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Lactococcal Bacteriophages Require a Host Cell Wall Carbohydrate and a Plasma Membrane Protein for Adsorption and Ejection of DNA

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The mechanism of the initial steps of bacteriophage infection in *Lactococcus lactis* subsp. *lactis* C2 was investigated by using phages c2, ml3, kh, l, h, 5, and 13. All seven phages adsorbed to the same sites on the host cell wall that are composed, in part, of rhamnose. This was suggested by rhamnose inhibition of phage adsorption to cells, competition between phage c2 and the other phages for adsorption to cells, and rhamnose inhibition of lysis of phage-inoculated cultures. The adsorption to the cell wall was found to be reversible upon dilution of the cell wall-adsorbed phage. In a reaction step that apparently follows adsorption to the cell wall, all seven phages adsorbed to a host membrane protein named PIP. This was indicated by the inability of all seven phages to infect a strain selected for resistance to phage c2 and known to have a defective PIP protein. All seven phages were inactivated in vitro by membranes from wild-type cells but not by membranes from the PIP-defective, phage c2-resistant strain. The mechanism of membrane inactivation was an irreversible adsorption of the phage to PIP, as indicated by adsorption of [^{35}S]methionine-labeled phage c2 to purified membranes from phage-sensitive cells but not to membranes from the resistant strain, elimination of adsorption by pretreatment of the membranes with proteinase K, and 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 host cell membrane protein PIP, which leads to ejection of the phage genome.

Lactococcus lactis is required for fermentation of milk during the manufacturing of many types of cheese. Because bacteriophage contamination of milk causes failures of the fermentation process, a considerable effort has been made to improve starter cultures of *L. lactis* by isolating bacteriophage-resistant strains. The defensive strategies employed by *L. lactis* against bacteriophage attack have generated much interest and include restriction and modification (2, 4, 8, 30), abortive infection (9, 13, 16, 20), blocking of phage adsorption (18, 31–33), loss of the cell surface phage receptor (7, 35), and alterations of plasma membrane components required for phage infection (36).

Research in our laboratory has focused on the interactions between phages and the lactococcal cell surface and mechanisms of phage resistance that affect them. Like other gram-positive bacteria, *L. lactis* adsorbs phage to the carbohydrates of its cell wall (11, 24, 33, 35, 37). A subsequent interaction between the phage or the phage DNA and the plasma membrane of the host cell is inferred, because the phage DNA must translocate across the plasma membrane for an infection to occur. In fact, interactions between the lactococcal plasma membrane and phages have been demonstrated by inactivating phage with plasma membranes in vitro (23, 36). However, the specific nature of the interactions has not been defined and they may involve irreversible adsorption, release of phage DNA from the phage particle, or both. In a related lactic acid bacterium, *Lactobacillus casei*, phage DNA penetration of the plasma membrane is dependent on cellular energy (40) and extracellular calcium (39) but little more is known about this reaction required for phage infection.

Recently, a gene (*pip*) from *L. lactis* that is required for phage infection and apparently codes for a membrane protein has been cloned and sequenced (6). Membranes from *pip* mutants do not inactivate phage in vitro, whereas membranes from wild-type cells do. The role of the *pip* gene product in phage infection is unknown.

Here we report that seven lactococcal phages adsorbed initially to the same cell wall carbohydrate receptors and subsequently to the same membrane protein encoded by the *pip* gene. Adsorption of phage to the *pip* gene product was required for temperature-dependent ejection of phage 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 (36) were grown and maintained on M17 medium (34), supplemented with 0.5% glucose (M17G), at 30°C. SLSD and SL1C media were used for growing cultures in the preparation of [^{3}H]thymidine-labeled phage c2 (26). Lactococcal bacteriophages were prepared from single plaques and plaque assayed as previously described (34). Phages were stored in M17 medium containing 20% glycerol at −70°C.

Monosaccharide inhibition of phage infection. Either α-L-(+)-rhamnose, α-D-(+)-glucose, D-(+)-galactose, or *N*-acetylglucosamine was added to an exponential-phase culture (optical density at 600 nm [OD_{600}], 0.1) to a final concentration of 0.5 M. $CaCl_2$ (15 mM) and phage (10^6 PFU/ml) were immediately added to the cultures. Control cultures for each sugar received only the saccharide, while another control received

