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A Membrane Protein Is Required for Bacteriophage c2 Infection of *Lactococcus lactis* subsp. *lactis* C2

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Phage-resistant mutants, isolated from cultures of *Lactococcus lactis* subsp. *lactis* C2 infected with phage c2, did not form plaques but bound phage normally. The mutants were sensitive to another phage, sk1, although the number of plaques was reduced ~56% and the plaques were four times smaller. Binding to phage sk1 was reduced about 10%. Another group of phage-resistant mutants, isolated from cultures infected with phage sk1, bound normally to both phages 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 cells, 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 nondenaturing conditions, the phage-inactivating protein has an apparent M_r of ≈350,000. The protein has an apparent subunit size of 32 kDa, which suggests that it normally exists as a multimer with 10 to 12 subunits or in association with other membrane components. It is proposed that this protein is required for phage c2 infection.

In lactococci, carbohydrates of the exopolysaccharide may be commonly used as phage receptors (8). Previous studies from our laboratory have shown that the binding determinants of phages for *Lactococcus lactis* subsp. *cremoris* KH and subsp. *lactis* C2 include the rhamnose of the extracellular wall polysaccharides (16, 17). An exception to this was reported by Oram and Reiter (12), who found that membranes from *L. lactis* subsp. *lactis* ML3 inactivated phage m13. The phage-inactivating material appeared to be a protein, because trypsin digestion of the plasma membrane destroyed the phage-inactivating activity. In our previous study (16), treatment of the cell envelope (cytoplasmic membrane plus peptidoglycan and exopolysaccharides) from *L. lactis* subsp. *lactis* C2 with either mutanolysin or sodium dodecyl sulfate (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 (peptidoglycan and exopolysaccharides).

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 the presence of a protein and was used to purify and identify a protein with an apparent M_r of ≈350,000 and a subunit size of 32 kDa. We propose that this protein is required for phage c2 infection, apparently at a step subsequent to adsorption.

MATERIALS AND METHODS

Bacterial strains, phages, and medium. Bacteriophages c2 and sk1 and the bacterial host, *L. lactis* subsp. *lactis* C2, were grown and maintained on M17 medium at 30°C (15). Phage and bacterial stocks were prepared as previously described (15) 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 an A_{600} of 1 to 1.2. Cytoplasmic membranes were prepared by differential centrifugation of lysed, lysozyme-treated protoplasts, essentially as previously described (13). Briefly, the cells were pelleted by centrifugation (10,000 × *g*, 10 min, 4°C) and washed once in 150 ml of 0.1 M potassium phosphate (pH 7.0)–0.2 mM phenylmethylsulfonyl fluoride (PMSF). The cells were again pelleted by centrifugation and resuspended in 150 ml of 0.1 M potassium phosphate (pH 7.0)–10 mM $MgSO_4$. Lysozyme (0.75 g) was added, and the mixture was incubated at 30°C for 30 min. Potassium sulfate and PMSF were added to final concentrations of 0.15 M and 0.2 mM, respectively. After 5 min at 30°C, the mixture was diluted to twice its volume with 0.1 M potassium phosphate (pH 7.0), and RNase and DNase were added to final concentrations of 50 μg/ml each. The mixture was incubated 20 min at 30°C. A 0.1 M solution of K-EDTA (pH 7.2) was added to a final concentration of 15 mM. The mixture was incubated 10 min at 30°C, and then $MgSO_4$ was added with gentle stirring so that the concentration was increased to 20 mM. The mixture was centrifuged at 48,000 × *g* for 30 min at 4°C. The pellet, containing the membranes, cells, and debris, was resuspended in 75 ml of 50 mM potassium phosphate (pH 7.0)–10 mM $MgSO_4$. The mixture was centrifuged at 750 × *g* for 70 min at 4°C to remove the unbroken cells and debris. The supernatant was removed and centrifuged at 48,000 × *g* for 30 min at 4°C. The pellet, containing the membranes, was resuspended in 10 mM [bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane], pH 6.5.

Membranes (21 mg of protein) were solubilized on ice in a buffer consisting of 10 mM bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (pH 6.5), 1% (wt/vol) Triton

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X-100 (Sigma Chemical Co.), 10% (vol/vol) 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 × *g* for 60 min; 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 more than 90% of phage-inactivating activity.

