The Adsorption of Phage to Group N Streptococci. The Specificity of Adsorption and the Location of Phage Receptor Substances in Cell-wall and Plasma-membrane Fractions

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SUMMARY

The specificities of adsorption and multiplication of phages attacking some strains of lactic streptococci (serological group N) were investigated. At multiplicities of infection (m.o.i.) of 0.1 to 1 p.f.u./coccus, the specificity of adsorption was similar to that of multiplication, but some strains also adsorbed one or more heterologous phages. At m.o.i. ~ 100 both homologous and heterologous strains of streptococci were lysed from without. The specificity of adsorption to cell walls was the same as to whole cocci and, with the exception of 2 phages, was irreversible at 30°. Three types of phage receptors, with different specificities, were recognized in the cell wall. Extraction of streptococci with lipid-solvents did not affect the adsorption of those phages which were irreversibly adsorbed to cell walls but reduced the adsorption of other phages. The plasma membrane of Streptococcus lactis strain ML 3, but not the cell wall, inactivated phage ml 3 but not heterologous phages in the presence of electrolytes. The plasma membrane of a phage-resistant mutant of this strain did not inactivate phage although it was similar in chemical composition to that of the parent strain.

INTRODUCTION

The concept of specific phage receptors in bacterial cell surfaces is now generally established, largely as a result of the pioneering studies of Burnet (1934), Jesaitis & Goebel (1952) and Weidel, Koch & Lohss (1954). However, specific phage receptor substances have as yet been demonstrated only in the cell-envelopes of the enterococci in the form of complex lipoprotein and lipopolysaccharides. Although phage-inactivating substances have been obtained from some Gram-positive bacteria, including a group C streptococcus (Krause, 1958), Staphylococcus aureus (Rosato & Cameron, 1964) and Streptococcus faecium (Vidaver & Brock, 1966), the specificities of the phage-inactivating processes have not been examined in detail.

Many of the phages attacking the lactic streptococci possess fairly restricted host ranges (Nichols & Hoyle, 1949; Whitehead & Bush, 1957) and adsorb well to the host cells. The effects of physical factors, e.g. electrolyte concentration, pH value and temperature on the adsorption of a Streptococcus lactis phage were established by Cherry & Watson (1949), and we have shown (Reiter & Oram, 1962) that suramin inhibits the adsorption of phage to S. lactis. However, the specificity of adsorption of phage to lactic streptococci and the nature of the phage receptor substances have not been previously investigated.

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This paper deals with the specificity of phage adsorption and the location of specific phage receptor substances within the surface layers of lactic streptococci. The isolation and chemical characterization of the different types of phage receptor substances will be reported later.

METHODS

Bacteria. The following strains of Group N streptococci were obtained from the National Collection of Dairy Organisms (catalogue numbers are given in parentheses):

- *Streptococcus cremoris* strains HP (607), KH (1198), TR (1200), E 8 (1196), 699 (699), 799 (799), 803 (803), D 9.
- *Streptococcus lactis* strains ML 3 (763), C 10 (509), 712 (712), M2S1 (702).

They were grown in glucose-Lemco broth (GL broth) or in Difco trypticase-soya broth (TS broth) at 30°C as described by Oram & Reiter (1965).

Phages. Phages for the streptococci (indicated as ml 3, c 10, 712, etc.) were also obtained from the National Collection of Dairy Organisms. Stock suspensions of phage were prepared by making serial dilutions of the dried phage preparation in 10 ml. of autoclaved litmus milk and inoculating these with 0.1 ml. of a 10^-2 dilution of a 24 hr culture in milk of the host streptococcus. The infected cultures were incubated at 30°C from 6 to 18 hr and those showing lysis were pooled, acidified with sterile n-lactic acid, centrifuged at 3000 rev./min. for 30 min. and the supernatant neutralized with sterile n-NaOH. After centrifuging, the supernatant was stored at 4°C. Phage counts were made as previously described (Oram & Reiter, 1965). The stock suspensions contained between 1 x 10^8 and 5 x 10^10 p.f.u./ml.