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only phage and $CaCl_2$. Growth was determined by measuring OD_{600}. Readings were taken every 30 min 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 used (10^6 PFU/ml) (see Table 1 for a list of the phages used) in 100 mM bis(2-hydroxyethyl) imino-tris(hydroxymethyl)methane-HCl (BisTris; pH 6.8)–20 mM $CaCl_2$. The mixtures were incubated at 0°C for 1 h. 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 h. The cells were a stationary-phase culture that was diluted in 100 mM BisTris (pH 6.8) to 10^8 CFU/ml (determined by plate count) and stored at −70°C. The mixtures were centrifuged at 14,000 × *g* for 5 min at 4°C, and the supernatant was assayed for plaques. 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. Percent inhibition by monosaccharides was determined by subtracting the percent adsorption in the presence of a monosaccharide from the percent adsorption with no added monosaccharide. Additional controls consisting of phage in the absence of cells, with and without monosaccharides, were included to monitor phage viability throughout the reaction. Less than 2% of the control PFU were lost during a typical 1-h incubation.

Unlabeled phage inhibition of ^{35}S-labeled phage c2 adsorption. Nonradiolabeled phages (1.5×10^8 PFU) were mixed on ice with 3×10^7 PFU of ^{35}S-labeled phage c2 in 90 μl of 100 mM BisTris (pH 6.8)–10 mM $CaCl_2$. Washed cells (10^5 CFU in 10 μl of 100 mM BisTris [pH 6.8]) were added, and the mixture was incubated for 16 h at 4°C with gentle shaking. This amount of cells was determined from previous experiments to adsorb about 20% of the radiolabeled phage in the absence of competing, nonradiolabeled phage. The mixtures were centrifuged at 14,000 × *g* for 5 min at 4°C. The supernatant and pellet were separated and counted in a liquid scintillation counter. In a control reaction without cells, the background adsorption measured was 7.5% of the total counts added, and this was subtracted from the other samples.

Preparation of cell walls and phage adsorption assay. Cell walls were prepared as previously described (35), and total carbohydrate content was quantified (3). Phage (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 h, and half of the mixture was centrifuged at 14,000 × *g* for 5 min. Both the supernatant and the mixture that had not been centrifuged were diluted 10^{-3}, and the phage titer was determined. A control in which phage without cell walls was incubated under the same conditions was used to monitor for any loss of PFU unrelated to adsorption. Percent adsorption was calculated as stated above for monosaccharide inhibition.

Preparation of [^{35}S]methionine-labeled phage c2. Labeled phage was prepared by a modification of the procedure of Kim and Batt (12). *L. lactis* subsp. *lactis* C2 was grown in M17G medium at 30°C to an OD_{600} of 0.3. A 1-ml culture volume was transferred to a microcentrifuge tube containing 0.5 mCi of [^{35}S]methionine. $CaCl_2$ (10 mM) and phage c2 (multiplicity of infection, 5) were immediately added to the culture, which was allowed to lyse at 30°C for 2 h. The lysate was centrifuged at 14,000 × *g* for 5 min at 4°C to remove cell debris. Phage in the supernatant was purified by polyethylene glycol precipitation and CsCl gradient centrifugation (29) with a 1.4-g/ml continuous gradient. The gradient was fractionated into 250-μl aliquots. Each fraction was titrated for phage and counted in a liquid scintillation counter. The fraction with the highest titer and radioactivity was dialyzed in buffer containing 10 mM BisTris (pH 6.8), 10 mM $MgSO_4$, and 20% glycerol. Phage was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (22) and fluorography (17). Major bands were seen at both 169 and 85 kDa. Several other, minor bands were also present. The positions of all of the bands were similar to those previously reported (25).

Preparation of cell membranes and adsorption of phage. Cytoplasmic membranes were prepared as previously described (36). 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 and 1.5 M sucrose in 100 mM BisTris [pH 6.8]) by centrifugation in an SW 41 rotor (Beckman Instruments) at 160,000 × *g* for 3 h. Membrane total phosphate contents were quantified (1).

[^{35}S]methionine-labeled phage c2 (10^7 PFU/ml and 10^4 cpm/ml) was mixed at 4°C with either wild-type strain C2 membranes (1.12 mg of phospholipid per ml) or an equal amount of membranes from phage-resistant mutant RMC2/4 in 100 mM BisTris (pH 6.8). The mixture was diluted 1:1 with M17G plus 20 mM $CaCl_2$, agitated at 4°C for 1 h, and centrifuged at 14,000 × *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 to calculate the percent phage adsorption. Percent inactivation (see Table 4) 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.