Preparation of cell walls for gas chromatography. Cells were harvested at an A_{600} of 1.8 to 2 and suspended in 10 mM KH_2PO_4 (pH 6.8) to a final concentration of 150 mg/ml (wet weight). Cell walls (peptidoglycan and exopolysaccharides) were isolated as previously described (16). Briefly, the resuspended cells were mechanically disrupted by vigorous agitation with glass beads. The beads were removed by filtration through paper, and the whole cells and debris were removed by centrifugation at 1,400 × *g* for 5 min at 4°C. Cell walls were sedimented by centrifugation at 15,000 × *g* for 10 min at 4°C. Resuspended cell walls were treated with DNase and RNase (40 μg/ml [each]) and PMSF (0.2 mM). The cell wall preparation was extracted with hot (70°C) SDS (1 mg/ml) to remove the membrane. The cell walls were washed three times with water, collected by centrifugation, resuspended in water, frozen in liquid nitrogen, and stored at −70°C. 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_4OH by using 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 (5).

Gas chromatography and identification and quantitation of alditol acetates. The alditol acetate derivatives were separated on a 3% SP-2340 glass column (30.48 by 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 (14). Peak areas and retention times were determined by using a Hewlett-Packard model HW 3390A electronic integrator. The derivatized cell wall polymer saccharides separated by gas chromatography were quantified as previously described (16).

Isolation of resistant mutants. *L. lactis* subsp. *lactis* C2 cells (10^8 CFU) were mixed with 10^{10} PFU of phage c2 or 10^8 PFU of phage sk1 in M17 top agar (0.4%) plus 15 mM $CaCl_2$. 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 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 previously described (16). 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 (5×10^5 PFU/ml) in 10 mM bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (pH 6.5) at 25°C for 20 min. The mixtures were centrifuged (12,000 × *g*, 4 min), and titers of phage in the supernatant were assayed as previously described (15). 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 then multiplying by 100.

Our phage inactivation assay contained phage c2 (5×10^5 PFU/ml) in 10 mM [bis(2-hydroxyethyl)imino-tris(hydroxymethyl) methane] (pH 6.8) and an appropriate amount of sample to decrease the titer of the phage by 27 to 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 midpoint of the linear portion of the phage inactivation curve.

Treatments with mutanolysin and proteinase K. Membranes in a solution of 50 mM K_2PO_4 (pH 7.0) and 10 mM Mg_2SO_4 at concentrations which inactivated 90% of phage (5×10^5 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 16 h. The proteinase K-treated samples were further incubated with PMSF (0.2 mM) at 4°C for 30 min. The cell envelopes (membrane plus peptidoglycan and exopolysaccharides) were treated with mutanolysin as described previously (16).

Gel filtration chromatography. The solubilized membranes were applied to a Sephacryl S300 (Pharmacia LKB Biotechnology) column (1.6 by 60 cm) in a buffer consisting of 10 mM bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (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 (1.5 ml each) were collected and assayed for phage-inactivating activity and total protein (3; ovalbumin standard). Fractions with the highest phage-inactivating activity were combined.

Ion-exchange chromatography. A DEAE-cellulose (DE52; Whatman Biosystems Ltd., Kent, England) column (0.8 by 6 cm) was equilibrated with an equilibration buffer consisting of 10 mM bis(2-hydroxyethyl)imino-tris(hydroxymethyl) methane (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 NaCl step. Fractions (1 ml each) were collected, and aliquots were assayed for phage-inactivating activity and total protein.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (9). The gels were silver stained as previously described (2).

Phospholipid addition to column fractions. Attempts to increase the phage-inactivating activity of the DEAE-purified fractions were done as follows: acetone- and ether-extracted *Escherichia coli* phospholipids (50 μg) were lyophilized and then dissolved by adding 200 μl of the combined peak activity fractions from the DEAE column. The mixture was assayed for phage-inactivating activity as described above.

Materials. Mutanolysin, lysozyme (EC 3.2.1.17), bovine serum albumin, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), carbonic anhydrase (EC 4.2.1.1), soybean trypsin inhibitor, α-lactalbumin, thyroglobulin, apoferritin, β-amylase (EC 3.2.1.2), and ovalbumin were purchased from Sigma Chemical Company. Proteinase K (EC 3.4.21.14) was from Boehringer Mannheim Biochemicals. Total *E. coli* phospholipids were purchased from Avanti Polar Lipids, further purified by acetone and diethyl ether extraction in the presence of 2 mM 2-mercaptoethanol as previously described (7), and stored in chloroform under N_2 at −70°C.