Preparation of cell walls. Cocci grown in GL or TS broth at 30°C were harvested in the late exponential phase of growth and washed twice with and resuspended in ½ strength Ringer’s solution in 0.01 M-potassium phosphate buffer, pH 6.8 (Ringer-phosphate solution). The cocci were broken by shaking with glass beads, either for 30 min. at 0°C in a centrifuged shaker head (Shockman, Kolb & Toennies, 1957) or for 4 min. in the Braun disintegrator (Shandon Ltd, London), cooled by intermittent jets of CO₂. The broken suspension was diluted with 4 vol. of ice-cold Ringer-phosphate solution and filtered through a sintered glass funnel to remove the glass beads. The glass beads were washed with cold Ringer-phosphate solution and the washings added to the filtrate and the combined suspension of broken cocci centrifuged at 10,000 rev./min. (the M.S.E. rotor no. 2407 was used throughout in a Superspeed 40 centrifuge at 5°C) for 30 min. The deposit was washed twice with cold M-KCl, resuspended in 0.1 M-potassium phosphate buffer (pH 6.8) and then centrifuged several times at 2000 rev./min. for 10 min. to deposit unbroken cocci. The cell walls were then washed once with 0.1 M-potassium phosphate buffer (pH 6.8), suspended in 0.05 M-tris+HCl buffer, (pH 8.0) containing 50 μg./ml. of deoxyribonuclease and incubated at 37°C for 3 hr in the presence of chloroform. The cell walls were then washed once with 0.05 M-tris+HCl buffer (pH 8.0), resuspended in the same buffer containing 50 μg./ml. of trypsin and incubated at 37°C for 18 hr, in the presence of chloroform. They were finally washed three times with deionized water and freeze-dried.

Preparation of plasma membranes. The supernatant obtained by centrifuging the disintegrated cocci at 10,000 rev./min. for 30 min. was centrifuged at 21,000 rev./min. for 2 hr. The deposit, which contained membranes, cell wall fragments and much nucleic acid, was washed once with 0.2 M-KCl in 0.01 M-tris+HCl buffer (pH 7.5) and
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resuspended in 50 ml. of a 20% (w/v) solution of sucrose in 0.01 M-tris + HCl buffer (pH 7.5) and 12.5 ml. portions were layered on to 65 ml. of a 35% (w/v) solution of sucrose in the same buffer in 100 ml. polystyrene centrifuge tubes. The tubes were centrifuged at 21,000 rev./min. for 2 hr. and the material which layered at the density boundary was carefully removed with a syringe, without disturbing the deposit. The deposit, which contained mostly cell wall but some membrane material, was discarded. The low-density material was dialysed against 3 l. of 0.001 M-tris + HCl buffer (pH 7.5) at 4°C for 18 hr and deposited by centrifuging at 21,000 rev./min. for 2 hr. After two more cycles of this procedure (centrifuging in sucrose solutions, followed by dialysis and re-centrifugation), a homogeneous, translucent and slightly orange-coloured preparation of membranes was obtained from which characteristic cell wall constituents, e.g. rhamnose, glucosamine and muramic acid, were absent. The membrane preparations contained 3% to 6% of ribonucleic acid and although most of this could be removed by further washing with deionized water, extensive washing appeared to change the properties of the membrane, which became opaque in appearance and sedimented in much smaller centrifugal fields (10,000 rev./min. for 1 hr). As similar changes occurred on freeze-drying, membrane preparations were normally suspended in deionized water and stored at -20°C.

Extraction of streptococci with lipid solvents. Cells from 50 ml. of a 16 hr culture in GL broth were washed successively with 100 ml. of Ringer-phosphate solution and 100 ml. of deionized water and resuspended in 2.0 ml. of deionized water. One half of the suspension (the 'control') was diluted with Ringer-phosphate solution to an E₁₆₅₀ value of 0.4 and stored at 4°C and the other portion was extracted by one or other of the following procedures.

Alcohol and ether. The suspension (1.0 ml.) was cooled to -20°C and mixed with 25 ml. of absolute alcohol at -20°C. After 30 min. the mixture was placed in a water bath at 37°C for 60 min. After centrifuging, the cocci were resuspended in 25 ml. of peroxide-free diethyl ether and incubated at 37°C for a further 60 min. After a second centrifugation, the cocci were suspended in the same volume of Ringer-phosphate solution as the 'control' and ether removed by bubbling with N₂.

n-Butanol. The cell suspension was diluted to 5.0 ml. with deionized water and shaken at room temperature with 5 ml. of n-butanol saturated with water for 30 min. The extracted cells were deposited by centrifugation and washed successively with 10 ml. each of n-butanol saturated with water, ethyl alcohol and ether and then resuspended in Ringer-phosphate solution as described above.

Gel filtration. A 90 x 2.5 cm. column of Sephadex G-200 was prepared and used at 4°C as described by Andrews (1965). For the determination of molecular weights, a standard curve was prepared by plotting the logarithms of published molecular weights of crystalline proteins against their elution volumes, measured either by their enzymic activities or extinctions at 225 mµ. The following proteins were used to calibrate the column (molecular weights x 10⁻² are given in parentheses), β-Galactosidase from Escherichia coli (510 to 530), bovine γ globulin (150 to 170), alkaline phosphatase from E. coli (75 to 80), ovalbumin (44 to 46) and cytochrome c from horse heart (12.4).