The specific activity of the membranes was determined by mixing 2×10^3 to 1.5×10^8 PFU of phage with 0.12 μg of membranes in 100 μl of 100 mM BisTris (pH 6.8). The mixtures were incubated for 16 h at 4°C with shaking and centrifuged at 14,000 × *g* for 5 min at 4°C. Further incubation did not increase the amount of phage inactivation. The phage titer of the supernatant was determined by plaque assay, and the amount of bound phage was calculated by subtracting the titer after incubation from that of a control reaction without membranes.

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 the phage titer was determined. In addition, half of each aliquot was centrifuged at 12,000 × *g* for 5 min at 4°C and the phage titer of the supernatant was determined. In separate experiments, the length of time between dilution and plating was varied from 5 min to 1 h, but this was found to have no effect. Because the time it took to precipitate 99% of our membranes under our assay conditions was 5 min, it was important to determine if a significant amount of inactivation occurred during centrifugation. In separate experiments, the time of centrifugation was shortened to 0.5 min and the force was increased to 150,000 × *g* at 4°C in an air-driven ultracentrifuge (Airfuge; Beckman Instruments). The difference in centrifugation time had no effect on the results.

Membrane treatment with mutanolysin and proteinase K. Membranes were treated as previously described (36), except that the concentration of mutanolysin (Sigma) was 40 U/ml and that of proteinase K (Boehringer Mannheim) was 0.5 mg/ml. The treated membranes (0.12 μg) were mixed with [^{35}S]methionine-labeled phage c2 (10^6 PFU and 10^3 cpm) in 50

μl of 100 mM BisTris (pH 6.8) and then immediately diluted with 50 μl of M17G plus 20 mM $CaCl_2$. The mixture was agitated at 4°C for 1 h and then centrifuged at 14,000 × *g* for 5 min. 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 previously described (26), except that 8 mCi of [^{3}H]thymidine per ml was used. After addition of phage (10^8 PFU/ml; multiplicity of infection, 2), the medium was supplemented with 3.5 mCi of [^{3}H]thymidine per ml. The phage was further purified as described above for the ^{35}S-labeled phage.

[^{3}H]thymidine-labeled phage c2 adsorption to membranes and DNA ejection. [^{3}H]thymidine-labeled phage c2 (10^8 PFU/ml and 1.5 × 10^4 cpm/ml) was mixed with wild-type membranes (1.12 mg/ml) in 100 mM BisTris (pH 6.8) and diluted 1:1 with M17G plus 20 mM $CaCl_2$. The mixture was agitated for 30 min at 4°C, and aliquots were taken at 1, 5, 15, and 30 min. The reaction tube was transferred to a 30°C shaker for 60 min, and aliquots were taken at 1, 5, 15, 30, and 60 min after the temperature shift. The aliquots were centrifuged at 14,000 × *g* for 5 min at 4°C, and the pellet and supernatant were quantified separately in a scintillation counter. The supernatants were also titrated for phage 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 (19). 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 μg of snake venom phosphodiesterase I (Sigma Chemical Co.) per ml plus 0, 20, or 100 μg of bovine pancreatic DNase I (Sigma Chemical Co.) per ml and 20 mM $MgCl_2$ for 3 h at 15°C. At 0, 1, and 3 h, the treated aliquots were precipitated with 10% trichloroacetic acid (TCA) for 1 h at 0°C. The TCA precipitations were then centrifuged at 14,000 × *g* for 10 min 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

Initial absorption of phage is to the cell wall carbohydrate. To show that phage c2 and six other phages (ml3, kh, l, h, 13, and 5) that infect *L. lactis* subsp. *lactis* C2 adsorb to the carbohydrates of the cell wall in a manner similar to other previously characterized phages and hosts, a competitive inhibition assay was employed. Monosaccharides that compose the cell wall of this strain (35) were mixed with cells and phage, and the amount of adsorption was determined (Table 1). Rhamnose inhibited adsorption of all seven phages from 64 to 85%. Glucose, galactose, and *N*-acetylglucosamine inhibited the adsorption of all of the phages to lesser extents than rhamnose. These results are consistent with the suggestion that the cell wall, and specifically the rhamnose moieties, acts as an adsorption site for the seven phages tested. This is similar to the adsorption of other lactococcal phages (11, 35, 37).

To ascertain further the role of the cell wall carbohydrates in phage adsorption, different monosaccharides were added to exponential-phase cultures immediately before their infection with phage. Growth was determined by measuring OD_{600}. For phages c2 and kh, rhamnose significantly delayed or prevented lysis for at least 2 h (Fig. 1). Glucose, galactose, or *N*-acetylglucosamine did not delay lysis, and addition of sugars had no effect on the growth of uninfected cultures (data not shown). Nearly identical results were obtained with 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.

