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The mechanism of bacteriophage infection in *Lactococcus lactis* subsp. *lactis* C2 was investigated using phages c2, ml3, kh, l, h, 5, and 13. All seven phages adsorbed reversibly to rhamnose moieties on the host cell wall. This was indicated by rhamnose inhibition of phage adsorption to cells, rhamnose inhibition of lysis of phage-inoculated cultures, and the dissociation of adsorbed phage from cell walls upon dilution. All seven phages were inactivated *in vitro* by membranes from wild-type cells, but not by membranes from a strain selected for resistance to phage c2. Because the phage c2-resistant strain was also resistant to the other six

phages, this suggests that all seven phages share a requirement for a host membrane protein, named PIP, which is defective in the phage-resistant strain. The mechanism of membrane inactivation was found to be an irreversible adsorption of the phage to PIP, as indicated by the adsorption of [^{35}S]methionine-labeled phage to purified membranes from phage-sensitive cells but not to membranes from the resistant strain, the elimination of adsorption by pre-treatment of the membranes with proteinase K, and the lack of dissociation of ^{35}S from the membranes upon dilution. Following membrane adsorption, ejection of phage DNA occurred rapidly at 30°C, but not at 4°C. These results suggest that many lactococcal phages adsorb initially to the cell wall and subsequently to the host cell membrane protein PIP, which leads to the ejection of the phage genome.

Mechanisms Involved in Lactococcal Phage Adsorption and DNA Ejection

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INTRODUCTION

The following paper investigates the mechanism of phage infection of *L. lactis* subsp. *lactis* C2, and focuses on the role of a host cell membrane protein, named PIP, in this process. The initial recognition of lactococcal phages c2, ml3, kh, l, h, 5, and 13 to the cell wall will be studied by competitive binding assays as well as lytic inhibition studies using the monosaccharides that are naturally found on the cell wall. The mechanism of phage DNA ejection will also be investigated using ^{35}S- and ^{3}H-labeled phage c2. The results will help define the reaction steps that occur at the host cell surface prior to phage genome entry into the cell. The results may also serve as a model for future studies of the mechanism of DNA translocation across the bacterial plasma membrane.

The practical aspect of the research is that we are constructing phage resistant strains that will be used for cheddar cheese production. Because we must engineer genetic changes in the host cell's gene that codes for PIP, without negatively affecting growth or favorable cheese making characteristics, it is advantageous to know the mechanism of interaction between phages and PIP.

REVIEW OF LITERATURE

Lactococci are lactic acid forming bacteria that are used as starter cultures for the fermentation of milk during cheese production. These gram-positive bacteria convert lactose to lactic acid thus reducing the pH, and coagulating the milk. In addition, these bacteria break down milk proteins, and produce fermentation products with favorable aroma and flavor associated with good cheese (50).

Over the years, a major problem that cheese manufacturers have had is the destruction of starter cultures by bacteriophage infection. The amount and size of all the equipment used in the production of cheese makes it virtually impossible to keep the system entirely aseptic. Based on this fact, bacteriophage have the opportunity to enter the facility during all phases of production (48, 51). Even if aseptic production was possible, it would not allow for the monitoring of temperate phages within lysogenic strains (20, 31, 41, 54). When lysogenic strains are induced by various stress conditions, phage may appear in the starter culture at any time. Regardless of the path of phage contamination, the problem which arises is the continuous cycle of phage infection and lysis of cells leading to the destruction of the bacterial starter culture. In turn fermentation slows and possibly ceases. It is quite easily understood that this magnitude of loss can lead to financial disaster.

The elimination of phage infection has been approached in several different ways. One way is to infect a starter culture strain with a phage and select for naturally occurring phage resistant mutants in the

population (44, 58). The resistant cells are constantly pressured by phage-laden whey in order to maintain growth of only the cells that continue to be resistant (55). However, a limitation to this approach is that mutations within the phages may lead to their ability to eventually infect cells that were previously considered resistant. It is important to continually pressure the cells in order to select only those which remain truly phage-resistant over time.

Another strategy to avoid phage infections of starter cultures involves the use of undefined blends of both *L. lactis* subsp. *lactis* and subsp. *cremoris* which have not been characterized for flavor, aroma, and phage sensitivity (56, 57). The theory behind the use of these blends of strains is that the emergence of phage during fermentation will only allow for the destruction of a limited amount of bacteria, since all strains are presumably not susceptible to the same phages. The problem with this method is that the batch of undefined strains alters the levels of acid production as well as flavor characteristics of the cheese. An additional problem is that once phage is discovered, the strain that it is growing on is hard to pinpoint because it is, in fact, undefined (45, 56, 57).

Cheese manufacturers have eliminated the problems of undefined strains by using a blend of characterized strains (51). This "defined strain" method uses strains of *L. lactis* subsp. *lactis* and subsp. *cremoris* each of which is resistant to the phages that can infect the other strains in the blend. If, during the fermentation process, phage is found within the whey, the susceptible defined strain on which it is growing is identified and removed. This strain can then be used to make resistant mutants. If

a resistant mutant is found which has favorable characteristics then it can once again be used in the blend of defined strains. The importance of multiple strain use is that even though one strain is removed, the others can continue to be used for fermentation and cheese-production. Another advantage to using defined strains is that they can also be selected for the best acid production and flavor quality. This method of multiple defined strains has been used quite successfully around the world. For instance, it is used in the United States for cheddar, monterey jack, cottage cheese, and buttermilk manufacturing (45, 56, 57).

Generally speaking, phage infection is avoided by using blends of strains and by rotating the use of strains as they become phage-sensitive. Perhaps if the mechanism of phage infection in lactococcal strains was better understood it would be possible to combat the problem on the molecular level. With genetic engineering techniques, phage-resistant strains might be constructed that could not be isolated using traditional methods of selection.

Most of the research on the molecular mechanism of phage infection has been done on the gram-negative bacteria *Escherichia coli* . Because many of the steps of phage infection are similar in all bacteria studied, a review of the *E. coli* literature on the relevant steps of phage infection is in order.

The general sequence of events involved in phage replication include the following (6):

1. Attachment of phage to the host cell
2. Penetration or injection of phage DNA or RNA into the cell

3. Early steps in replication of phage genome (phage-specific enzyme production)
4. Replication of phage nucleic acid
5. Synthesis of phage structural proteins
6. Assembly of new phage particles (packaging phage genome into phage proteins)
7. Release of progeny phage

E. coli phages initially recognize either the lipopolysaccharide (LPS) or specific proteins of the outer membrane. An example of this is bacteriophage T4 infection of *E. coli* strains B and K-12. Mutoh *et al.* have shown that cell envelopes of strain B are capable of acting alone as a receptor for phage adsorption (35). However, strain K-12 requires both the lipopolysaccharide and the OmpC protein to function as receptors, allowing the distal ends of the phage tail fibers to attach to the cell (16, 19, 29). Once adsorption occurs, there is a contraction of the tail and a penetration of the internal tube across the outer membrane.

The adsorption step is followed by an ejection of phage DNA. The exact mechanism of DNA translocation through the host cell plasma membrane is unknown (5). However, the ejection requirements have been studied by Furukawa *et al.*, who labeled phage DNA with the fluorescent dye 4', 6-diamidino-2-phenylindole (DAPI). Artificially contracted and DAPI-labeled phage particles were incubated with spheroplasts and examined under a fluorescent microscope. The fluorochrome was observed entering the host cell cytoplasm, suggesting that phage DNA translocated across the plasma membrane.

