSK1/4	0	0	ND[c]	32.2±3.3	14.2±2.0	36.9±7.0

[a] Rha, rhamnose; Gal, galactose; Glu, glucose; GlcN-Ac, N-acetylglucosamine
[b] C2, Wild type strain C2
[c] ND, Not detectable

Fig. 2.1. (A) The effects of lectins from *Concanavalin A* (□), *V. sativa* (■), *M. charantia* (•) and *B. simplicifolia* (O) on phage skl binding to the cell walls of *L. lactis* subsp. *lactis* C2. Cell walls (100 μg) pre-incubated with different concentrations of lectins were mixed with phage skl (10^6 PFU/ml). The phage-bound cell walls were removed by centrifugation and the titer for free phage was assayed. (B) Glucose reverses lectin inhibition of phage binding. Cell walls (200 μg) pre-incubated with lectin from *Concanavalin A* (30 μg) were mixed with different concentrations of glucose (□) or galactose (■) prior to the addition of phage (10^6 PFU). The percentages of phage binding were assayed as described in panel A.
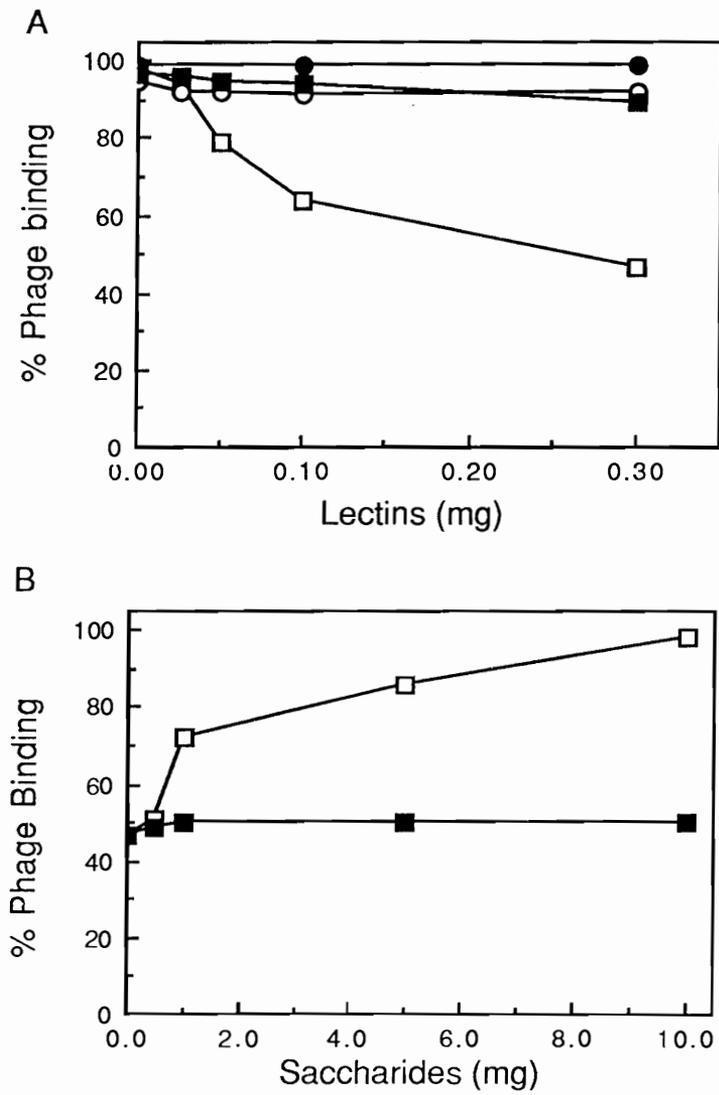
FIGURE 2.1

Fig. 2.2. (A) The effects of L-(+)rhamnose and D-(+)glucose on the growth of phage-infected liquid cultures of *L. lactis* subsp. *lactis* C2. Mid-exponential phase cultures containing no rhamnose or glucose (■), 0.4 M rhamnose (▲), 0.4 M rhamnose and 0.4 M glucose (O), or 0.4 M glucose (Δ) were infected with phage skl (at time zero) at a multiplicity of infection = 0.1. The control (●) was not infected. Growth was measured by A_{600}. (B) Growth curve of phage-infected cultures of *L. lactis* subsp. *lactis* C2. The procedures was the same as described in (A), except the control contained 0.4 M rhamnose, and phage skl was added at a multiplicity of 0.01
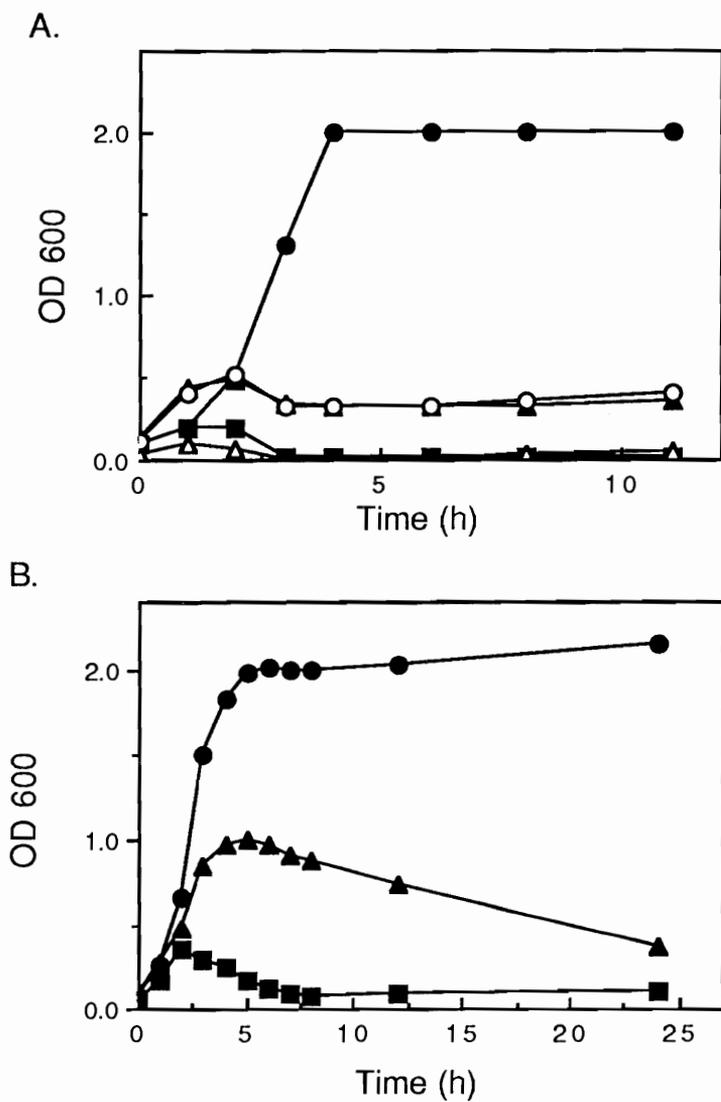
FIGURE 2.2