TABLE 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	sk1	c2	sk1	c2	sk1
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 strains, phage c2-resistant mutants; RMSK1 strains, phage sk1-resistant mutants.
[b] Percentage of total PFU in the supernatant of a mixture of phage and cell walls after a 20-min incubation and centrifugation.
[c] Number of plaques divided by the number of plaques formed by the same amount of sample on wild-type strain C2 and then multiplied by 100.
[d] Percentage of total PFU after incubation with membranes.
[e] Plaque diameters reduced by 50%.

TABLE 2. Carbohydrates present in acid-hydrolyzed cell wall preparations of wild type and phage-resistant mutants of *L. lactis* subsp. *lactis* C2 determined by gas chromatographic analysis of alditol acetate derivatives

Strain[a] (*n*)	Amt (μg/mg of cell wall) of carbohydrate[b] (mean ± SD)			
	Rha	Gal	Glc	GlcNAc
C2 (5)	234 ± 20	37.1 ± 2.2	66 ± 5.3	73.5 ± 6.2
RMC2/4 (3)	245 ± 40	40.9 ± 6.7	80.7 ± 18	90.5 ± 29
RMC2/8 (3)	255 ± 20	42.8 ± 7.4	76.0 ± 8.3	84.8 ± 8.8
RMC2/9 (3)	241 ± 8.4	40.6 ± 3.9	72.8 ± 3.1	86.0 ± 13
RMC2/13 (3)	255 ± 22	39.8 ± 7.0	73.4 ± 8.9	82.0 ± 7.1
RMSK1/1 (3)	251 ± 23	32.9 ± 2.6	63.9 ± 7.8	81.2 ± 14
RMSK1/2 (3)	262 ± 18	35.6 ± 1.8	68.6 ± 4.6	85.6 ± 4.9

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

RESULTS

Isolation and properties of phage-resistant mutants. Spontaneously occurring phage-resistant mutants of *L. lactis* subsp. *lactis* C2 were selected by infecting a mid-exponential-phase culture with phage c2 at a multiplicity of infection of 100. The frequency of the selected phenotype was 10^{-6}. 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 1 (RMC2/4, -2/8, -2/9, and -2/13). These phage c2-resistant mutants were challenged with another phage, sk1, which also infects this strain. There was a 54 to 58% reduction in the sensitivities to phage sk1 (Table 1) and a fourfold reduction in the plaque sizes.

None of the 50 randomly picked mutants had detectable defects in binding of whole cells to phage c2. To corroborate this, cell walls (we use this term to mean the peptidoglycan and exopolysaccharides) were isolated from 4 of the 50 mutants and tested for phage binding. All were found to bind phage c2 to the same extent as the phage-sensitive parental strain, although binding to phage sk1 was reduced by approximately 10% (Table 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 infecting a mid-exponential-phase culture with phage sk1 at a multiplicity of infection of 1. The frequency of occurrence of the phage sk1-resistant mutant was 10^{-6}. Of the 30 mutants that were randomly picked, all but three bound the same amount of phages c2 and sk1 as the phage-sensitive parental strain. Further analysis of the three binding-defective mutants will be presented elsewhere (17). All of the mutants that had no decrease in the amount of phage which could bind were tested for sensitivity to phages sk1 and c2 by plaque assay and were found to be totally insensitive to both phages. Two representative analyses are listed in Table 1 (RMSK1/1 and RMSK1/2). Cell walls were prepared from 3 of the 27 mutants which appeared to bind the phages normally and tested for binding to phage sk1 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 an *L. lactis* subsp. *cremoris* strain that we have characterized previously (16). It also shows that the loss of the phage receptor is not a common 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 carbohydrate components (Table 2). This confirmed that the insensitivity to phage sk1 and c2 infections is not related to any changes in the cell wall carbohydrate components, which contain the receptors for phage sk1 and c2 in this strain (16, 17). The mechanism of phage resistance in these cases apparently involves a step subsequent to the binding of the phage to the exopolysaccharide receptor. It is also important to note that the alditol acetate derivatives of neither glycerol nor ribitol were detected, although internal standards of the two were readily detected and resolved from the other monosaccharides. This suggests that this strain lacks teichoic acids.