The energy requirements of phage DNA injection have also been studied using the DAPI-labeled phage. Whole cells were poisoned with potassium cyanide to disrupt the respiratory chain and eliminate the proton motive force. Phage that contained labeled DNA were able to bind to the cells, but significant amounts of fluorochrome were unable to traverse the cytoplasmic membrane. When spheroplasts were treated with carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP, which destroys the proton motive force) the DAPI-labeled phage DNA was still capable of being injected into the cytoplasm. The results suggest that the proton motive force is required for the inner and outer membranes of the host cell to contact each other at the site of phage adsorption, but not required for the translocation of phage DNA across the membranes (17).

In addition to the energy requirements for phage DNA translocation, the physical route that phage DNA uses to traverse the plasma membrane has been studied. Letellier and Boulanger have shown that phages T4 and T5 inject their DNA into the host cell cytoplasm via ion-channels. Using a potassium-selective electrode they have demonstrated that the injection of phage DNA is coupled with an efflux of cytoplasmic potassium. A linear relationship was found between the amount of adsorbed phage and the rate of potassium efflux, suggesting that each adsorbed phage leads to the opening of one ion-channel. It also has been shown that at low temperatures (4°C), phage were capable of irreversibly binding to the membrane receptor while no potassium efflux occurred. As the temperature was raised above 15°C the efflux was once again detected. These results suggest that while reversible

adsorption is not temperature dependent, DNA translocation is (3, 4, 13, 19, 32).

E. coli phage ΦX174 also recognizes the outer LPS for an initial, reversible adsorption. Following adsorption, the phage undergoes a conformational change during an eclipse stage where the phage genome is partially ejected. Newbold and Sinsheimer have shown the eclipse stage is a temperature dependent process not occurring at temperatures below 19°C. This was demonstrated by incubating phage with cells at different temperatures and monitoring the amount of viable phage that was reversibly bound. At 15°C all the phage remained in its infectious form, but as the temperature increased to a maximum of 37.5°C the amount of viable phage rapidly decreased (37). During the eclipse stage some of the phage particles were spontaneously released from the cell surface but were found to be non-infectious. The remaining adsorbed phage became irreversibly bound and completed the injection of their genome across the cytoplasmic membrane (6, 15, 21, 36).

In gram-positive bacteria, the initial adsorption of phage almost always involves the cell wall carbohydrates (2, 33). This general rule includes phages of *Bacillus subtillus* (67), *Staphylococcus aureus* (33), *Streptococcus pyogenes* (7, 14), and various species of lactobacillus (9, 23, 65, 66). The phages of lactococcal strains are no exception. Competitive inhibition studies have shown that rhamnose moieties on the extracellular wall polysaccharide of both *L. lactis* subsp. *lactis* C2, subsp. *cremoris* KH, and subsp. *cremoris* EB7 play a major role in adsorption of phage sk1, kh, and eb7 respectively (25, 59, 61).

Although phage attachment is similar in both gram-negative and gram-positive bacteria, the penetration of phage DNA may be different because of the gross structural differences in the arrangement of the membranes. In gram-negative bacteria, the phage DNA must transfer across two membranes, whereas in gram-positive cells, the transfer involves only one membrane. Perhaps because of the more complex gram-negative membrane structure, an *in vitro* system to study the mechanism of phage DNA penetration has been elusive. For this reason, gram-positive membrane vesicles may provide a more facile system to study this particular step in the phage infection cycle.

The roles of the lactococcal plasma membrane in phage adsorption and subsequent steps leading to the ejection of phage DNA into the host cell are vague. An initial report showed that when purified membranes from *L. lactis* subsp. *lactis* ML3 and subsp. *lactis* D9 were mixed with phages ml3 and d9 respectively, the number of viable plaque forming units was significantly reduced. The membrane receptor activity of strain ML3 was shown to be highly specific based on its ability to inactivate only phage ml3 and not the other six phages used in the study. When membranes from the two strains were treated with lipid solvents the amount of receptor activity was greatly reduced. These results suggested that a plasma membrane component inactivated phage *in vitro* (39). In later experiments, membrane proteins from *L. lactis* subsp. *lactis* ML3 were extracted by sodium deoxycholate and passed through a Sepharose gel filtration column. A fraction containing several proteins was found to inactivate phage ml3 *in vitro* (40). Individual proteins within the

fraction were not further purified and characterized for their specific role in phage inactivation. However, this research provided initial evidence that a membrane protein was involved in lactococcal phage infection. Similar results were reported with membranes from *L. lactis* subsp. *lactis* C2 using various phages (60). Phage-resistant mutants have been isolated that apparently adsorb phage efficiently, but have defective membranes that were incapable of inactivating phage *in vitro* (60). This suggested that the membrane protein required for phage infection was not the receptor, but had a role in a step subsequent to adsorption. A gene (*pip*) that complemented the membrane defect was cloned and sequenced (18), and by use of hydropathy analysis it appears to code for a membrane protein. The exact role of the protein, however, is unknown.

In this paper, we report that lactococcal phages c2, ml3, kh, l, h, 5, and 13 recognize rhamnose moieties on the cell wall of *L. lactis* subsp. *lactis* C2 as initial receptors. We also report that all 7 phages tested share a similar mechanism of infection which requires a host membrane protein. This membrane protein causes an irreversible adsorption of the phage which is followed by the ejection of DNA from the phage particle.

MATERIALS AND METHODS

Bacterial strains, phages, medium, and plaque assay. *L. lactis* subsp. *lactis* C2 and its phage-resistant derivative, RMC2/4 (60), were grown and maintained on M17 medium (53), supplemented with 0.5% glucose (M17G), at 30°C. SLSD and SL1C media was used for growing cultures in the preparation of [^{3}H]thymidine-labeled phage c2 (63). Lactococcal bacteriophages were prepared from single plaques, and plaque-assayed as described (53). Phages were stored in 20% glycerol at -70°C.

Monosaccharide inhibition of phage infection. α-L-(+)-rhamnose, α-D- (+) - glucose, D -(+) - galactose, and N-acetylglucosamine were added to an exponential-phase culture (OD_{600} = 0.1) at a final concentration of 0.5 M. $CaCl_2$ (15mM) and phage (1 X 10^6 PFU/ml) were immediately added to the cultures. One control culture received only the saccharide, while another control received only phage and $CaCl_2$. Growth was measured by optical density at 600 nm. Readings were taken every 30 minutes until the control tube had completely lysed.

Monosaccharide inhibition of adsorption. The monosaccharides (0.5 M) listed in the previous paragraph were separately mixed with each of the phages (1 X 10^6 PFU/ml) listed in Table 1 in 100mM bis (2-hydroxyethyl) imino-tris (hydroxymethyl) methane-HCl (BisTris), pH 6.8, and 20mM $CaCl_2$. The mixtures were incubated at 0°C for 1 hour. A stationary phase culture of *L. lactis* subsp. *lactis* C2 was mixed with 9

volumes of the phage\saccharide mixture, and agitated at 4°C for 1 hour. The cells were a stationary phase culture that was diluted in 100mM BisTris (pH 6.8) to 1 X 10^8 CFU/ml (determined by plate count) and stored at -70°C. The mixtures were centrifuged at 14,000 X g for 5 minutes at 4°C, and the supernatant was assayed for plaques. The percent phage adsorption was calculated by dividing the titer of the supernatant after centrifugation by the phage titer in the control tube containing no cells. This number was then multiplied by 100 to get the percent adsorption. The percent inhibition by monosaccharides was determined by subtracting the percent adsorption in the presence of monosaccharide from the percent adsorption without added monosaccharides. Additional controls included phage in the absence of cells, with and without monosaccharides, in order to monitor phage viability throughout the reaction. Less than 2% of the control PFU were lost during a typical 1 h incubation.