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Chapter 3

A Membrane Protein is required for Bacteriophage c2 infection of

Lactococcus lactis subspecies *lactis* C2

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ABSTRACT

Phage-resistant mutants, isolated from superinfections (phage and cells mixed at MOI>1) of *L. lactis* subsp. *lactis* C2 with phage c2, did not form plaques upon infection with phage c2, but bound phage normally. The mutants were sensitive to another phage sk1, although the number of plaques was reduced ~56% and the plaque size was four times smaller. The binding to phage sk1 was reduced about 10%. Another group of phage-resistant mutants, isolated from superinfections with phage sk1, bound normally to both phage c2 and sk1, but did not form plaques with either phage. Carbohydrate analyses by gas chromatography of the cell walls showed no significant differences in saccharide compositions between the wild type and phage-resistant cells. However, a difference was observed in the interactions of the phage with the cytoplasmic membranes. Membranes from the wild-type, but not mutant cells, inactivated phage c2. Phage sk1 was not inactivated by membrane from either strain. Treatment of wild type membranes with proteinase K eliminated the ability of the membrane to inactivate the phage, whereas treatment with mutanolysin had no effect. On the basis of this ability to inactivate the phage, a membrane protein was partially purified by gel filtration and ion-exchange chromatography. Under non-denaturing conditions, the phage-inactivating protein appears to have an apparent $M_r \approx 350,000$. We propose that the protein has an apparent subunit size of 32 kDa. This suggests that it normally exists as a multimer with 10-12 subunits, or in association with other membrane components. It is proposed that this

protein is required for phage c2 infection.

INTRODUCTION

The initial stage of phage infection involves the adsorption of phage to a specific component on the surface of the host cell. Phages of *Escherichia coli*, such as those with the T-even structure, often require both the lipopolysaccharide and a specific membrane protein for adsorption (9, 13, 22). Following adsorption, the phage genome is transferred across the cell membranes. In the case of coliphage T4, the penetration of phage DNA requires an interaction between the core tip of the phage and the membrane phospholipids (6). Other coliphages require membrane proteins for phage DNA injection (5, 7).

In *lactococci*, carbohydrates of the exopolysaccharide may be commonly used as phage receptors (10). Previous studies from our laboratory have shown that the binding determinants of phages for *L. lactis* subsp. *cremoris* KH and subsp. *lactis* C2 include the rhamnose of the extracellular wall polysaccharides (20; Chapter 1, 21; Chapter 2). An exception to this was reported by Oram and Reiter (15), who found that membranes from *L. lactis* subsp. *lactis* ML3 inactivated phage ml3. The phage-inactivating material appeared to be a protein, because trypsin digestion of the plasma membrane destroyed the phage-inactivating activity. In our previous studies (20; Chapter 1), treatment of cell envelope from *L. lactis* subsp. *lactis* C2 with either mutanolysin or sodium dodecylsulfate (SDS) alone did not completely eliminate the adsorption of some phages (for example, c2). This suggested that the adsorption of certain phages may require both a membrane component and a saccharide unit on the cell wall.

In this paper, we report a difference in the cytoplasmic membranes of phage c2-sensitive and -resistant strains of *L. lactis* subsp. *lactis* C2. This difference correlates with, and was used to purify and identify a protein with an apparent $M_r \approx 350,000$, and a subunit size of 32 kDa. We propose that this protein is required for phage c2 infection, perhaps at a step subsequent to adsorption.

MATERIALS AND METHODS.

Bacterial strains, phages and medium. Bacteriophages c2 and skl and the bacterial host, *Lactococcus lactis* subsp. *lactis* C2, were grown and maintained on M17 medium at 30°C (19). Phage and bacterial stocks were prepared as previously described (19) and stored in 20% glycerol at -70° C.

Isolation and solubilization of phage-inactivating protein. Cells of *L. lactis* subsp. *lactis* C2 from a 9-liter culture were harvested at the mid-exponential growth phase at $A_{600} = 1$. Cytoplasmic membranes were prepared by differential centrifugation of lysed, lysozyme-treated protoplasts, essentially as previously described (16). Membranes prepared for purification were suspended in 10 mM bis-tris (pH 6.5).

Membranes (21 mg protein) were solubilized on ice in a buffer consisting of 10 mM bis-tris (pH 6.5), 1% (w/v) Triton X-100 (Sigma Chemical Co.), 10% (v/v) glycerol and 1 mM dithiothreitol (DTT) in a final volume of 1 ml. After 60 min, unextracted material was pelleted by centrifugation at 4° C (120,000 x g x 60 min; Beckman 30° A-100 rotor, Beckman Airfuge Ultracentrifuge). The supernatant was collected and stored at -70° C for later use. The extract usually contained about 40% of input protein and over 70% of phage-inactivating activity.

Preparation of cell walls for gas chromatography. Cells were harvested at $A_{600} = 2$, and suspended in 10 mM KH_2PO_4 (pH 6.8) to a final concentration

of 150 mg/ml. Cell walls were isolated as previously described (20; Chapter 1). Freeze-dried cell walls (10 mg/ml) were mixed with ribose (20 μg/ml) as an internal standard in 2 N HCl, flushed with nitrogen gas, and hydrolyzed at 100° C for 3 h. The hydrolysates were neutralized with 15 N NH$_4$OH with phenolphthalein as an indicator. Hydrolyzed cell walls (1 mg/ml) were mixed with xylose (50 μg/ml) and derivatized to alditol acetates as described previously (8).