TABLE 1. Competitive inhibition of phage adsorption by monosaccharides[a]

Phage	Mean % inhibition ± SD of phage adsorption by:			
	Rhamnose	Glucose	Galactose	*N*-Acetylglucosamine
c2	70 ± 1.4	48 ± 18	34 ± 19	22 ± 2.8
ml3	68 ± 16	27 ± 26	16 ± 19	11 ± 3.5
kh	69 ± 1.4	40 ± 39	22 ± 19	14 ± 2.8
l	71 ± 7.6	65 ± 9.0	64 ± 26	14 ± 0
13	64 ± 9.5	48 ± 10	37 ± 14	8 ± 1.4
5	86 ± 3.6	62 ± 19	25 ± 13	7 ± 0
h	65 ± 2.9	51 ± 11	57 ± 8.5	2 ± 1.4

[a] Each monosaccharide was tested at a concentration of 500 mM.

Phages inhibit adsorption of phage c2. Because the adsorption sites for each of the phages tested appear to have the same characteristics, it was important to determine if the sites are identical. Each phage was tested for the ability to inhibit adsorption of ^{35}S-labeled phage c2. A fivefold excess of each of the seven unlabeled phages was mixed separately with a constant amount of radiolabeled phage c2. The phage mixtures were incubated with a limiting amount of cells. Following incubation, the cell-adsorbed material was isolated by centrifugation and the amount of radiolabel in the pellet and

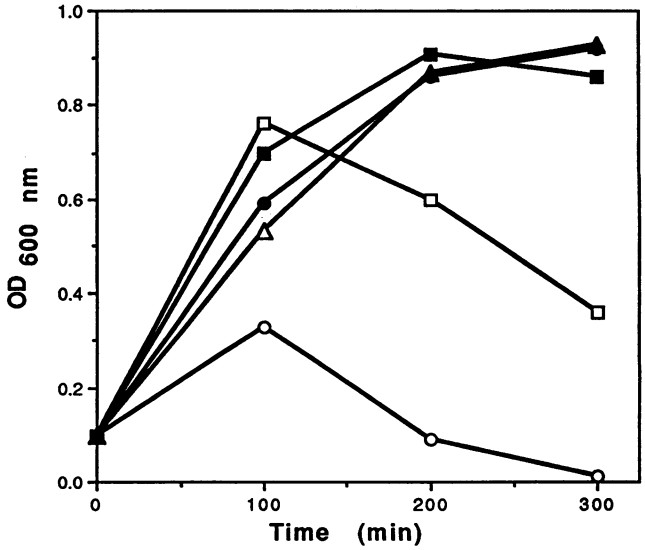

FIG. 1. Effects of L-(+)-rhamnose on the growth of phage-infected liquid cultures. Mid-exponential-phase cultures of *L. lactis* subsp. *lactis* C2 supplemented with $CaCl_2$ were mixed with 0.5 M rhamnose (△), phage c2 and rhamnose (●), phage c2 (○), phage kh and rhamnose (■), and phage kh (□). Growth was determined by measuring OD_{600} for 5 h. Each point represents the average of two or three experiments.

TABLE 2. Inhibition of adsorption of ^{35}S-labeled phage c2 to cells by unlabeled phages

Unlabeled phage[a]	Mean % inhibition ± SD of ^{35}S-labeled phage c2 adsorption
None	0[b]
c2	100 ± 0
ml3	104 ± 19
kh	102 ± 4
l	104 ± 19
h	66 ± 1
13	53 ± 4
5	98 ± 11

[a] A fivefold excess of unlabeled phage was added.
[b] Each percent inhibition value was calculated as a percent reduction in adsorption from the sample without added unlabeled phage.

supernatant was determined. The results (Table 2) show that each of the unlabeled phages, except phages h and 13, which were not as inhibitory, nearly eliminated phage c2 adsorption. This suggests that all of the phages tested, except perhaps phages h and 13, adsorb to the same sites.

Initial adsorption to the cell wall is a reversible process. Each of the seven phages was mixed separately with purified cell walls to determine whether adsorption was irreversible and caused inactivation of the phages. Initially, 85 to 98% of each phage was adsorbed to the cell walls at 4°C. The adsorbed phage-cell wall suspensions were then diluted and titrated for phage. For phages c2, ml3, kh, l, h, 13, and 5, the percentages of reversible adsorption were 97, 99, 88, 100, 98, 81, and 99%, respectively.