Preparation of cell walls and phage adsorption assay. Cell walls were prepared as described (59), and quantified by total carbohydrate content (10). Phage (1 X 10^6 PFU/ml) was mixed with cell walls (0.96 mg/ml) in 100 mM BisTris (pH 6.8) and diluted 1:1 with M17G plus 20 mM $CaCl_2$. The mixture was agitated at 4°C for 1 hour, and half the mixture was centrifuged at 14,000 X g for 5 minutes. Both the supernatant and the mixture that had not been centrifuged were diluted 1 X 10^{-3} and titered. A control to monitor for any loss of PFU unrelated to adsorption was used, in which phage without cell walls was incubated under the same

conditions. The percent adsorption was calculated as stated in the above paragraph.

Preparation of [$^{S}35$]methionine-labeled phage c2. Labeled phage was prepared by a modified procedure of Kim *et al.* (26). *L. lactis* subsp. *lactis* C2 was grown in M17G media at 30°C to OD_{600} = 0.3. One ml of culture was transferred to a microfuge tube containing 0.5 mCi [^{35}S]methionine. $CaCl_2$ (10mm) and phage c2 (moi = 5) were immediately added to the culture, which was allowed to lyse at 30°C for two hours. The lysate was centrifuged at 14,000 X g for 5 minutes at 4°C to remove cell debris. Phage in the supernatant was purified by polyethylene glycol precipitation and CsCl gradient centrifugation (47), using a 1.4 g/ml continuous gradient. The gradient was fractionated into 250ul aliquots. Each fraction was titered and counted in a liquid scintillation counter (Fig. 1). The fraction with the highest titer and radioactivity was dialyzed in buffer containing 10mM BisTris (pH 6.8), 10mM $MgSO_4$, and 20% glycerol (Fig. 1). Phage was analyzed by SDS-PAGE (23) and fluorography (Fig. 2) (30). Major bands were seen at both 169 and 85 kDa. Several other minor bands were also present. The positions of all the bands were similar to those previously reported (42).

Preparation of cell membranes and adsorption of phage. Cytoplasmic membranes were prepared as described (60). Briefly, cells were lysed with lysozyme, and the membranes were isolated by differential centrifugation. The membranes were purified further on a two-step sucrose gradient (0.5

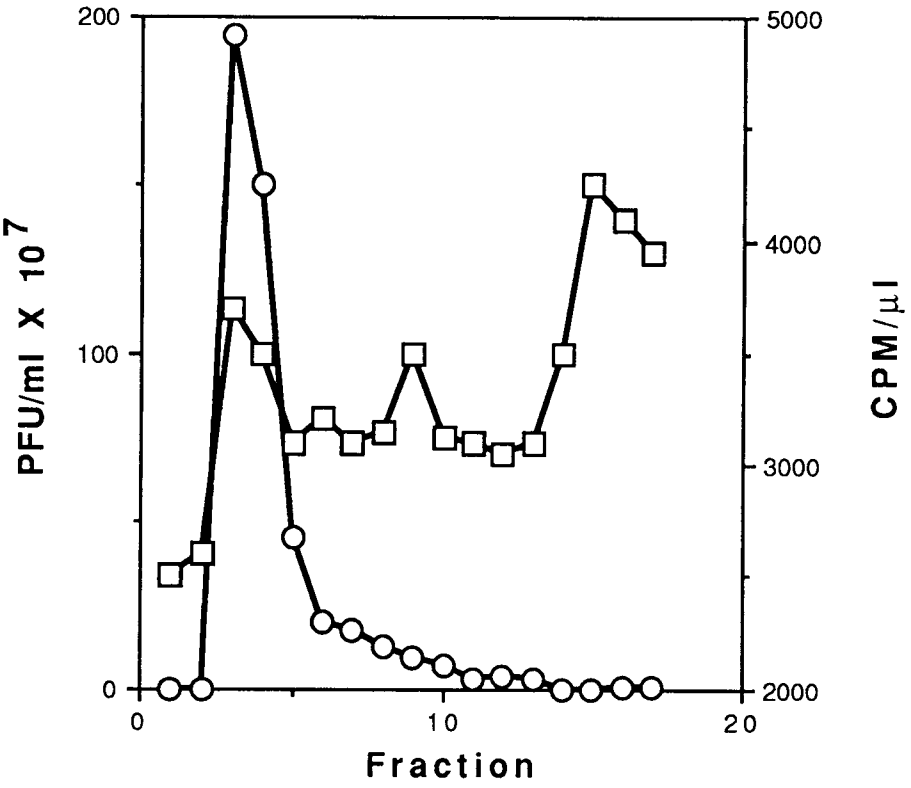

Fig. 1. **CsCl purification of radiolabeled phage c2.** [^{35}S]methionine-labeled phage c2 was purified on a CsCl gradient and fractionated. PFU/ml (O) and radioactivity (□) were each measured.

and 1.5 M sucrose in 100mM BisTris pH 6.8) by centrifuging in a SW 41 rotor (Beckman Instruments) at 160,000 x g for 3 hours. Membranes were quantified by total phosphate content (1).

[^{35}S]methionine-labeled phage c2 (1 X 10^7 PFU/ml and 1 X 10^4 cpm/ml) was mixed at 4°C with either wild-type strain C2 membranes (1.12 mg phospholipid/ml), or an equal amount of membranes from the

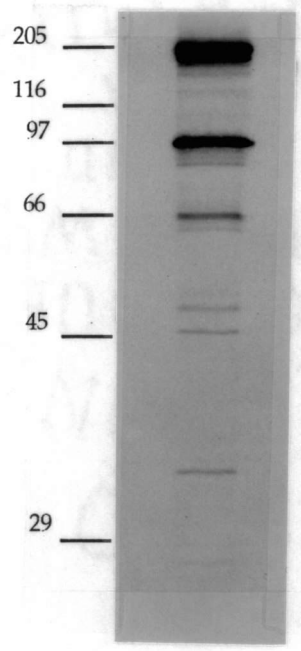

Fig. 2. **Fluorograph of phage c2.** (standards in kDa)

phage-resistant mutant RMC2/4, in 100mM BisTris (pH 6.8). The mixture was diluted 1:1 with M17G plus 20 mM $CaCl_2$, agitated at 4°C for 1 hour, and centrifuged at 14,000 X g for 5 min at 4°C. The membrane pellet and supernatant were counted separately in a liquid scintillation counter. Membrane-bound radioactivity was divided by the sum of the radioactivity in the supernatant and pellet, and multiplied by 100 in order to calculate the percent phage adsorption. The percent inactivation in Table 3 was calculated by dividing the titer of the supernatant after the 1 h incubation by the titer of the mixture before addition of the membranes, and multiplying by 100.