Gas chromatography and identification and quantitation of alditol acetates. The alditol acetate derivatives were separated on a 3% SP-2340 glass column (30.48 X 1.2 cm; Supelco Inc., Bellefort, Pa.) connected to a model 5710A gas chromatograph (Hewlett-Packard Co., Palo Alto, Calif.) equipped with a flame ionization detector. Chromatographic conditions were adjusted according to the specifications of the manufacturer (GLC/HPLC bulletin 774B, Supelco). Peak areas and retention times were determined using a Hewlett-Packard model HW 3390A electronic integrator. The derivatized cell wall polymer saccharides separated by gas chromatography were quantified as previously described (20; Chapter 1).

Isolation of Resistant Mutants. Cells of *L. lactis* subsp. *lactis* C2 (10^8 cfu) were superinfected with 10^{10} PFU of phage c2 or 10^8 PFU of phage skl in M17 top agar (0.4%) and 15mM CaCl$_2$. Superinfection is defined as phage and cell were mixed at the multiplicity of infection greater than 1. The phage-resistant mutant cells were collected by washing cells off the top

agar with M17 broth. Single colonies were isolated on M17 agar containing phage c2 (10^8 PFU/ml) or phage sk1 (10^7 PFU/ml) and tested for their abilities to bind phage and form plaques. The cell walls of selected mutants were prepared and tested for losses in phage binding as described (20; Chapter 1). The ability of cell walls to bind phage is expressed as the percentage of phage inactivated by the same weight of cell walls from the wild type strain C2.

Phage inactivation assay and units of phage inactivation. Membranes were mixed and shaken with phage c2 (5 x 10^5 PFU/ml) in 10 mM bis-tris (pH 6.5) at 25° C for 20 min. The mixtures were centrifuged (12,000 x g, 4 min), and titers of phage in the supernatant were assayed as described (19). The amount of phage inactivated was calculated by dividing the difference of the total phage added minus the phage in the supernatant by the total phage added, and multiplying by 100.

Our phage-inactivation assay mixture contained phage c2 (5 x 10^5 PFU/ml) in 10 mM bis-tris (pH 6.8) and an appropriate amount of sample to decrease the titer of the phage by 27-77% when shaken at 25°C for 20 min. The amount of phage inactivated under these conditions was a linear function of the amount of sample added. One unit of phage-inactivating activity was defined as the amount of sample required to inactivate 52% of the phage added, which is the mid-point of the linear portion of the phage-inactivation curve.

Treatments with mutanolysin and proteinase K. Membranes in 50mM K_2PO_4 (pH

7.0) and 10 mM Mg_2SO_4 at concentrations which inactivated 90% of phage (5 x 10^5 PFU/ml), were treated with mutanolysin (20 U/ml) at 25° C for 16 h or proteinase K (0.1 mg/ml) at 4° C for 16h. The proteinase K-treated samples were further incubated with phenylmethylsulfonyl fluoride (0.2 mM) at 4° C for 30 min. The cell envelopes were treated with mutanolysin as described previously (20; Chapter 1).

Gel filtration chromatography. The solubilized membranes were applied to a Sephacryl S300 (Pharmacia LKB Biotechnology) column (1.6 X 60 cm) in 10 mM bis-tris (pH 6.5), 0.1% Triton X-100, 10% glycerol and 1 mM DTT. The same buffer was used for elution at a flow rate of 20 ml/h. Fractions of 1.5 ml were collected and assayed for phage-inactivating activity and total protein (4; ovalbumin standard). Fractions with the highest phage-inactivating activity were combined.

Ion Exchange Chromatography. A diethylaminoethyl cellulose (DE52, Whatman Biosystems Ltd, Kent, England) column (0.8 X 6 cm) was equilibrated with 10 mM bis-tris (pH 6.5), 0.1% Triton X-100, 10% glycerol and 1 mM DTT. The combined fractions from the gel filtration column which contained phage-inactivating activity were applied to the column. The column was developed with a three step gradient at 10 ml/h. Each step was 1 bed volume of equilibration buffer, plus 0.15, 0.25, and 0.50 M NaCl. The phage-inactivating activity eluted in the 0.25 M NaCl step. Fractions of 1 ml were collected and aliquots were assayed for phage-inactivating activity and total protein.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. SDS polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (11). The gels were silver-stained as described (2).

RESULTS

Isolation and properties of phage-resistant mutants. Spontaneously occurring phage-resistant mutants of *Lactococcus lactis* subsp. *lactis* C2 were selected by superinfection with phage c2. Fifty randomly picked mutants were tested for their sensitivities to phage by plaque assay, and all were found to be totally insensitive to phage c2 (no plaques were formed). Four of these mutants were studied further, and the results are listed in Table 3.1 (RMC2/4, /8, /9 and /13). These phage c2-resistant mutants were challenged with another phage, skl, which also infects this strain. There was a 54-58% reduction in the sensitivities to phage skl (Table 3.1) and a four-fold reduction in the plaque sizes (not shown).

None of the 50 randomly picked mutants had detectable defects in binding of whole cells to phage c2. To corroborate this, cell walls were isolated from 4 of the 50, and tested for phage binding. All were found to bind phage c2 normally, although binding to phage skl was reduced by approximately 10% (Table 3.1). This suggests that the loss of sensitivity to these phages was not the result of a loss of the cell surface phage receptors.

To analyze further the cellular requirements for phage infection, spontaneously occurring phage-resistant mutants of the same host were selected by superinfection with phage skl. Of the 30 mutant cells that were randomly picked, all but three bound phage c2 and skl normally. Further analysis of the three binding-defective mutants will be presented elsewhere (21; Chapter 2). All of the mutants which bound phage normally

were tested for sensitivity to phage skl and c2 by plaque assay, and found to be totally insensitive to both phages. Two representative analyses are listed in Table 3.1 (RMSK1/1 and /2). Cell walls were prepared from 3 of the 27 mutants which appeared to bind the phages normally, and tested for binding to phage skl and c2. In all three mutants tested, there was no reduction in binding to either phage. These results demonstrate that phage-binding mutants of this strain are much less frequent than those in a *cremoris* subspecies that we have characterized previously (20; Chapter 1), relative to the total number of phage-resistant isolates. It also shows that the loss of the phage receptor is not a frequent mechanism of phage-resistance for this host and these two phages.