Thin-layer chromatography of lipids. Samples of lipid dissolved in benzene were applied to thin layers (0.5 mm.) of silica gel HF 254 and 366 (E. Merck Ag. Darmstadt, German Federal Republic) spread on 20 x 20 x 0.3 cm. glass plates and previously
activated by heating at 105 °C for 1 hr. The plates were chromatographed for 30 min. using benzene + diethyl ether + ethylacetate + acetic acid (80:10:10:0.2, v/v) as the solvent in the narrow solvent chamber system of Storry & Tuckley (1967). The plates were dried and sprayed with a solution of 0.005% rhodamine B and 0.025% dichloro-R-fluorescein in methanol and examined under ultraviolet light (254 and 366 nm).

Paper chromatography of sugars. Portions (5 to 10 mg.) of the plasma membranes of streptococci were hydrolysed with 1.0 ml. of 0.5 N-HCl in an atmosphere of N₂ at 100 °C for 18 hr and dried in vacuo over P₂O₅ + NaOH. The residue was dissolved in 25 to 50 µl. of deionized water and 5 µl. portions spotted on to Whatman No. 1 paper and chromatographed in n-butanol + pyridine + acetic acid + water (60:40:3:20, v/v) or ethyl acetate + pyridine + water (2:1:1, v/v, top phase). Carbohydrates were located by the reagent of Trevelyan, Proctor & Harrison (1950).

Adsorption of phage. (a) By viable streptococci. Cultures of streptococci in GL broth were harvested after incubation at 30 °C for 18 hr, washed once in 1/4 strength Ringer’s solution and resuspended in 0.1 M-NaCl + 0.001 M-CaCl₂ + 0.01 % (w/v) gelatin to give an E₆₀₀ nm value of 0.4, which corresponded to about 3·10⁸ cocci/ml. The suspension of cocci (2.0 ml.) and a suspension of phage (about 10⁸ p.f.u./ml) in the same medium were separately equilibrated at 30 °C for 10 min. and 1.0 ml. of phage suspension added to the cocci. The adsorption mixture was shaken gently for 15 min., centrifuged at about 8,000 g for 3 min. and dilutions of the supernatant in 1/4 strength Ringer’s solution counted in duplicate to determine the number of unadsorbed p.f.u.

(b) By cell walls. This procedure was similar to that employed for viable cocci except that, as the number of unadsorbed p.f.u. was, with the exceptions reported in the ‘Results’ section, unaffected by centrifuging the adsorption mixture, the centrifugation step was omitted.

(c) By plasma membranes. In this procedure the volume of the adsorption mixture was reduced in order to conserve material. After temperature equilibration, 0.1 ml. of phage suspension (about 10⁷ p.f.u.) was added to 0.4 ml. of membrane suspension and the mixture incubated at 30 °C for 30 min. The titre of the residual phage was determined without centrifugation. In preliminary experiments more consistent results were obtained when the NaCl + CaCl₂ + gelatin medium was replaced by GL broth and although later found to offer no advantage, the latter medium was employed, except where stated in the text. A unit of phage receptor activity was defined as the amount of material which adsorbed or inactivated 10⁶ p.f.u. under these conditions and the specific activity as units of phage receptor activity/mg.

Chemical analyses. Before estimating the protein and RNA contents of membrane preparations, the membranes were suspended (4 mg./ml.) in deionized water and dispersed by ultrasonic disintegration (Salton & Freer, 1965). Protein was estimated by the method of Itzhaki & Gill (1964), except that the mixtures of membranes and reagents were centrifuged at about 5000 g for 10 min. to deposit insoluble material before the extinctions were measured. RNA was measured, after hydrolysis with 5% (w/v) trichloracetic acid at 90 °C for 20 min., by the orcinol reaction (Mejaun, 1939). The lipid contents of membrane preparations were determined by extracting 20 mg. of membrane, dispersed in 1.0 ml. of deionized water, with 25 ml. of chloroform + methanol (2:1, v/v) at 60 °C for 20 min. The extract was filtered through a sintered glass filter, washed with 0.2 vol. of 0.04% MgCl₂ (Folch, Leese & Sloan-Stanley, 1957),
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evaporated to dryness in a stream of N₂ and then dissolved in chloroform + methanol (2:1, v/v) and transferred to tared weighing bottles. After drying with N₂ and then in a desiccator over P₂O₅, the weight of the extracted lipid was estimated gravimetrically. After hydrolysis of samples with 4 N-HCl at 100° for 1 hr, glucosamine and muramic acid were separated on charcoal + celite columns (Perkins, 1965) and determined by the method of Strominger, Park & Thompson (1959). Rhamnose was determined by the method of Gibbons (1955). After hydrolysis of samples with 0·5 N-HCl at 100° for 18 hr, glucose was determined with glucose oxidase (Huggett & Nixon, 1957) and galactose with the 'galactostat' reagent (Worthington Biochemical Corporation, Freehold, N.J., U.S.A.).