Determination of reversibility of adsorption. Phage and membranes were mixed as described in the previous section, and aliquots of the

mixture were taken at 0, 1, 5, 15, 30, and 60 min. The aliquots were immediately diluted into M17G at 0°C, and titered. In addition, half of each aliquot was centrifuged at 12,000 x g for 5 min at 4°C, and the supernatant titered. In separate experiments, the length of time between diluting and plating was varied from 5 min to 1 h, but was found to have no effect. Because the length of time it takes to precipitate 99% of our membranes under our assay conditions was measured to be 5 min, it was important to determine if a significant amount of inactivation occurred during centrifugation. In separate experiments, the length of time of centrifugation was shortened to 0.5 min and the force increased to 150,000 x g at 4°C in an air-driven ultracentrifuge (Airfuge, Beckman Instruments). The length of centrifugation time had no effect on the results.

Membrane treatment with mutanolysin and proteinase K. Membranes were treated as described (60), except the concentration of mutanolysin was 40 U/ml, and proteinase K was 0.5 mg/ml. [^{35}S]methionine-labeled phage c2 (1 X 10^7 PFU/ml and 1x 10^4 cpm/ml) and the treated membranes were diluted 1:1 with M17G plus 20 mM $CaCl_2$. The mixture was agitated at 4°C for 1 hour and then centrifuged at 14,000 X g for 5 minutes. The pellet and supernatant were separated and analyzed by liquid scintillation counting. The change in adsorption as a result of the treatments was calculated by subtracting the percent adsorbed radioactivity on the treated membranes from that of the untreated membranes.

Preparation of [^{3}H]thymidine-labeled phage c2. Labeled phage c2 was prepared as described (43), except 8 μCi/ml [^{3}H]thymidine was used. After the addition of phage (1 x 10^8 PFU/ml, moi = 2), the medium was supplemented with 3.5μCi/ml [^{3}H]thymidine. The phage was further purified as described above for the ^{35}S-labeled phage.

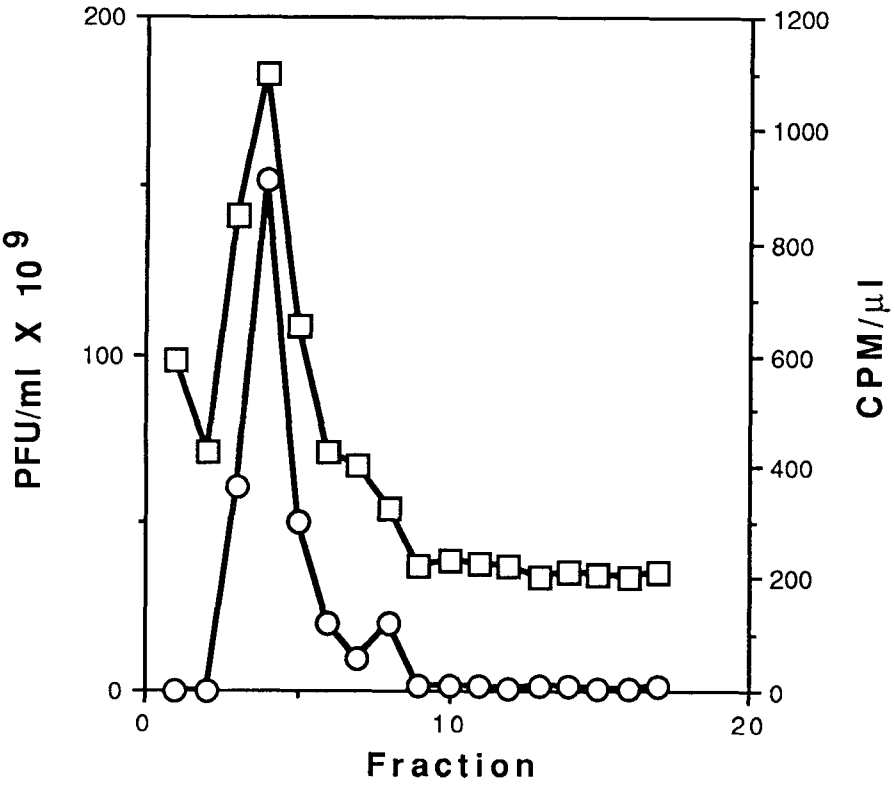

Fig. 3. **CsCl purification of ^{3}H-labeled phage c2.** [^{3}H]thymidine-labeled phage c2 was purified on a CsCl gradient and fractionated. PFU/ml (O) and radioactivity (□) were each measured.

[^{3}H]thymidine-labeled phage c2 adsorption to membranes and DNA ejection. [^{3}H]thymidine-labeled phage c2 (1 X 10^8 PFU/ml and 1.5 X 10^4 cpm/ml) was mixed with wild type membranes (1.12 mg/ml) in 100mM BisTris (pH 6.8) and diluted 1:1 with M17G plus 20 mM $CaCl_2$. The mixture was agitated at 4°C, and aliquots were taken at 1, 5, 15, and 30 minutes. The reaction tube was transferred to a 30°C shaker and aliquots were taken at 1, 5, 15, 30, and 60 min after the temperature shift. The aliquots were centrifuged at 14,000 X g for 5 min at 4°C, and the pellet and supernatant were quantified separately in a scintillation counter. The supernatants were also titered at each time point to monitor the percent phage inactivation. In a separate but similar reaction, [^{35}S]methionine-labeled phage c2 was used instead to measure the location of the phage proteins under the same reaction conditions.

Nuclease degradation of ejected DNA. The ^{3}H released from the membrane-adsorbed phage at 30°C was analyzed by nuclease digestion as previously described (34). Reactions identical to those described in the previous section were incubated at 30°C for 1 h, without prior incubation at 4°C, and the pellets and supernatants were separated by centrifugation. The supernatant was treated with 0, 10, or 50 ug/ml snake venom phosphodiesterase I (Sigma Chemical Co.), plus 0, 20, or 100 ug/ml bovine pancreatic DNase I (Sigma Chemical Co.), and 20mM $MgCl_2$ for 3 hours at 15°C. At 0, 1, and 3 h, the treated aliquots were precipitated with 10% TCA for 1 h at 0°C. The TCA precipitations were then centrifuged at 14,000 X g for 10 minutes at 4°C. The pellet and supernatant were quantified in a

liquid scintillation counter. A control reaction without membranes was analyzed in an identical procedure.

RESULTS

Inhibition of phage adsorption by rhamnose. Bacteriophage adsorption sites on the envelope of *L. lactis* subsp. *lactis* C2 were investigated for phages c2, ml3, kh, l, h, 13, and 5, using a competitive inhibition assay. Monosaccharides that compose the cell wall of this strain (59) were mixed with cells and phage, and the amount of adsorption was determined (Table 1). Rhamnose inhibited adsorption of all 7 phages from 64 to 85%. Glucose, galactose, and N-acetylglucosamine inhibited adsorption to lesser extents for all phages, as compared to rhamnose. These results are consistent with the suggestion that the cell wall, and specifically the rhamnose moieties, act as adsorption sites for the 7 phages tested.