Chemical analyses of the cell walls from the mutants by gas chromatography showed that there were no significant changes in the cell wall components (Table 3.2). This confirmed that the insensitivity to phage skl and c2 infections is not related to any changes in the cell wall carbohydrate components, which contain the receptor for phage skl and c2 in this strain (20; Chapter 1, 21; Chapter 2). The mechanism of phage resistance in these cases must have to do with a step subsequent to the phage binding.

Membrane inactivation of phage. Plasma membranes were prepared from parental and mutant cells. When phage c2 was mixed with membranes from the phage-sensitive, parental strain, the phage was inactivated, and no longer had the ability to infect sensitive cells (Table 3.1). The

inactivation was specific for phage c2, as other phages, sk1, kh and 18-16, were not inactivated by the parental membranes. Moreover, membranes from the phage-resistant mutants (selected from superinfections with phage c2) did not inactivate phage c2 (Table 3.1), sk1, kh or 18-16 (data not shown). These results suggested that a membrane component of the parental, but not the mutant strains, irreversibly interacts with phage c2, resulting in phage inactivation.

To test if the phage-inactivating membrane component was proteinaceous, parental membranes were treated with proteinase K. This destroyed the phage-inactivating activity of the membranes (Table 3.3). Incubation of the same membranes with mutanolysin, which hydrolyses cell wall glycosidic bonds, did not inactivate the membrane. As a control to show that the mutanolysin treatment would have effectively hydrolyzed any contaminating cell wall components in the membrane preparation, the cell envelope (wall + membrane) from the parental strain was similarly treated with mutanolysin. This essentially destroyed the polysaccharide, which is the phage receptor (20; Chapter 1), as indicated by a 76% reduction in phage binding to the mutanolysin-treated cell envelopes, as compared to the untreated cell envelopes. These results suggest that a membrane protein inactivates the phage.

Identification and purification of phage-inactivating membrane protein. Fractionation of the phage-inactivating protein is summarized is Table 3.4. The amount of membrane material necessary to inactivate 50% of the 5×10^5 phage/ml in 20 min was defined as one unit of activity. Membranes

prepared from an exponential phase culture of *L. lactis* subsp. *lactis* C2 had a specific activity of 205 units/mg protein. To purify the phage-inactivating protein, membranes were solubilized in 1% Triton X-100, 10% glycerol, 10 mM bis-tris (pH 6.5), and 1 mM DTT. The insoluble proteins were removed by centrifugation (100,000 x g, 1 h). About 70% of phage-inactivating activity was retained in the supernatant. Because 64% of the total protein was not solubilized under these conditions, the specific activity after solubilization increased about two fold, to 384 units/mg protein.

The solubilized material was passed through a Sephacryl S300 gel filtration column. Fractions were collected and assayed for phage-inactivating activity and total protein (Fig. 3.1). One broad peak of activity eluted from the column, centered at a position corresponding to a $M_r = 350,000$. The fractions with peak activity were pooled for further purification. The pooled fractions contained about 36% of the total activity applied to the column. The specific activity of the pooled fractions was 701 units/mg, an increase of about 2-fold from the applied material.

The pooled fractions from the gel filtration column were applied to a DEAE-cellulose column. After washing with 2 volumes of equilibration buffer, phage-inactivating activity was eluted with a three step gradient of 0.15, 0.25, and 0.50 M NaCl in elution buffer. Most (>90%) of the phage-inactivating activity eluted in a single peak in the 0.25 M salt step (Fig. 3.2). Usually this was the only detectable activity, although occasionally a small amount of activity eluted in the

0.15 M step. Unfortunately, about 97% of the activity was lost during this step, reducing the specific activity to 52 units/mg, or about 25% less than the crude membranes.

A number of attempts were made to reconstitute the activity to the pooled fractions from the DEAE column, or discover the reason for the loss of activity: 1. Aliquots of equal volume from all fractions were mixed and assayed for activity. Another mixture contained just the peak protein-containing fractions from each step, including the initial wash. The total activity of the mixtures did not change. 2. *E. coli* phospholipids were added to the peak fractions, but had no effect on the activity. 3. Solubilized membranes from the phage-resistant strain were mixed with the purified material, but this did not increase the activity. 4. A sample of Triton-solubilized phage-inactivating material was adsorbed to a small amount of equilibrated DEAE-cellulose in a tube. The unadsorbed material had less than 2% of the activity. When 0.25 M NaCl was added, 76% of the activity was recovered in the unadsorbed supernatant. Increasing the NaCl concentration from 0.25 to 0.5 M did not increase the activity in the unadsorbed fraction. 5. The crude membranes, Triton-extracted material, and pooled fractions from the gel filtration column were stored at 4°C for 1 week without loss of activity.

Electrophoretic analysis. Fractions from the gel filtration and DEAE columns were analyzed by SDS-polyacrylamide gel electrophoresis and silver staining (Figs. 3.1B and 3.2B, respectively). In each gel, only one band (arrowhead) correlated with the activity peak. This protein had

an apparent size of about 32 kDa. It was the only major silver-stained band present after the DEAE chromatography, although several minor bands could be seen. Considering the size of the active material which eluted from the gel filtration column under non-denaturing conditions, this suggests that the 32 kDa phage-inactivating protein exists normally in a multimeric complex.

DISCUSSION

All of the phage-resistant mutants isolated from a superinfection of *L. lactis* subsp. *lactis* C2 with phage c2 adsorbed phage normally, even though all were incapable of forming plaques (Table 3.1). The mutation in phage c2-resistant cells also affected the sensitivity to another phage, sk1. Although the phage c2-resistant cells formed plaques when challenged with phage sk1, the plaques were ~56% fewer and four-fold smaller. The phage c2-resistant cells were reduced ~10% in binding phage sk1. A similar result was also observed with the phage-resistant mutants isolated from superinfection of cells with phage sk1. All the mutant cells analyzed with the exception of three, were found to bind phages c2 and sk1 normally, but none formed plaques when challenged with either phage (Table 3.1). The three mutants with loss of adsorption to phage have changes in their exopolysaccharide composition (21; Chapter 2).