To test if the incubation temperature had an effect on the reversibility of adsorption, a suspension of phage c2-adsorbed cell wall was warmed from 4 to 30°C before being diluted and titrated for phage. The result was that 103% of the phage was reversibly adsorbed. These results show that adsorption to the cell wall is a reversible process that does not inactivate these phages and, at least for phage c2, is not affected by temperature in the range from 4 to 30°C.

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 (36). 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 phage-resistant mutant RMC2/4. To test if phages kh, ml3, l, h, 13, and 5 have the same requirement as phage c2 for infection, first the efficiency of plating of each phage on RMC2/4 was determined. For each phage, high titers ($>10^{10}$ PFU/ml) were grown on wild-type strain C2 but no plaques were formed on RMC2/4, indicating an efficiency of plating of $<10^{-9}$ for each. Second, purified membranes from the wild-type and strain RMC2/4 were mixed and incubated with the seven different phages. Wild-type membranes inactivated each phage, whereas RMC2/4 membranes did not (Table 3). These results suggest that for infection, all of the seven phages tested have a requirement for the same host cell membrane component, which is apparently defective in RMC2/4 and which we named PIP (an acronym for phage infection protein).

Interaction of phage with the plasma membrane involves adsorption to a membrane protein. To determine whether the inactivation involved 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 (87%) of the radiolabeled phage was found in the membrane pellet (Table 4). A corresponding amount of PFU (98%) was also adsorbed. The small difference between adsorbed radiolabeled phage and adsorbed PFU is probably the amount of noninfectious (damaged) phage particles in the phage preparation (28) which could not adsorb to the membrane protein. This shows that membranes adsorb phage.

TABLE 3. Inactivation of phages by wild-type (C2) and phage-resistant (RMC2/4) membranes

Phage	Mean % ± SD of total PFU inactivated by:	
	C2 membranes[a]	RMC2/4 membranes[a]
c2	90 ± 4.2	10 ± 8.5
ml3	86 ± 6.4	4 ± 3.5
kh	79 ± 7.6	8 ± 2.1
l	70 ± 1.4	14 ± 2.1
13	81 ± 0.0	14 ± 2.8
5	82 ± 2.1	14 ± 14
h	72 ± 16	10 ± 15

[a] The membrane concentration used was 1.12 mg of phospholipid per ml.

The composition of the membrane adsorption site was investigated by treating the membranes with proteinase K. After inactivation of the proteinase K with phenylmethylsulfonyl fluoride, the level of phage adsorption was measured and found to be 18% of that of the control without membranes (Table 4). Membranes treated with mutanolysin (an *N*-acetylmuramidase; 14), which hydrolyzes the lactococcal cell wall and destroys the cell wall adsorption site for phage c2 (36), adsorbed 84% of the phage. A control reaction using the same concentration of mutanolysin and purified cell walls lowered phage adsorption to cell walls 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 (36), it was not known if they adsorb phage c2. Therefore, radiolabeled phage c2 was mixed with an equal amount (compared to the wild-type membranes used in the experiment described above) of membranes from RMC2/4. The results (Table 4) 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 4. Adsorption of ^{35}S-labeled phage c2 to plasma membranes in vitro

Membrane source	Treatment	Mean % ± SD of:	
		Phage adsorbed[a]	PFU inactivated
C2	None	87 ± 3.6	98 ± 2.0
	Proteinase K	18 ± 4.2	12 ± 13
	Mutanolysin	84 ± 0	98 ± 0.7
RMC2/4	None	11 ± 4.2	8 ± 5.0
	Proteinase K	ND[b]	ND
	Mutanolysin	ND	ND

[a] Determined by liquid scintillation counting.
[b] ND, not determined.