Table 1

Competitive inhibition of phage adsorption

	% inhibition of phage adsorption			
Phage	Rhamnose*	Glucose*	Galactose*	N-acetylglucosamine*
c2	69	35	34	22
ml3	74	18	14	11
kh	71	40	22	15
l	71	58	58	14
13	64	47	37	8
5	85	62	25	7
h	65	51	56	2

* 500 mM.

Delay of infection by rhamnose. To investigate further the role of the cell wall carbohydrates in phage adsorption, different monosaccharides

were added to exponential-phase cultures immediately before infecting them with phage. Growth was measured by optical density. For phages c2 and kh, rhamnose significantly delayed or prevented lysis for at least two hours (Fig. 4). Glucose, galactose, or N-acetylglucosamine did not delay lysis, and the addition of sugars had no effect on the growth of uninfected cultures (data not shown). Nearly identical results were found for phages ml3, l, h, 13, and 5 (data not shown). These results suggest that rhamnose is a major component of the receptor on the cell wall, while glucose and galactose may play lesser roles.

Initial adsorption to the cell wall is a reversible process. Each of the seven phages was mixed with purified cell walls in order to determine whether adsorption was irreversible and caused inactivation of the phages. Initially, 90-99% of each phage was adsorbed to equal amounts of cell walls at 4°C. The adsorbed phage-cell wall suspension was then diluted and titered. It was found that for each phage, 100% of the bound phage formed plaques.

To test if the incubation temperature had an effect on the reversibility of adsorption, the adsorbed phage-cell suspension was warmed from 4°C to 30°C before being diluted and titered. For each phage, all of the adsorbed phage formed plaques. These results show that adsorption to the cell wall is a reversible process that does not inactivate these phages, and is not affected by temperature in the range from 4°C to 30°C.

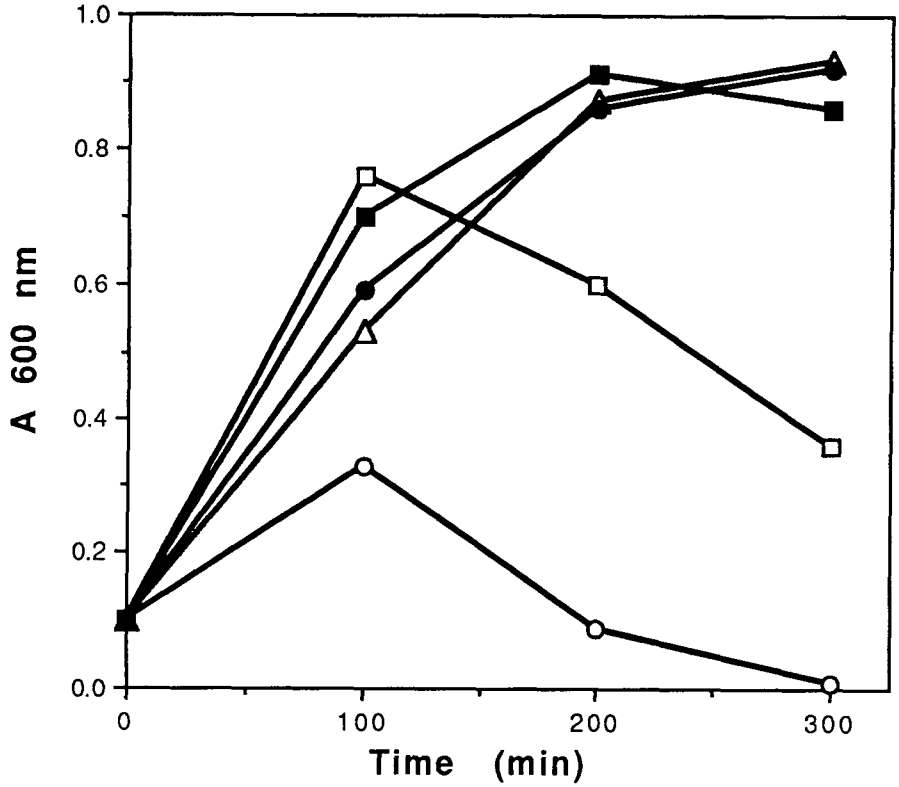

Fig. 4. **L-(+)-rhamnose inhibition of cell lysis.** Mid-exponential-phase cultures of *L. lactis* subsp. *lactis* C2, supplemented with $CaCl_2$, were mixed with 0.5 M rhamnose (△), phage c2 and rhamnose (●), phage c2 (○), phage kh and rhamnose (■), and phage kh (□). Growth was measured at $O.D._{600}$ for 5 h. Each point represents the average of 2-3 experiments.

Phages share a mechanistic requirement for a host cell membrane component. It was previously found that a host cell plasma membrane protein is required for phage c2 infection (60). This can be measured *in vitro* by mixing phage and purified plasma membrane, which inactivates

the phage. The membrane requirement for phage infection was inferred from results that showed no *in vitro* inactivation of phage by membranes from a phage-resistant mutant, RMC2/4. In order to test if the phages kh, ml3, l, h, 13 and 5 share this same requirement as phage c2 for infection, first the efficiency of plating (EOP) of each phage on RMC2/4 was determined. For each phage, high titers (>1 X 10^{10} PFU/ml) were grown on wild type strain C2, but no plaques were formed on RMC2/4, indicating an EOP of <1 X 10^{-9} for each. Second, purified membranes from wild type and RMC2/4 strains were mixed and incubated with the seven different phages. For each phage, wild type membranes inactivated the phage, whereas RMC2/4 membranes did not (Table 2). These results suggest that all seven phages tested share a requirement for infection for the same host cell membrane component, apparently defective in RMC2/4, which we name PIP (an acronym for phage infection protein).

Table 2

Inactivation of phages by C2 and RMC2/4 membranes

	% of total PFU inactivated by:	
Phage	C2 Membranes*	RMC2/4 Membranes*
c2	90	10
ml3	91	2
kh	82	9
l	70	14
13	81	16
5	83	14
h	72	10

*Membrane concentration = 1.12 mg phospholipid/ml.

Interaction of phage with the plasma membrane involves adsorption to a membrane protein. To determine whether the inactivation involved an adsorption of the phage to the membrane, [^{35}S]methionine-labeled phage c2 was mixed and incubated with purified host plasma membranes, and the membranes were precipitated by centrifugation. Nearly all (89%) of the radiolabeled phage was found in the membrane pellet (Table 3). A corresponding amount of plaque forming units (99%) was also adsorbed. The small difference between adsorbed radiolabeled phage and adsorbed plaque forming units is probably the amount of non-infectious (damaged) phage particles in the phage preparation (46). This shows that membranes adsorb phage. Moreover, an equal amount of membranes from RMC2/4 adsorbed only 8% of the radiolabeled phage.

The composition of the membrane adsorption site was investigated by treating the membranes with proteinase K. After inactivating the proteinase K with PMSF, the level of phage adsorption was measured, and found to be 18% of the control without membranes (Table 3). Membranes treated with mutanolysin (an N-acetylmuramidase) (27), which hydrolyzes the lactococcal cell wall and destroys the cell wall adsorption site for phage c2 (60), adsorbed 84% of the phage. A control reaction using the same concentration of mutanolysin and purified cell walls lowered phage adsorption from 81% to 28%. Additionally, when rhamnose was included in the adsorption mixture, there was no decrease in adsorption of the phage to the membranes. These results suggest that phage c2 adsorbs to a membrane protein or proteins, and this cannot be due to contaminating cell walls in the membrane preparation.