The chemical analyses of the cell walls from six of the phage-resistant mutants by gas chromatography showed that there were no significant changes in the cell wall components as compared to the wild type (Table 3.2). This suggests that phage resistance was not due to mutations of the phage receptor on the cell wall, but rather that phage replication is blocked at a step subsequent to adsorption.

Although there are many possible mechanisms of phage resistance, one is a change in the membrane proteins required for the steps of phage infection following adsorption, and leading to phage DNA entry into the

host cell. Our results demonstrate that membranes from phage-sensitive cells inactivate phage c2. In addition, proteinase K treatment of the membranes completely eliminated the ability of the membranes to inactivate the phage. Although these data alone might suggest a non-specific effect of the membranes to inactivate the phage, the fact that membranes from phage-resistant strains did not inactivate the phage makes this very unlikely. At present, there is no evidence that a carbohydrate moiety is involved in the inactivation of phage, because mutanolysin did not reduce the phage-inactivating activity of the membranes. However, we can not rule out the possibility that small, hydrolytic fragments of the exopolysaccharide, or glycoproteins are involved in the phage-inactivating activity. We can speculate that the adsorption of phage c2 to the cell surface involves at least two steps: the reversible binding to the cell wall extracellular polysaccharide and an irreversible step as the phage interacts with the proteinaceous membrane component. It is not known if the interaction of phage with the membrane protein results in the release of phage DNA.

Although there is a lack of evidence, it is possible that the phage-inactivating protein functions as a channel for phage DNA to enter the cell. Its large size is not inconsistent with this idea. Recently, a model was proposed for the entry of phage DNA through the cytoplasmic membrane of *E. coli*, based on a proteinaceous transmembrane channel (12). Supportive evidence includes a correlation between the time of entry of phage DNA and a release of cytoplasmic potassium (3).

Incubation of the membranes from *L. lactis* subsp. *lactis* C2 did not

inactivate phage skl, yet membranes from strains that were completely resistant to phage skl were also incapable of inactivating phage c2. This suggests that phage c2 and skl require the same membrane protein for infection, but that phage skl does not interact irreversibly with this protein *in vitro*, whereas phage c2 does. Consistent with this idea are the results that phage c2-resistant cells were partially resistant to phage skl, and phage skl-resistant cells were completely resistant to phage c2. However, the mechanism of interaction between the phage-inactivating activity and both phages needs further study.

Recently, membranes from wild-type strain C2 were found to inactivate phage 5, whose host-range includes strain C2. Phages c2 and 5 are morphologically similar, both being prolate-headed (17, 18), whereas phage skl is small isometric-headed (17). Perhaps the mechanisms of interactions between phages and the membrane are divided along morphological lines.

Attempts to purify the phage-inactivating protein from crude membranes involved Triton X-100 extraction and gel filtration and ion-exchange chromatography. The molecular mass of the phage-inactivating protein under non-denaturing conditions was estimated to be 350,000. The size is in the same range as that of the phage-inactivating protein of strain ML3, reported previously (14). The broad elution profile may suggest some heterogeneity of structure. This could result from attached lipids or carbohydrate, or a loss of some of the components of such a large membrane complex. Although the specific activity decreased as a result of the ion-exchange step, the purity of one protein was improved

after each step. This protein has an apparent size of 32 kDa, based on its migration on SDS-polyacrylamide electrophoretic gels. The mass yield of the 32 kDa protein was estimated from densitometer scans of silver-stained gels to be 54%. The elution pattern of the 32 kDa protein is the only apparent one which coincides with the phage-inactivating activity. Each of the other contaminating proteins are from side fractions of incompletely resolved peaks which did not co-elute with the phage-inactivating activity. Attempts to re-activate the protein have thus far been unsuccessful. This is not uncommon for membrane proteins with biological activity, including other lactococcal membrane proteins in general (1), and phage-inactivating proteins specifically (14). Although we have taken some precaution to avoid inactivation, such as the use of glycerol as an osmolyte and the addition of phospholipids during detergent-solubilization (1), we have not yet prevented inactivation, and further attempts to maintain activity after solubilization are currently in progress.

The loss in specific activity during the last step of purification and the lack in perfect correlation of phage inactivating activity to the silver-stained band intensity in some fractions from the gel filtration chromatography (fractions 32, 33 and 35) and ion-exchange column (lanes 1 and 3) leave open the possibility that the 32 kDa protein may not be the phage inactivating protein. We cannot rule out the possibility that the phage inactivating protein is another minor protein band which is not readily visible from the silver-stained gel. However, any slight discrepancies in correlation of phage inactivating activity to the band

intensity may be explained as follows: first, the protein in these fractions may not stain equally well due to the difference in the efficiencies in silver binding. It is highly unbelievable to compare slight differences in band intensity on silver-stained gels.

Secondly, the loss of activity particularly after the DEAE step, suggests that the activity is irreversibly denatured during the purification. It is possible that the broad elution profile from the gel filtration step is a result of a dissociation and inactivation of the 32 kDa protein. This could cause the inactivated 32 kDa subunit to elute in fractions following the peak activity.

Future attempts at purification without loss of activity should include the addition of lactococcal phospholipids during the detergent solubilization step (1). Recently the gene for the membrane protein apparently has been cloned by genetic complimentation (B. Hettinger-Smith and B.L. Geller, unpublished data), and this may confirm the identity of the phage inactivating protein.

ACKNOWLEDGMENTS

Table 3.1: Analysis of phage binding to cell wall, sensitivity to infection and phage inactivation by membrane

Strain[a]	% cell wall binding of phage[b]		% sensitivity to phage[c]		% membrane inactivation of phage[d]	
	c2	skl	c2	skl	c2	skl
C2	96	99	100	100	94	0
RMC2/4	98	84	0	46[e]	0	0
RMC2/8	96	86	0	43[e]	0	0
RMC2/9	96	87	0	42[e]	0	0
RMC2/13	95	83	0	42[e]	0	0
RMSK1/1	96	99	0	0	0	0
RMSK1/2	94	99	0	0	0	0

[a] C2, Wild-type strain C2; RMC2, phage c2-resistant mutant; RMSK1, phage skl-resistant mutant.