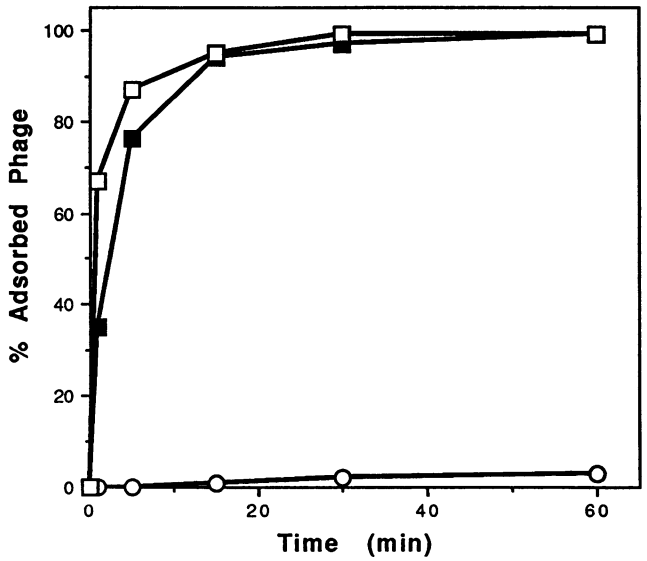

FIG. 2. Irreversible adsorption of phage to membranes. Phage c2 was mixed with wild-type membranes and $CaCl_2$. The mixture was incubated at 4°C for 1 h. Aliquots were removed at the times indicated and analyzed for reversibly and irreversibly adsorbed PFU. Total (reversibly plus irreversibly) adsorbed PFU (□), irreversibly adsorbed PFU (■), and phage without membranes (○) were measured. Each point represents the average of two experiments. Each experiment was done in duplicate.

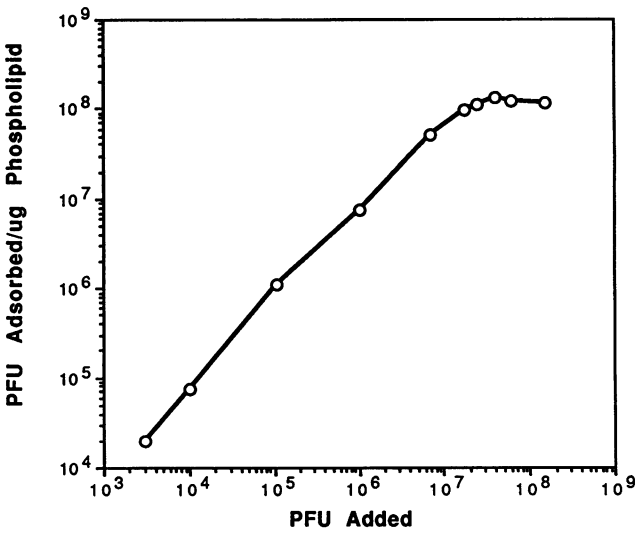

FIG. 3. Saturation of membrane adsorption sites. Various concentrations of phage c2 were mixed with wild-type membranes. The mixtures were incubated at 4°C for 1 h and centrifuged to remove the membrane-adsorbed phage. The titer of the supernatants were determined before and after incubation, and the amounts of adsorbed phage (○) were calculated by subtraction. Each point is the average of two experiments. Each experiment was done in duplicate.

Membrane adsorption is irreversible. The correlation between inactivation and adsorption suggested that an irreversible reaction between phage and membranes had occurred. The reversibility of 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 1,000-fold. The supernatant from the centrifuged half sample was diluted, and the phage titers of 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 reactions (see below). The adsorption kinetics showed a typical exponential rate (Fig. 2). Within the first minute of incubation, more than half of the phage was adsorbed and about half of the adsorbed phage was irreversibly bound. By 5 min, more of the phage was adsorbed but the difference between the total and irreversibly adsorbed phage was smaller than at 1 min. After 15 min, nearly all of the phage was irreversibly adsorbed. This pattern of adsorption, in which the difference between the total and irreversibly bound phage was greatest at 1 min and essentially zero by 15 min, was reproducible, despite the fact that the absolute values at the 1-min time point differed by as much as 40% between experiments. Neither increasing the time of incubation after dilution from 15 min to 1 h nor reducing the centrifugation time (from 4 min to 30 s) while increasing the force of the centrifugation (from 12,000 to 150,000 × *g*) had any effect on the results. This shows that phage adsorption to the membranes is initially reversible but becomes irreversible within 15 min.

Specific activity of membrane inactivation of phage. A constant amount of membrane was incubated with various amounts of phage c2, and the amount of phage that was inactivated was determined by plaque assay. The results (Fig. 3) show that the maximum amount of inactivation was 1.2×10^8 phage per μg of phospholipid.

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 1 h of 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 about 80% adsorption by 1 h (data not shown). However, when the incubation temperature was 30°C, the results were entirely different (Fig. 4). The amount of membrane-associated ^{3}H steadily decreased by about half between 5 min and 1 h. In a similar reaction mixture 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 nearly 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. 5) show that at 4°C both ^{3}H- and ^{35}S-labeled phage c2 rapidly associated with the membranes in nearly identical manners. 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 a shift to 30°C, about