Although membranes from RMC2/4 do not inactivate phage c2, as do wild type membranes (60), it was not known if they adsorb phage c2. Therefore, radiolabeled phage c2 was mixed with an equal amount (as compared to the wild type membranes used in the experiment described above) of membranes from RMC2/4. The results (Table 3) show that RMC2/4 membranes did not adsorb or inactivate phage. These results demonstrate that PIP is responsible for the adsorption of phage c2 to wild type membranes.

Table 3

Adsorption of ^{35}S-labeled phage c2 to membranes

Membranes	Treatment	% phage adsorbed:[a]	% PFU inactivated:
C2	None (untreated)	89	99
	Proteinase K	18	2
	Mutanolysin	84	98
RMC2/4	None (untreated)	8	3
	Proteinase K	NA[b]	NA
	Mutanolysin	NA	NA

[a] determined by liquid scintillation counting.
[b] NA, not applicable.

Membrane adsorption is irreversible. The correlation between inactivation and adsorption suggested that an irreversible reaction between phage and membranes had occurred. The reversibility of the membrane adsorption was tested by mixing phage c2 with membranes, and analyzing samples after different times of incubation. Half of each sample at each time point was centrifuged to remove the membranes and

any adsorbed phage. The other half was not centrifuged, but immediately diluted 1000-fold. The supernatant from the centrifuged half-sample was diluted, and the phage titers for both diluted half-samples were determined. Care was taken to maintain the samples and dilutions at 4°C until they were plated, and avoid any temperature-dependent reaction (see below). The adsorption kinetics show a typical exponential rate (Fig. 5). In the first 1 min of incubation, 67% of the phage was adsorbed. However, only 36% was irreversibly bound. By 5 min, 88% was adsorbed, and 76% irreversibly so. After 15 min, 96% of the phage was irreversibly adsorbed. Neither increasing the time of incubation after dilution from 15 min to 1 h, nor reducing the centrifugation time (from 4 min to 30 sec) while increasing the force of the centrifugation (from 12,000 to 150,000 X g) had any effect on the results. This shows that phage adsorption to the membranes is initially reversible, but within 15 min becomes irreversible.

Ejection of phage DNA is a temperature dependent process. The irreversibility of phage adsorption to the plasma membrane suggested that either the association constant was high, or a subsequent, irreversible step in the infection process had occurred, making desorption of an infectious phage impossible. To investigate these possibilities, [^{3}H]thymidine-labeled phage c2 was prepared and mixed with wild-type membranes. During a 1 h incubation at 4°C, the amount of ^{3}H associated with the membranes increased rapidly to about 50% within the first 5 min, and asymptotically approached 80% adsorption by 1 h (data not

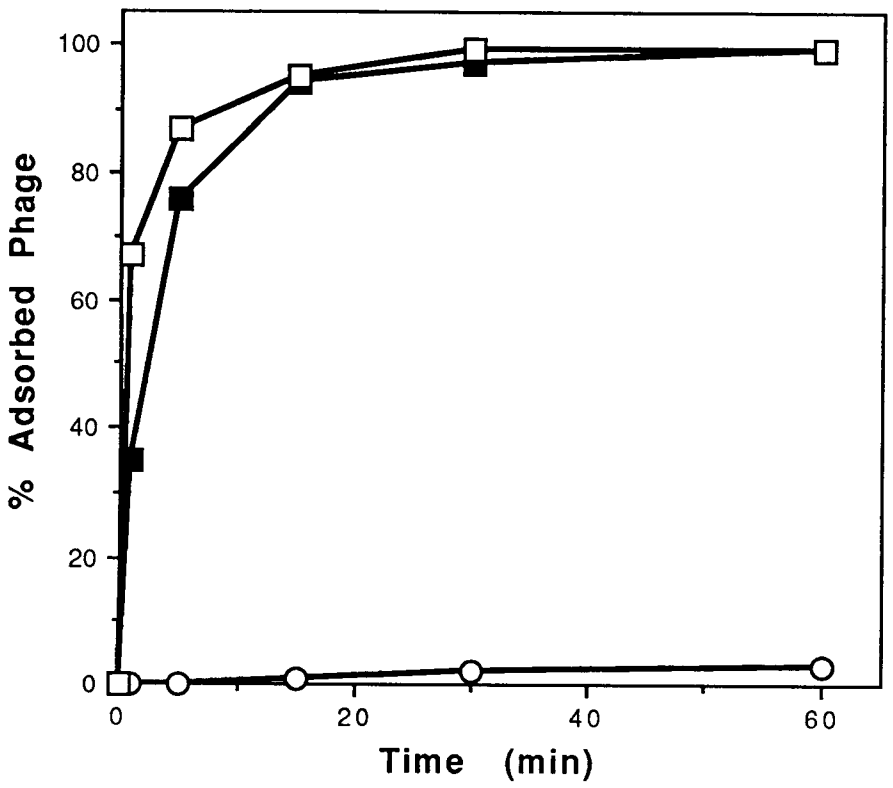

Fig. 5. **Irreversible binding of phage to membranes.** Phage c2 was mixed with wild-type membranes and $CaCl_2$. The mixture was incubated at 4°C for 1 hour. Aliquots were removed at the indicated times, and analyzed for reversibly and irreversibly adsorbed PFU. Total (reversible + irreversible) adsorbed PFU (□), irreversibly adsorbed PFU (■), and phage without membranes (O) were measured. Each point represents the average of 2-3 experiments. Each experimental value was done in duplicate.

shown). However, when the incubation temperature was 30°C, the results were entirely different (Fig. 6). The amount of membrane associated ^{3}H steadily decreased from 45% at 5 min, to 26% at 1 h. In a similar reaction that contained ^{35}[S]methionine-labeled phage, the ^{35}S associated with the membranes increased with time. During the 30°C incubation, the amount of PFU not associated with the membranes decreased to near 0% by 1 h. The data suggest that phage DNA was ejected from the adsorbed phage particles while the phage proteins remained associated with the membrane.

The kinetics of the apparent temperature-dependent release of the phage DNA from adsorbed phage particles was investigated further. ^{3}[H]thymidine- and ^{35}[S]methionine-labeled phage preparations were incubated separately at 4°C with purified membranes. After 30 min, the reactions were rapidly warmed to 30°C. At different time points, aliquots were centrifuged, and analyzed for membrane-associated ^{3}H and ^{35}S, and for PFU not associated with membranes. The results (Fig. 7) show that at 4°C both ^{3}H- and ^{35}S-labeled phage c2 rapidly associated with the membranes in a nearly identical manner. Adsorption of both ^{3}H- and ^{35}S-labeled phage c2 increased with time at 4°C, asymptotically approaching maximal adsorption (80%) by about 30 min. Upon shifting to 30°C, about 20% of the phage DNA dissociated from the membranes within 1 min, whereas the phage proteins remained adsorbed. Following the initial, rapid release of DNA, an additional 20% of the phage DNA dissociated during the ensuing 60 min, while all of the phage proteins remained adsorbed. In both reactions, the PFU were similarly inactivated

and were not released from the membranes upon shifting to 30°C. These results suggest that at 30°C, but not at 4°C, membrane-adsorbed phage eject their DNA from the phage particle.