[b] The % of total PFU in the supernatant of a mixture of phage and cell walls after a 20 min incubation and centrifugation.

[c] The number of plaques divided by the number of plaques formed by the same amount of sample on wild type strain C2, times 100.

[d] The % of total PFU after incubation with membranes

[e] Plaque diameters reduced by 50%

Table 3.2.   Carbohydrates present in acid hydrolyzed cell wall
preparations of wild type and phage-resistant mutants of *Lactococcus
lactis* subsp. *lactis* C2 as determined by gas chromatographic
analysis of alditol acetate derivatives.

Strain[b]	Carbohydrates[a] (μg/mg of cell wall ± SD)			
	Rha	Gal	Glc	GlcNAc
C2	234±20	37.1±2.2	66±5.3	73.5±6.2
RMC2/4	245±40	40.9±6.7	80.7±18	90.5±29
RMC2/8	255±20	42.8±7.4	76.0±8.3	84.8±8.8
RMC2/9	241±8.4	40.6±3.9	72.8±3.1	86.0±13
RMC2/13	255±22	39.8±7.0	73.4±8.9	82.0±7.1
RMSK1/1	251±23	32.9±2.6	63.9±7.8	81.2±14
RMSK1/2	262±18	35.6±1.8	68.6±4.6	85.6±4.9

[a] Rha, rhamnose; Gal, galactose; Glu, glucose; GlcNAc,
   N-acetylglucosamine.
[b] C2, wild-type strain C2; RMC2, phage c2-resistant mutants; RMSK1,
   phage sk1-resistant mutants.

Table 3.3. Inactivation of phage c2 by membranes
 and cell envelope[a] treated with proteinase K and
mutanolysin

Treatment	% of total PFU after incubation with treated:	
	membranes	envelopes
Untreated	92	94
Proteinase K	4	NA[b]
Mutanolysin	93	18

[a] Envelopes preparations contain cell wall + cytoplasmic membrane.
[b] NA, not applicable

TABLE 3.4. Purification of the phage-inactivating protein isolated from *L. lactis* subsp. *lactis* C2.

Purification step	Total protein (mg)	Total activity (units)	Sp. Activity (units/mg)	Yield[a] (%)	Purification (fold)
Crude membrane	21	4300	205	100	--
Soluble membrane	7.5	2880	384	67	2
Sephacryl S-300	1.5	1052	701	24	3
DE-52[b]	0.83	43	52	<1	<1