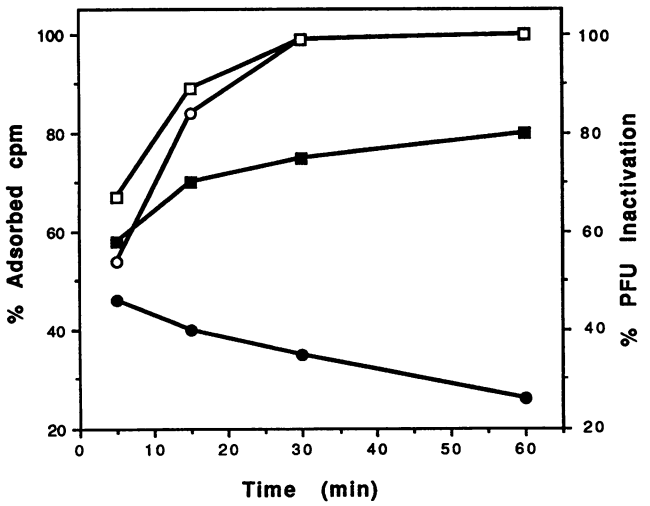

FIG. 4. Adsorption of phage to membranes at 30°C. [^{3}H]thymidine- and [^{35}S]methionine-labeled phage c2 were mixed separately with wild-type membranes and $CaCl_2$ and incubated at 30°C. The membrane-adsorbed radioactivity, ^{3}H (●) and ^{35}S (■), was determined at the times indicated. Percent 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 two experiments. Each experiment was done in duplicate.

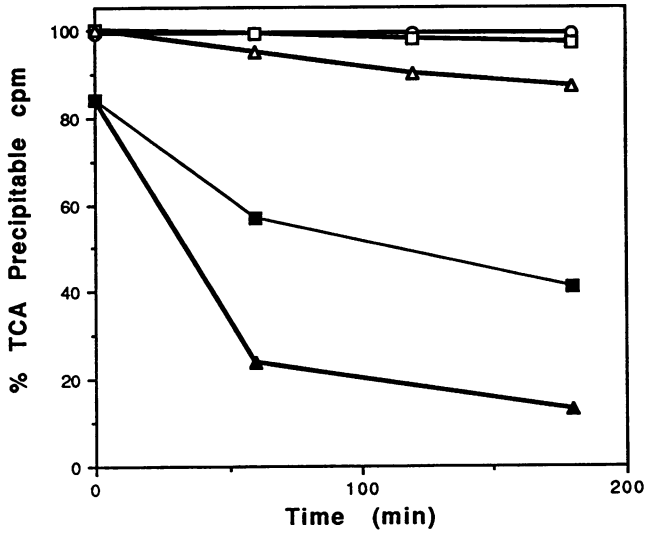

FIG. 6. Nuclease degradation of DNA released from the membrane-adsorbed phage. [^{3}H]thymidine-labeled phage c2 was mixed with (■, ▲) and without (○, □, △) wild-type membranes and $CaCl_2$. The mixtures were incubated at 30°C for 1 h and centrifuged. The supernatants were removed and treated at 15°C with 10 μg of phosphodiesterase I per ml and 20 μg of DNase I per ml (■, □) or 50 μg of phosphodiesterase I per ml and 100 μg of DNase I per ml (▲, △) for 0, 1, and 3 h. The amount of TCA-soluble ^{3}H in each digest was determined. Each point represents the average of two experiments.

one-fourth of the phage DNA dissociated from the membranes within 1 min whereas the phage proteins remained adsorbed. Following the initial, rapid release of DNA, one-fourth 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 the shift 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.