The tritiated material that dissociated from the membranes was large molecular weight DNA that was exposed to the aqueous solution. This was evident by adding DNAse and phosphodiesterase I to the membrane-dissociated material (Fig. 8). The DNAse and phosphodiesterase I converted membrane-dissociated, ^{3}H-labeled material from 84% trichloroacetic acid (TCA) precipitable to 59% TCA soluble. The ^{3}H-labeled material in intact phage remained 97% TCA precipitable after an identical treatment with the enzymes. Increasing the concentration of the two enzymes hastened the degradation of the ^{3}H-labeled, membrane dissociated material to a final value of 88% TCA soluble, but had little effect on intact phage. These results suggest that the ^{3}H-labeled material is large molecular weight phage DNA, and that the phage DNA is released from the phage particle into the aqueous medium upon interaction with the plasma membrane at 30°C.

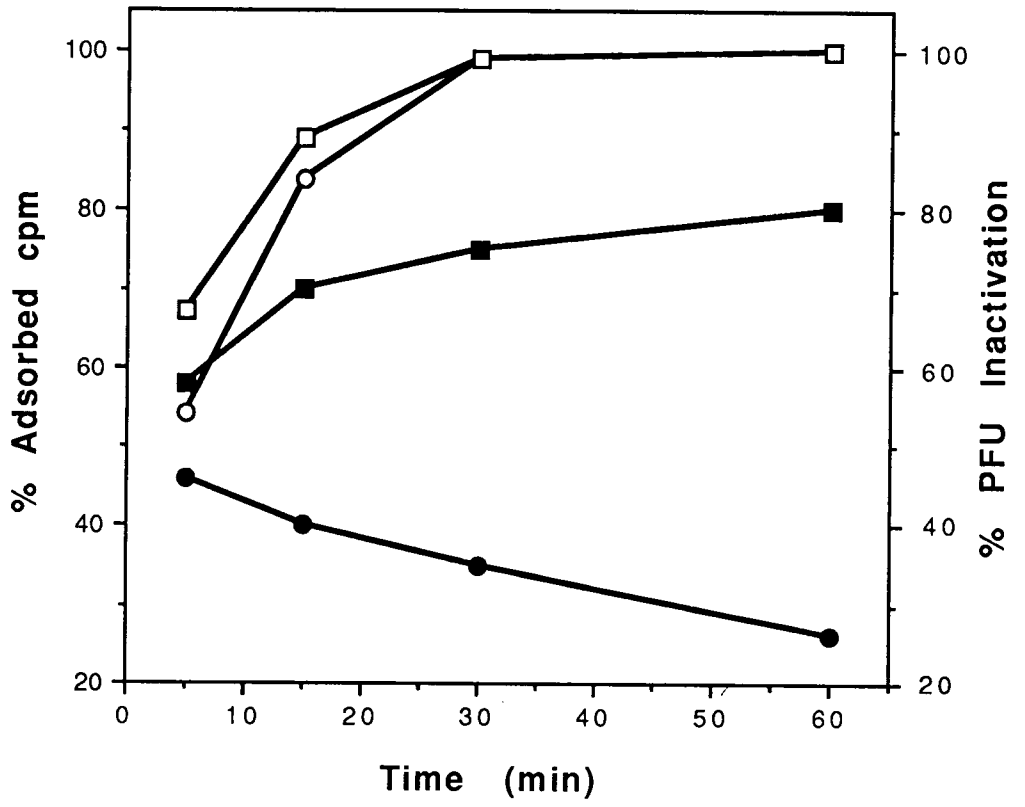

Fig. 6. **Adsorption of phage to membranes at 30°C.** [^{3}H]thymidine- and [^{35}S]methionine-labeled phage c2 were mixed separately with wild-type membranes and $CaCl_2$, and incubated at 30°C. The membrane-adsorbed radioactivity, ^{3}H (●) and ^{35}S (■), was determined at the indicated times. The % PFU inactivation was also determined at each time point for the reactions containing [^{3}H]thymidine- (○) and [^{35}S]methionine-labeled (□) phage. Each point represents the average of 2-3 experiments. Each experimental value was done in duplicate.

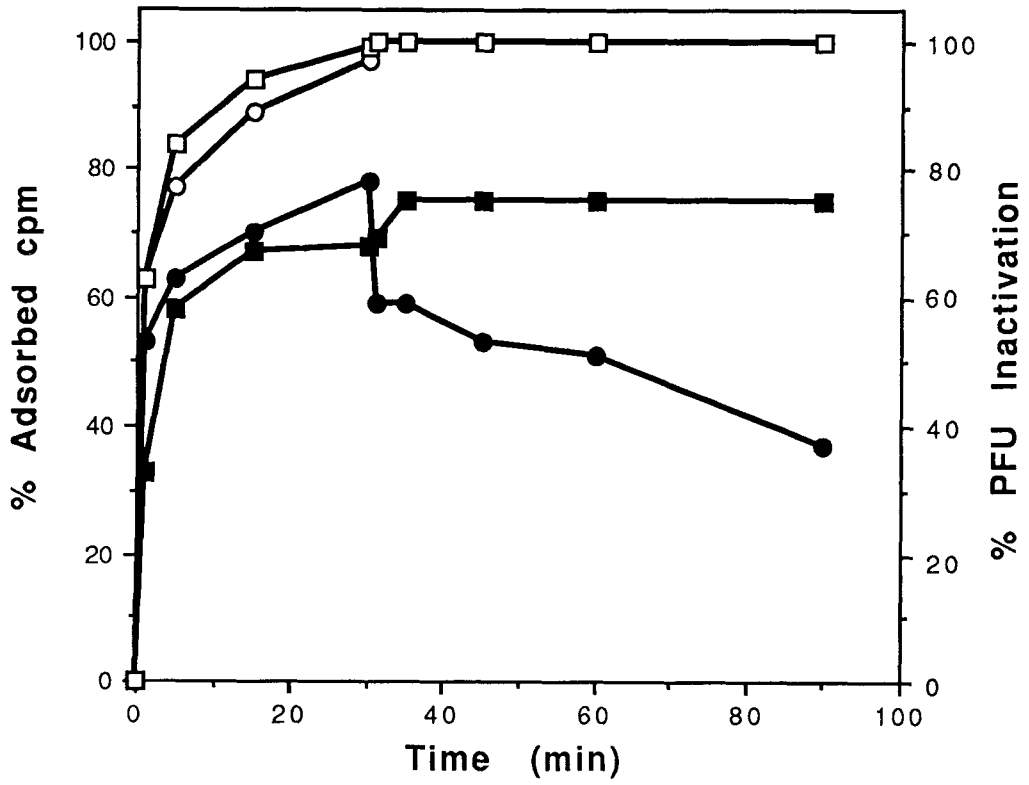

Fig. 7. **Effects of temperature shift from 4°C to 30°C on membrane-adsorbed phage.** [^{3}H]thymidine- and [^{35}S]methionine-labeled phage c2 were mixed separately with wild-type membranes and $CaCl_2$. The mixture was incubated at 4°C for 30 minutes followed by a temperature shift to 30°C, and an additional 1 h incubation. Aliquots were removed at the indicated times, and the % of membrane adsorbed radioactivity, ^{3}H (●) and ^{35}S (■), was determined. The % PFU inactivation was also determined at each time point for the reactions containing [^{3}H]thymidine (○) and [^{35}S]methionine-labeled (□) phage. Each point represents the

average of 2-3 experiments. Each experimental value was done in duplicate.