RESULTS

Comparison of the specificities of adsorption and multiplication of phage

Initially, we sought to establish the importance of phage adsorption patterns on the specificity of phage multiplication. The latter was established by examining the ability of a phage to produce plaques rather than to lyse cultures of streptococci since some of these were lysed from without by high concentrations of phage or phage-associated muralytic enzymes (Oram & Reiter, 1965). The ability of a streptococcal strain to support the multiplication of the various phages was examined by plating the strepto-

Table 1. Phage relationships of some lactic streptococci; the specificities of phage multiplication

<table>
<thead>
<tr>
<th>Strain of streptococcus used in phage assay</th>
<th>Titration values (log. p.f.u./ml.) of phages using different strains of streptococcus as host</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>699</td>
</tr>
<tr>
<td>S. cremoris 699</td>
<td>9·10</td>
</tr>
<tr>
<td>S. cremoris KH</td>
<td>8·78</td>
</tr>
<tr>
<td>S. cremoris TR</td>
<td>0</td>
</tr>
<tr>
<td>S. cremoris 799</td>
<td>0</td>
</tr>
<tr>
<td>S. cremoris 799</td>
<td>0</td>
</tr>
<tr>
<td>S. cremoris ++ 9</td>
<td>0</td>
</tr>
<tr>
<td>S. cremoris HP</td>
<td>0</td>
</tr>
<tr>
<td>S. lactis ML 3</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Adsorption of phage to streptococci

Residual p.f.u. as percentage of 'control' titre.

<table>
<thead>
<tr>
<th>Strain of streptococcus</th>
<th>Phages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>699</td>
</tr>
<tr>
<td>S. cremoris 699</td>
<td>19·5</td>
</tr>
<tr>
<td>S. cremoris KH</td>
<td>4·8</td>
</tr>
<tr>
<td>S. cremoris TR</td>
<td>70·5</td>
</tr>
<tr>
<td>S. cremoris 799</td>
<td>95·7</td>
</tr>
<tr>
<td>S. cremoris ++ 9</td>
<td>6·8</td>
</tr>
<tr>
<td>S. lactis ML 3</td>
<td>97·9</td>
</tr>
</tbody>
</table>

Adsorption mixture containing about 3×10⁸ cocci and 2 to 13×10⁴ p.f.u. in 3·0 ml. of adsorption medium were shaken at 30° for 15 min., centrifuged, and the supernatant fluids titrated for residual p.f.u. C cocci were omitted from the 'controls'. Residue of phages adsorbed are shown in boxes. Results are averages of duplicate experiments.
coccus with dilutions of the different phages. Each phage had a fairly restricted host range, none multiplying in more than two of the streptococcal strains (Table 1). However, a lower specificity was found for the adsorption of phage, most phages being adsorbed by several strains of streptococcus (Table 2). Thus, \textit{Streptococcus cremoris TR} adsorbed phage 799 in addition to its homologous phages \textit{tr} and \textit{d 9} and \textit{S. cremoris} 799 also adsorbed the heterologous phages \textit{tr} and \textit{d 9}. Similarly, \textit{S. lactis} ML 3 adsorbed the heterologous phage \textit{hp}. Some homologous strains of phage were, however, adsorbed with a much lower efficiency under these conditions. Thus, although the titre of phage \textit{tr} was the same when plated on strain \textit{TR} or \textit{d 9}, only about 30\% of the phage was adsorbed by the latter strain. The low efficiency of plating of phage \textit{hp} on strain \textit{d 9} appeared to be due to the very poor adsorption of the phage to this strain. Some strains of lactic streptococci, other than those included in Tables 1 and 2, appeared to adsorb a wide variety of heterologous phages but this was an extremely variable phenomenon which was possibly the result of a non-specific inactivation of phage and not phage adsorption.

\textit{Lysis-from-without of streptococci by heterologous phage}

It was previously shown that \textit{Streptococcus lactis} ML 3 was lysed-from-without by high multiplicities of phage \textit{ml 3} (Oram & Reiter, 1965) and it was of interest to determine the effect of this phage on heterologous strains of streptocci. Some strains of lactic streptococci were found to be even more susceptible to lysis-from-without than \textit{S. lactis} ML 3 (Fig. 1). The relative susceptibilities of the strains to lysis-from-without by \textit{ml 3} phage were very similar to those previously found for their lysis by \textit{ml 3} phage lysin or the lytic enzyme associated with the phage particles (Oram & Reiter, 1965).