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, 94% of the PFU were inactivated and no longer had the ability to infect sensitive cells (Table 1). The inactivation was specific for phage c2, as phages sk1, kh, and 18-16 were not inactivated by the parental membranes. Moreover, membranes from the phage-resistant mutants (selected from infections with phage c2) did not inactivate phage c2 (Table 1), sk1, kh, or 18-16 (data not shown). These results suggested that a membrane component of the parental strain, but not the mutant strains, irreversibly interacts with phage c2, resulting in phage inactivation.

To test whether 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). Incubation of the same membranes with mutanolysin, which hydrolyzes cell wall glycosidic bonds, did not inactivate the membrane. For 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 plus membrane) from the parental strain was similarly treated with mutanolysin. This destroyed the polysaccharide, which is the phage receptor (16), as indicated by a 76%

TABLE 3. Inactivation of phage c2 by membranes and cell envelope[a] treated with proteinase K and mutanolysin

Treatment	% of total PFU inactivated by:	
	Treated membranes	Treated envelopes
None (untreated)	92	94
Proteinase K	4	NA[b]
Mutanolysin	93	18

[a] Envelope preparations contain cell wall plus cytoplasmic membrane.
[b] NA, not applicable.

reduction in phage binding to the mutanolysin-treated cell envelopes compared with that of the untreated cell envelopes. These results suggest that a membrane protein inactivates the phage.

Identification and purification of phage-inactivating membrane protein. The purification of the phage-inactivating protein is summarized in Table 4. The amount of membrane material necessary to inactivate 50% of the 5×10^5 phage per ml in 20 min was defined as 1 U of activity. Membranes prepared from an exponential-phase culture of *L. lactis* subsp. *lactis* C2 had a specific activity of 205 U/mg of protein. To purify the phage-inactivating protein, membranes were solubilized in a solution consisting of 1% Triton X-100, 10% glycerol, 10 mM bis(2-hydroxyethyl)imino-tris (hydroxymethyl)methane (pH 6.5), and 1 mM DTT. The insoluble proteins were removed by centrifugation (120,000 $\times$ *g*, 1 h). More than 90% of phage-inactivating activity was retained in the supernatant. Because 48% of the total protein was not solubilized under these conditions, the specific activity after solubilization increased about twofold, to 384 U/mg of 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. 1). One broad peak of activity eluted from the column, centered at a position corresponding to an M_r of 350,000. The fractions with peak activity were pooled for further purification. The pooled fractions contained >90% of the eluted activity, which accounted for about 24% of the total activity applied to the column. The specific activity of the pooled fractions was 701 U/mg, an increase of about twofold from the applied material.

The pooled fractions from the gel filtration column were applied to a DEAE-cellulose column. After being washed with 2 volumes of equilibration buffer (see Materials and Methods), phage-inactivating inactivity was eluted with a three-step gradient of 0.15, 0.25, and 0.50 M NaCl in equilibration buffer. Most (>90%) of the phage-inactivating activity eluted in a single peak in the 0.25 M salt step (Fig. 2).