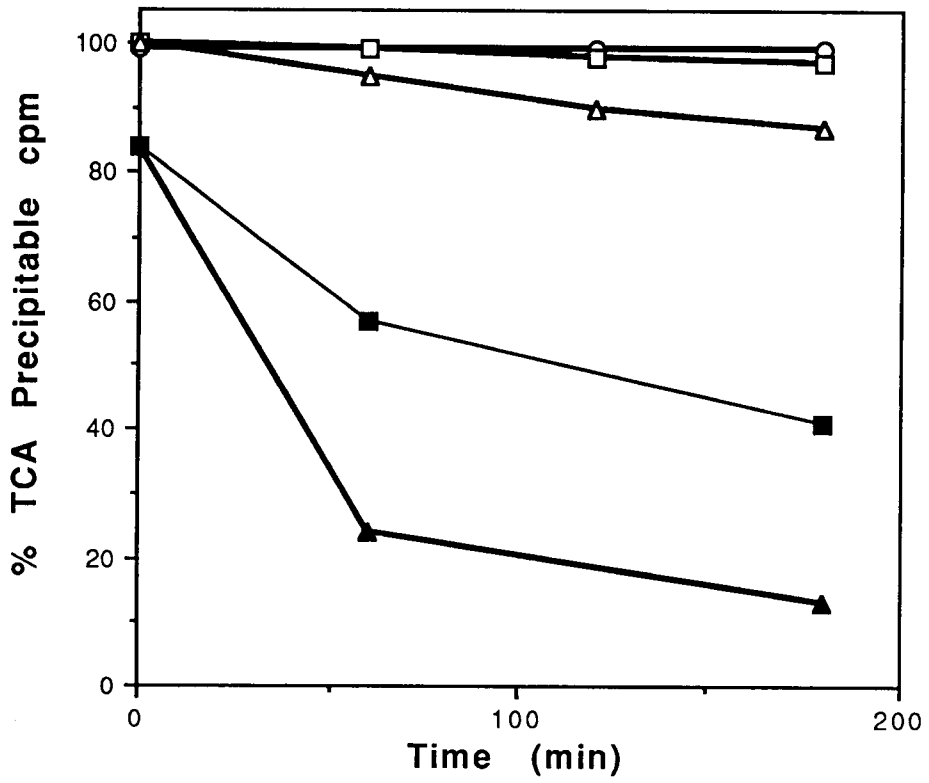

Fig. 8. **Nuclease degradation of ejected phage DNA.** [^{3}H]thymidine-labeled phage c2 was mixed with (■, ▲) and without (O, □, △) wild-type membranes and $CaCl_2$. The mixtures were incubated at 30°C for 1 h and centrifuged. The supernatants were removed and treated at 15°C with 10ug/ml phosphodiesterase I and 20ug/ml DNAse I (■, □), or 50ug/ml phosphodiesterase I and 100ug/ml DNAse I (▲, △) for 0, 1, and 3 h. The amount of TCA-soluble ^{3}H in each digest was determined. Each point represents the average of 2-3 experiments.

CONCLUSIONS

The results of the competitive inhibition between phages and different monosaccharides suggest that phages c2, ml3, kh, l, h, 5, and 13 adsorb to rhamnose moieties on the cell wall of *L. lactis* subsp. *lactis* C2 (Table 1). The lower amount of inhibition by glucose and galactose suggests that the adsorption sites may also include, or be influenced by, glucose and galactose moieties, but to a lesser extent than rhamnose. Therefore, the initial adsorption of these phages is similar to that of phage sk1 to the same strain (61), and phage kh to *L. lactis* subsp. *cremoris* KH (59). Rhamnose may be the cell wall determinant of most lactococcal phage adsorption sites. Similar results have been reported for the adsorption of phages to another lactic acid bacterium, *Lactobacillus casei* (63, 65). The adsorption to the lactococcal cell wall was found to be fully reversible for each of the phages tested.

Productive phage infections of *L. lactis* require that the phage adsorb to both the cell wall carbohydrates and the plasma membrane of the host. However, the initial site of phage attachment is the cell wall carbohydrates. This is evident because: 1) rhamnose, and to a lesser extent other monosaccharides that compose the cell wall of *L. lactis*, inhibit adsorption of phages to host cells (61, this work, Table 1), 2) lactococcal phages adsorb specifically to purified cell walls of their hosts (25, 59, 61), and 3) the addition of rhamnose to a growing culture inhibits phage infection (59, 61, this work, Fig. 4). We infer from this that the adsorption to the membranes occurs after adsorption to the cell wall.

All of the examined phages have a similar mechanism of infection that requires PIP, because none of the examined phages infected RMC2/4 (a strain that has a defective PIP). *In vitro* experiments (Table 2) with purified membranes confirmed the requirement of each phage for PIP. We previously reported (60) that phage kh was not inactivated by wild type C2 membranes *in vitro*, but additional experiments (Table 2) now indicate that this is so. In addition, we also determined that phage sk1 is inactivated *in vitro* by membranes from either wild type C2 or RMC2/4, suggesting that all phages do not require PIP for infection of strain C2.

A more detailed investigation of the mechanism of the interaction between PIP and phage c2 (and presumably the other phages) found that the amount of membrane-adsorbed phage correlated with the amount of inactivated phage (Table 3). Both adsorption and inactivation required PIP. Other experiments (Fig. 5) showed that the inactivation was apparently caused by an irreversible adsorption. The irreversible adsorption did not occur immediately upon mixing phage and membranes, and some reversibly adsorbed and infectious phages were evident during the first 5 min of incubation.

Figs. 5 and 7 show that irreversible phage adsorption occurs at 4°C not as a result of phage DNA ejection from the phage particle, but as a result of an interaction between the phage and the membrane that occurs before DNA ejection. A high thermodynamic association constant between phage and PIP is consistent with our results. However, other explanations are possible, such as a conformational change in the phage structure upon interacting with the membrane, or the ejection of a part of

the genome without full release from the capsid. Examples of the latter mechanisms are known to occur with coliphage ΦX174 (22, 37).

While PIP is clearly involved in phage adsorption to the membrane, it is not known if PIP plays a direct role in triggering the ejection of phage DNA. Perhaps other membrane and phage components are required for ejection, such as the 32 kDa membrane protein important for the inactivation of phage c2 by membranes of *L. lactis* (60), or the phospholipids. We speculate that the temperature effect may be related to the fluidity of the membrane, which in turn may influence the interaction between the phage and the host cell components required for phage DNA ejection and entry into the cell.

The phage DNA released at 30°C from the membrane-adsorbed phage was not associated with the membranes, and the release did not require ATP or the proton motive force. If the mechanism of phage DNA translocation across the plasma membrane is to be accurately reproduced *in vitro*, the ejected phage DNA should traverse the membrane. However, the DNA translocation step is known to require the proton motive force in intact cells (29, 62), and without it, the DNA is only partially released from the phage capsule. Moreover, *in vitro* interactions between coliphages and their receptors in the absence of the proton motive force result in the ejection of phage DNA free into the medium (16, 44, 46, 64). Experiments are underway to test the effects of energy on phage DNA translocation across the lactococcal plasma membrane.

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