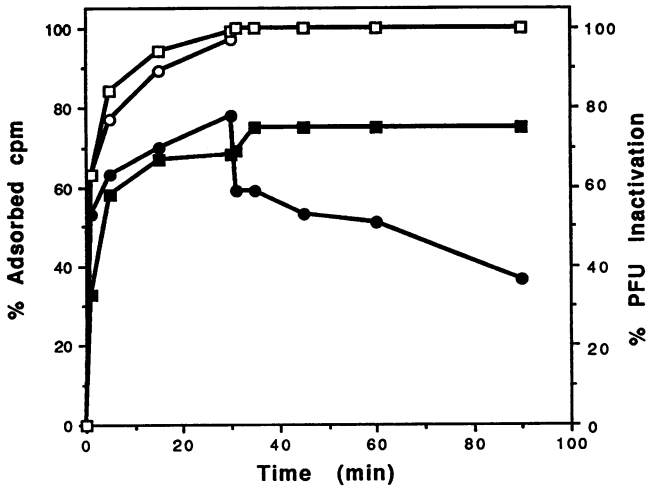

FIG. 5. Effects of a temperature shift from 4 to 30°C on membrane-adsorbed phage. [^{3}H]thymidine- and [^{35}S]methionine-labeled phage c2 were mixed separately with wild-type membranes and $CaCl_2$. The mixture was incubated at 4°C for 30 min, the temperature was shifted to 30°C, and the mixture was incubated for 1 h. Aliquots were removed at the times indicated, and the percentages of membrane-adsorbed radioactivity, ^{3}H (●) and ^{35}S (■), were determined. Percent PFU inactivation was also determined at each time point for the reaction mixtures containing [^{3}H]thymidine (○)- and [^{35}S]methionine (□)-labeled phage. Each point represents the average of two experiments. Each experiment was done in duplicate.

The tritiated material that dissociated from the membranes was high-molecular-weight DNA that was exposed to the aqueous solution. This was evident when DNase and phosphodiesterase I were added to the membrane-dissociated material (Fig. 6). The DNase and phosphodiesterase I converted about half of the membrane-dissociated, ^{3}H-labeled material from TCA precipitable to TCA soluble. The ^{3}H-labeled material in intact phage remained nearly all TCA precipitable after an identical treatment with the enzymes. Increasing the concentration of the two enzymes resulted in nearly total degradation of the ^{3}H-labeled, membrane dissociated material but had little effect on intact phage. These results suggest that the ^{3}H-labeled material is high-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.

DISCUSSION

The results indicate that phages requiring *pip* are not unusual in their adsorption mechanism but adsorb initially to the cell wall carbohydrates and not to the cell membrane. Competitive-inhibition experiments 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 also include, or are 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 (37) and that of phage kh to *L. lactis* subsp. *cremoris* KH (35). Similar results have been reported for the adsorption of phages to another lactic acid bacterium, *L. casei* (38, 42). The adsorption to the lactococcal cell wall was found to be fully reversible and temperature independent for each of the phages tested.

Unlabeled phages inhibited adsorption of radiolabeled phage c2 (Table 2), suggesting that all seven of the phages tested adsorb to the same cell wall receptor. However, phages h and 13 were not as inhibitory as the other phages and may not adsorb to the same sites as the other five. Because two of the phages did not inhibit phage c2 adsorption to the same extent as the other phages, this suggests that the inhibition by phages c2, ml3, kh, l, and 5 was competitive and not merely the result of steric interference.

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 (i) rhamnose and, to a lesser extent, other monosaccharides that compose the cell wall of *L. lactis* inhibit adsorption of phages to host cells (37; this work; Table 1), (ii) lactococcal phages adsorb specifically to purified cell walls of their hosts (11, 35, 37), and (iii) addition of rhamnose to a growing culture inhibits phage infection (35, 37; this work; Fig. 1). We infer from this that adsorption to the membranes occurs after adsorption to the cell wall.

All of the phages examined have similar mechanisms of infection that require PIP, because none of the examined phages infected RMC2/4 (a strain that has a defective PIP protein). In vitro experiments (Table 3) with purified membranes confirmed the requirement of each phage for PIP. The specific activity of the membrane inactivation of phage c2 was measured (Fig. 3) and found to be 1.2×10^8 phage per μg of phospholipid, which is about 2 orders of magnitude higher than that reported for membranes from another strain of *L. lactis* (24). We previously reported (36) that phage kh was not inactivated by wild-type C2 membranes in vitro, but additional experiments (Table 3) with more highly purified membranes (contaminating cell walls were removed) indicate that this is so.

Fig. 2 and 5 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 interaction with the membrane or 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 (10, 21).

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 (i) the 32-kDa membrane protein important for inactivation of phage c2 by membranes of *L. lactis* (36) or (ii) the phospholipids. We speculate that the temperature effect is 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 (15, 40), 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 ejection of phage DNA free into the medium (5, 27, 28, 41). Experiments are under way to test the effects of energy on phage DNA translocation across the lactococcal plasma membrane.

By identifying and understanding the different ways that *L. lactis* naturally avoids phage infection, molecular biological strategies may be employed to introduce these mechanisms into a variety of commercially important strains. Of all of the genes identified from *L. lactis* that are capable of conferring phage resistance, *pip* is the only chromosomal gene (the rest are from plasmids). The advantage to this is that *pip* mutants are stably maintained without constant selection pressure. Thus, in vitro-mutated copies of *pip* could be introduced into the chromosome of wild-type hosts by homologous recombination and used to replace wild-type *pip* without the need to maintain recombinant plasmids or selectable markers in a food grade organism. Such *pip*-replaced strains are currently being constructed.

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