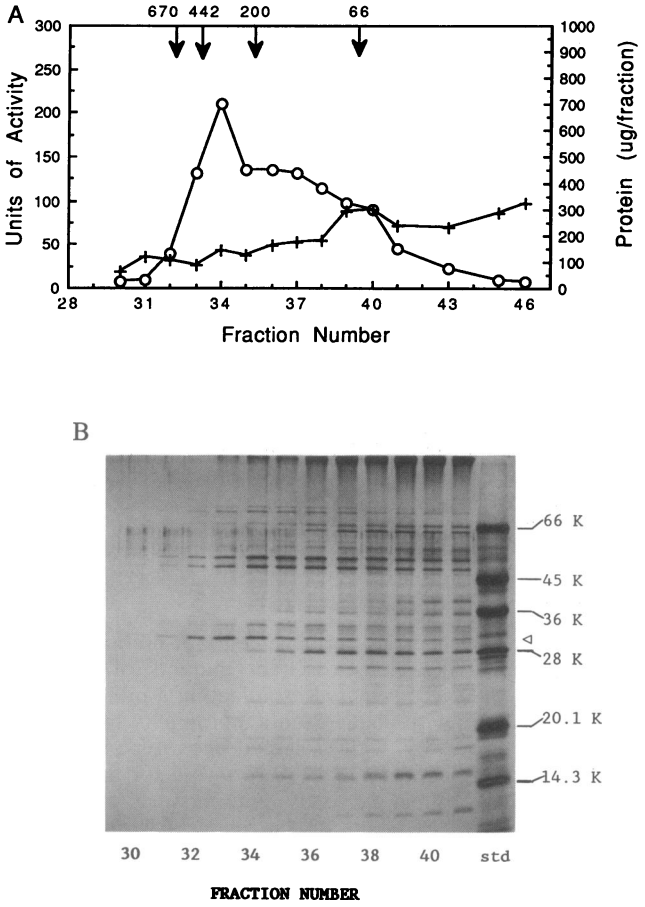

FIG. 1. (A) Sephacryl S300 chromatography of the Triton X-100-solubilized membrane preparation. Samples (7.5 mg each) of Triton X-100-soluble membrane components were applied to a column of Sephacryl S300 (1.6 by 60 cm) equilibrated in a buffer consisting of 10 mM bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (pH 6.5), 0.1% Triton X-100, 10% glycerol, and 1 mM DDT. Fractions (1.5 ml each) were collected and analyzed for phage-inactivating activity (○) and total protein (+). Molecular weight standards eluted in the positions indicated at the top of panel A: thyroglobulin (670,000 [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 S300 column. The gel was silver stained. The open arrowhead indicates the position of the 32-kDa protein which coelutes 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), ovalbumin (45K), glyceraldehyde-3-phosphate dehydrogenase (36K), carbonic anhydrase (29K), soybean trypsin inhibitor (20.1K), and α-lactalbumin (14.3K).

TABLE 4. Purification of the phage-inactivating protein isolated from *L. lactis* subsp. *lactis* C2

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield[a] (%)	Purification (fold)
Crude membrane	21	4,300	205	100	
Soluble membrane	7.5	2,880	384	67	2
Sephacryl S300	1.5	1,052	701	24	3
DE-52[b]	0.83	43	52	<1	<1

[a] Expressed in percent activity.
[b] Average of two values.

Usually this was the only detectable activity, although occasionally a small amount of activity eluted in the 0.15 M step. Unfortunately, about 96% of the activity was lost during this step, reducing the specific activity to 52 U/mg, or about 75% less than the crude membranes.

A number of attempts were made to reconstitute the activity to the pooled fractions from the DEAE column and to discover the reason for the loss of activity. (i) An equal volume from all fractions was mixed and assayed for activity. Another mixture contained just the peak protein-containing fractions from each step, including the initial wash.

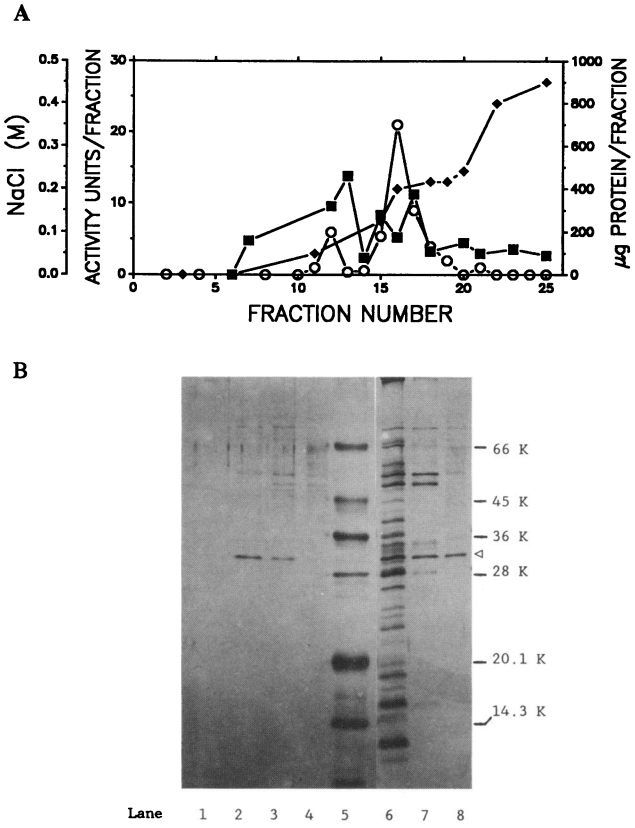

FIG. 2. (A) DEAE-cellulose chromatography of the phage-inactivating protein from the Sephacryl S300 column. The combined, peak activity fractions 32 to 36 (inclusive) from the Sephacryl column (1.5 mg of protein) were applied to a DEAE-cellulose column (0.8 by 6 cm) and eluted with a three-step NaCl gradient. Fractions were collected and analyzed for phage-inactivating activity (○), total protein (■), and conductivity (◆). (B) SDS-polyacrylamide gel electrophoresis and silver staining. Lanes 1 to 4 contain 15-μl samples of fractions 15 to 18, respectively, from the DEAE column shown in panel A. Lanes 6 to 8 contained pooled fractions from the Triton X-100 extraction, Sephacryl S300, and DEAE columns, respectively. The amounts of protein loaded in lanes 6 to 8 were 4, 1.5, and 1 μg, respectively. Lane 5 contains the same molecular weight standards used in Fig. 1. The open arrowhead indicates the 32-kDa protein band which coelutes with the phage-inactivating activity.