[a] expressed in % activity
[b] average of two values

FIG. 3.1. (A) Sephacryl-S300 chromatography of the Triton-solubilized membrane preparation. Seven and one half mg of Triton X-100-soluble membrane components were applied to a column of Sephacryl-S300 (1.6 x 60 cm) equilibrated in 10 mM bis-tris, pH 6.5, 0.1% Triton X-100, 10% glycerol, and 1 mM DTT. Fractions (1.5 ml) were collected and analyzed for phage-inactivating activity (O) and total protein (+). Molecular weight standards eluted in the positions indicated: thyroglobulin (670K), apoferritin (442K), β-amylase (200K), and bovine serum albumin (66K). (B) SDS-polyacrylamide gel electrophoretic analysis of fractions containing phage-inactivating activity from the Sephacryl S-300 column. The gel was silver-stained. The arrowhead indicates the position of the 32 kDa protein which co-elutes with the phage-inactivating activity. The fraction numbers are shown below each lane. The lane on the right contained molecular weight standards: bovine serum albumin (66K), glyceraldehyde-3-phosphate dehydrogenase (63K), ovalbumin (45K), carbonic anhydrase (29K), soybean trypsin inhibitor (20.1K), and α-lactalbumin (14.3K).
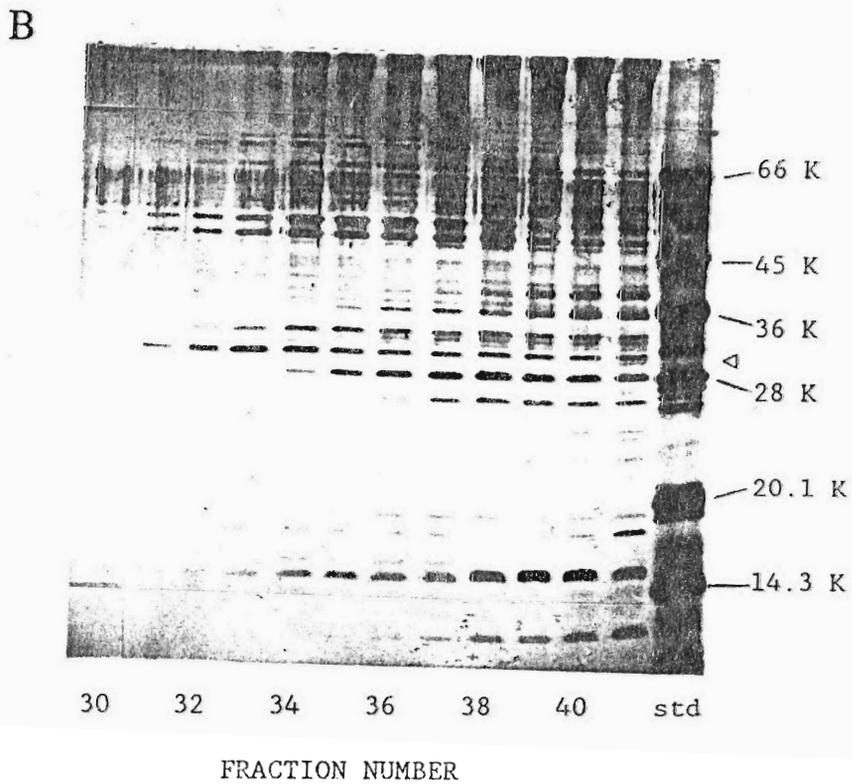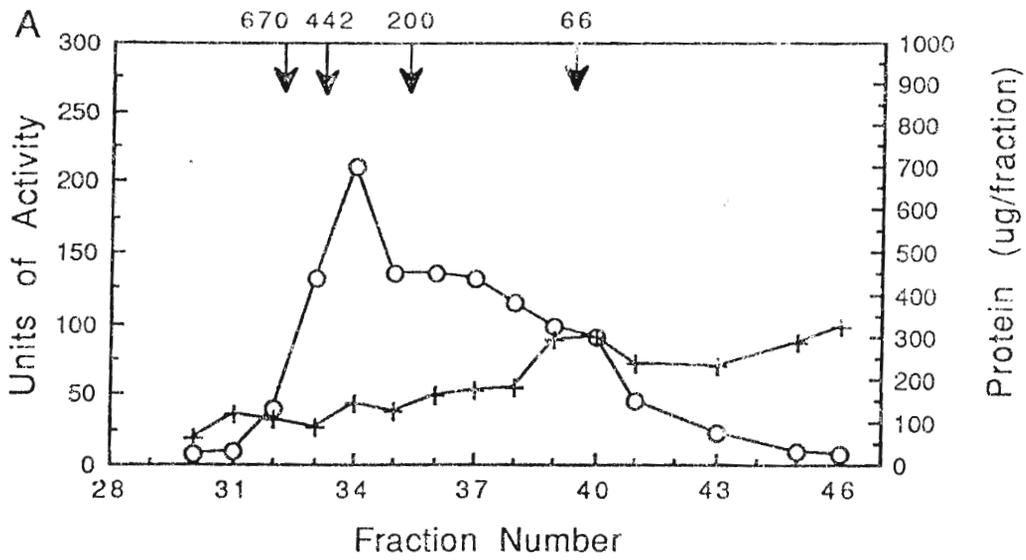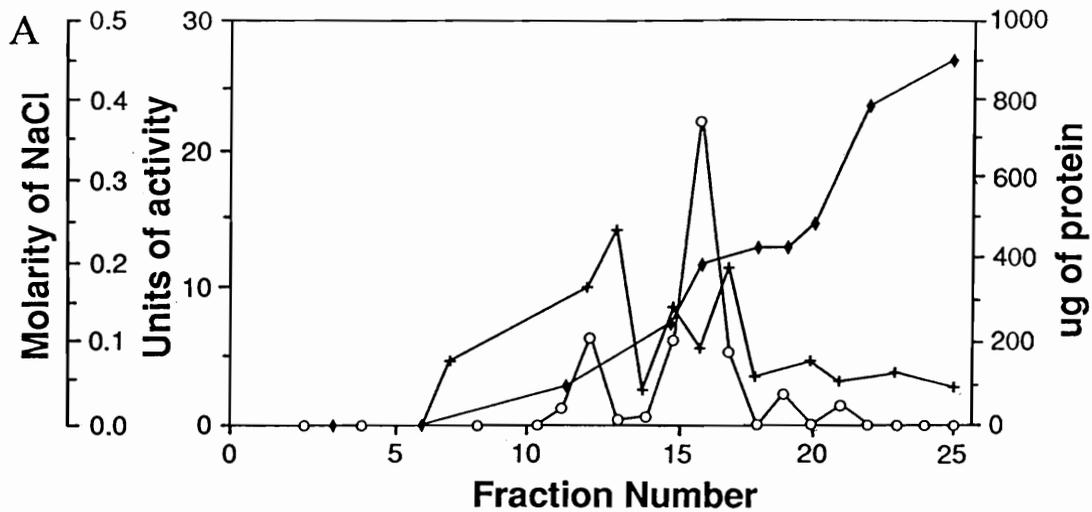
FIGURE 3.1

FIG. 3.2. (A) DEAE-cellulose chromatography of the phage-inactivating protein from the Sephacryl S-300 column. The combined, peak activity fractions from the Sephacryl column (1.5 mg protein) were applied to a DEAE-cellulose column (0.8 x 6 cm) and eluted with a three step NaCl gradient. Fractions were collected and analyzed for phage-inactivating activity (O), total protein (+), and conductivity (♦). (B) SDS-polyacrylamide gel electrophoresis and silver-staining. Lanes 1-4 correspond to 15 μl each of fractions 15, 16, 17 and 18 from the DEAE column. Lanes 6-8 are pooled fractions from the Triton extraction, Sephacryl S300 and DEAE columns, respectively. Amounts of protein loaded in lanes 6-8 were 4, 1.5, and 1 μg, respectively. Lane 5 contains the same molecular weight standards used in Fig. 3.1. The arrowhead indicates the 32 KDa protein band which co-elutes with the phage-inactivating activity.
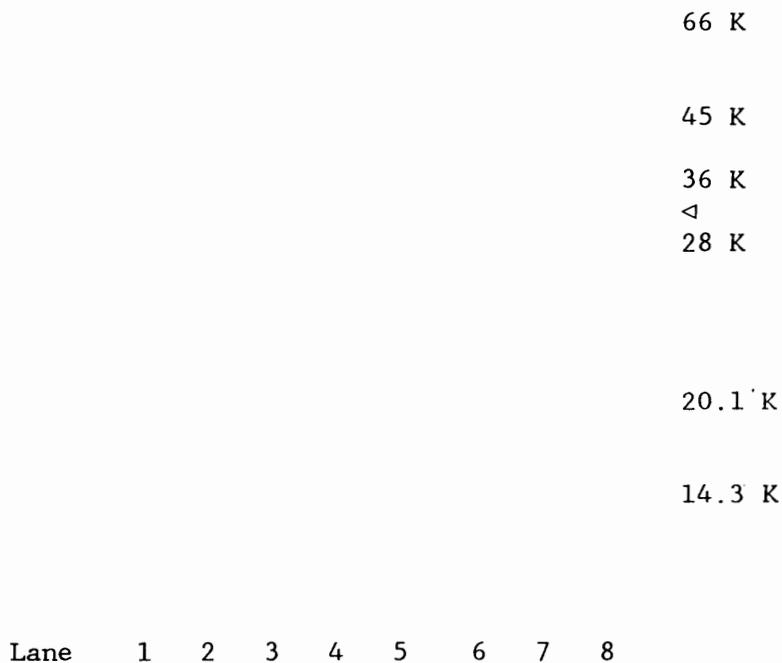
A

B

66 K

45 K

36 K
◁
28 K

20.1 K

14.3 K

Lane   1   2   3   4   5   6   7   8

FIGURE 3.2

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CONCLUDING REMARKS

The adsorption of bacteriophage to the surface of the host cell is an essential step for phage infection. Cells which lack a component of the receptor are insensitive to the phage (Chapter 1 and chapter 2). Although these phage resistant mutant cells have been used extensively to combat bacteriophage attack in commercial cheese-production, the molecular events leading to the resistance are not known. A better understanding of the precise molecular interaction of the phage and the host cell surface will provide crucial information to develop a more effective phage prevention program to reduce the incidence of phage infection of lactococcal starter cultures.