The total activity of the mixtures did not change. (ii) *E. coli* phospholipids were added to the peak fractions but had no effect on the activity. (iii) Solubilized membranes from the phage-resistant strain were mixed with the purified material, but this did not increase the activity. (iv) A sample of Triton X-100-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 desorbed from the resin and recovered in the supernatant after the resin was pelleted by centrifugation. Increasing the NaCl concentration from 0.25 to 0.5 M did not increase the activity in the unadsorbed fraction. (v) The crude membranes, Triton X-100-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 (Fig. 1B and 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. This suggests that the 32-kDa protein is required for phage c2 infection. Considering the size of the activity which eluted from the gel filtration column under nondenaturing conditions, the 32-kDa phage-inactivating protein is apparently associated with other components or itself in a multimeric complex.

DISCUSSION

All of the phage-resistant mutants isolated from an infection of *L. lactis* subsp. *lactis* C2 with phage c2 adsorbed phage normally, even though all were incapable of forming plaques (Table 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 number of plaques was ~56% less and the plaques were fourfold 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 infection with phage sk1. Almost all the mutant cells analyzed, with the exception of three, bound phages c2 and sk1 normally, but none formed plaques when challenged with either phage (Table 1). The three mutants with loss of adsorption to phage have changes in their exopolysaccharide composition (17).

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 carbohydrate components compared with the wild type (Table 2). This suggests that phage resistance was not due to mutations of the phage receptor on the cell wall but rather to a change in a cellular component required for phage infection at a step subsequent to adsorption.

Our results demonstrate that membranes from phage-sensitive cells inactivate phage c2 (Table 1). Proteinase K treatment of the membranes completely eliminated the ability of the membranes to inactivate the phage (Table 2), strongly suggesting that a protein is involved in the inactivation of phage. Although these data alone might suggest a nonspecific 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 associated with the membrane is involved in the inactivation of phage, because mutanolysin did not reduce the phage-inactivating activity of the membranes. However, mutanolysin has a restricted hydrolytic specificity, and we cannot rule out the possibility that small, hydrolytic fragments of the exopolysaccharide, glycoproteins, or membrane-bound lipoteichoic acids are involved in the phage-inactivating activity. We speculate that the adsorption of phage c2 to the cell surface may involve at least two steps, in a process similar to that of many well-characterized gram-negative bacteriophages (10). An initial, reversible binding to the cell wall polysaccharide may be followed by an irreversible step, as the phage interacts with the proteinaceous membrane component. In *E. coli*, the second, irreversible step that leads to phage inactivation is sometimes associated with the release of the phage DNA from the capsid (4, 6, 10).

Incubation of the membranes from *L. lactis* subsp. *lactis*

C2 did not inactivate phage sk1, yet membranes from strains that were completely resistant to phage sk1 were also incapable of inactivating phage c2. This suggests that phage c2 and sk1 require the same membrane protein for infection but that phage sk1 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 sk1 and phage sk1-resistant cells were completely resistant to phage c2. However, the mechanism of interaction between the phage-inactivating activity and both phages needs further study.

The phage-inactivating protein was purified from crude membranes by Triton X-100 extraction, gel filtration, and ion-exchange chromatography. The molecular mass of the phage-inactivating protein under nondenaturing 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 (11). The broad elution profile may suggest some heterogeneity of structure. This could result from the presence of attached lipids or carbohydrate or a loss of some of the components of such a large membrane complex during purification. 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, on the basis of 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 45%. The elution pattern of the 32-kDa protein is the only apparent protein which coincides with the phage-inactivating activity. Each of the other contaminating proteins are from side fractions of incompletely resolved peaks which did not coelute with the phage-inactivating activity. Attempts to reactivate 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 (11). 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), further attempts to maintain activity after solubilization are currently in progress.

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