The results described in this thesis identified the molecular components on the host cell surface which interact with phage. All phages for lactococcus bacteria appeared to bind to the extracellular wall polysaccharide. There is evidence that certain phages of *L. lactis* subsp. *lactis* C2 required a membrane component for infection in addition to the cell wall polysaccharide. Further analysis to identify the molecular components of the receptor for phage kh of *L. lactis* subsp. *cremoris* KH showed that both rhamnose and galactose are required for phage adsorption. Phage kh appeared to bind directly to the rhamnose, but not to galactose. Galactose may be important in determining the conformation of the receptor. Furthermore, phages for *L. lactis* subsp. *cremoris* KH were inhibited from infecting the growing cells by adding rhamnose to the medium. There is evidence that other phages, ml3, 1,

643, and C2 which are infectious to *L. lactis* subsp. *cremoris* KH, may also require galactose and rhamnose for adsorption. However, these phages may bind to a rhamnose unit that is different from that used by phage kh. One of the results from the studies reported in this thesis has an industrial significance. Phage kh-resistant mutants of *L. lactis* subsp. *cremoris* KH (chapter 1), were much more sensitive to other species of phages, 643, c2 and 1, than the wild type strain. This illustrates that the use of spontaneous phage resistant mutant cells in the defined strain program may cause catastrophic results, because they may be hyper-sensitive to phages other than the one used for selection.

Using a similar strategy to identify the molecular components of the phage receptor of *L. lactis* subsp. *lactis* C2, the results show that phage skl binds to the rhamnose and glucose of the extracellular wall polysaccharide. Inhibition of phage binding by lectins specific to glucose suggested that phage skl did not bind directly to glucose. Addition of rhamnose to the growing culture partially inhibited phage skl infection whereas glucose did not. It is proposed that the phage skl binds directly to rhamnose and that glucose plays an indirect role, perhaps by influencing the conformation of the receptor.

During the screening of *L. lactis* subsp. *lactis* C2 for mutations in phage c2 binding, it was found that all mutant cells isolated from superinfection of phage c2 did not form plaques but bound phage normally. These mutants also had a reduction in sensitivities to phage skl. The phage resistant mutants isolated by superinfection cells with phage skl were found to be totally insensitive to both phages but bound normally

to phages c2 and sk1. Further analysis of these mutants suggested that the resistant mechanism was due to the mutations of a membrane protein required for infection by phage c2 and sk1. Membranes from the wild type, but not the resistant mutant cells inactivated phage c2. The membrane protein was partially purified by gel filtration and ion exchange chromatography. The phage inactivating protein (PIP) was found to have a molecular weight of 350,000 under the non denaturing conditions with an apparent subunit size of 32 KDa. This suggested that the protein is multimer of 10-12 subunits.

It is our goal that the results from these studies will be used to construct a strain of starter culture with a defense mechanism at the phage adsorption step. Our results showed that rhamnose appeared to be the receptor for phages of diverse morphologies both in *L. lactis* subspecies *lactis* and *cremoris*. Certain phages of *L. lactis* subsp. *lactis* may also require a membrane protein in addition to the cell wall polysaccharide for infection. This suggests a strategy for constructing a better phage-resistant strain to minimize the chance that mutations in the phage could overcome the host's resistance. The target sites for genetic manipulation would include both the polysaccharide receptor and the phage-inactivating protein.

To construct a strain resistant to phage at the adsorption step, a gene for the phage receptor needs to be identified. The gene may be isolated by shotgun cloning fragments of the chromosome from the phage-sensitive strain directly into the isogenic phage resistant mutants which have been isolated (chapter 1). The cloned fragment containing the phage

receptor gene would be identified by screening for phage sensitivity. Once the gene is identified, two strategies could be used to construct a strain which does not express phage receptor. The first strategy would be to disrupt the wild type chromosomal receptor gene by insertion of the deleted copy of the gene. The deleted derivative of the gene would have to be cloned on a plasmid that integrates into the chromosome as described by Leenhouts et al. (5,6). The integration of the homologous DNA fragment by recombination would disrupt the receptor gene (5,6,1).

A second approach would be to clone the phage receptor gene into a plasmid DNA in an antisense orientation. Once the plasmid is transformed into the parental phage sensitive cell, the antisense transcription may specifically inhibit the expression of the phage receptor gene by forming a specific intermolecular RNA duplex. Antisense inhibition has successfully prevented the expression of thymidine kinase (2), myosin heavy chain (4), and bacteriophage infection in *L. lactis* subsp. *lactis* (3). The gene for phage inactivating protein could be isolated and mutated as described above. Alternately, a genomic library could be screened with an oligonucleotide probe complementary to the predicted coding sequence of the N-terminus of the phage inactivating protein. To develop the DNA probe, the phage-inactivating protein would have to be partially sequenced.

At presence it is not well understood what is the precise role of PIP during phage infection. A better knowledge of the role of PIP would not only give a better understanding of how phage interacts with the cell surface but it may provide information crucial to the design of a defense

system against all lactococcus phages.

As scientists, we are interested in the mechanism of the phage-infection cycle. Studies are continuing to determine if the phage binds to the phage-inactivating protein and if DNA is released as a result. These studies may be important in identifying other components of the infection cycle, which may in turn be targets for constructing phage-resistant strains. It is our belief that a better understanding of the molecular events of the phage infection cycle will produce the information necessary to eliminate the problem of phage infections in the dairy industry.

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