\textit{Specificity of phage adsorption to cell walls}

Cell walls of several strains of lactic streptococci adsorbed phage irreversibly at 30\°. The specificity of adsorption to the cell walls was, with some exceptions, similar to that observed with washed streptococci (Table 3). The main exceptions were phages \textit{ml 3} (Fig. 2) and \textit{d 9}, neither of which appeared to adsorb to the cell wall of the host strain if the adsorption mixtures were not centrifuged before plating. Hence, the adsorption of these phages to the host cell wall appeared to be reversible at 30\°. However, the cell wall of \textit{Streptococcus lactis} ML 3 adsorbed the heterologous phage \textit{hp} and that of \textit{S. cremoris} \textit{D 9} adsorbed the homologous phage \textit{tr} and the heterologous phage 799.

Weidel (1958) proposed that the adsorption of coliphage T 1, which is unable to adsorb irreversibly to the isolated cell envelope of \textit{Escherichia coli} B, involves a mechanism which functions in the living cell but not in the isolated cell envelope. However, before digestion with trypsin or nucleases, the crude cell wall preparations of \textit{S. lactis} ML 3 adsorbed phage \textit{ml 3} irreversibly, so this possibility was excluded. It seems more likely that the receptor substances for phages \textit{ml 3} and \textit{d 9} were destroyed or removed during the isolation and purification of the cell walls.

\textit{Isolation of ml 3 phage receptor substances}

The phage receptor activity of crude cell wall preparations of \textit{S. lactis} ML 3 was not affected by digestion with RNase or DNase but appeared to be destroyed by digestion with trypsin. Subsequently, however, it was found that the supernatant fluid of the trypsin-digested cell wall inactivated phage \textit{ml 3} irreversibly at 30\°. No loss in phage
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Fig. 1. Lysis-from-without of strains of group N streptococci by phage \textit{ml} 3. One ml. of a suspension of phage in 0.1 M-potassium phosphate buffer (pH 6.8) was added to 40 ml. of suspensions of cocci in the same buffer at 37°. Multiplicity of infection = 100 p.f.u./coccus; \(E_{580\text{ mg}}\) = extinction at 580 \text{mg/cm}^2. ○, \textit{S. cremoris} 803; ○, \textit{S. lactis}, c 10; □, \textit{S. lactis}, \textit{ml} 3.

Fig. 2. Adsorption of phage by cell walls of some lactic streptococci. Mixtures of cell wall and phage in 3.0 ml. of 0.1 M-NaCl + 0.01 M-CaCl\textsubscript{2} + 0.01 % gelatin were shaken at 30° for 15 min. Mixtures were not centrifuged before diluting and plating of residual p.f.u. The cell wall+phage mixtures were ○, \textit{S. cremoris} HP + hp; ○, \textit{S. cremoris} KH + kh; □, \textit{S. lactis} ml 3 + ml 3.

Table 3. Adsorption of phage to streptococcal walls

<table>
<thead>
<tr>
<th>Strain of streptococcus</th>
<th>Phages</th>
<th>699</th>
<th>kh</th>
<th>tr</th>
<th>799</th>
<th>d 9</th>
<th>hp</th>
<th>ml 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{S. cremoris} 699</td>
<td></td>
<td>4.5</td>
<td>0</td>
<td>82.7</td>
<td>103.8</td>
<td>90.9</td>
<td>107.8</td>
<td>111.0</td>
</tr>
<tr>
<td>\textit{S. cremoris} KH</td>
<td></td>
<td>3.9</td>
<td>0</td>
<td>81.2</td>
<td>96.3</td>
<td>111.6</td>
<td>85.2</td>
<td>106.1</td>
</tr>
<tr>
<td>\textit{S. cremoris} TR</td>
<td></td>
<td>87.0</td>
<td>109.1</td>
<td>27.3</td>
<td>32.2</td>
<td>103.5</td>
<td>87.0</td>
<td>104.2</td>
</tr>
<tr>
<td>\textit{S. cremoris} 799</td>
<td></td>
<td>104.4</td>
<td>76.4</td>
<td>30.1</td>
<td>41.0</td>
<td>80.8</td>
<td>82.0</td>
<td>86.1</td>
</tr>
<tr>
<td>\textit{S. cremoris} d 9</td>
<td></td>
<td>109.6</td>
<td>103.0</td>
<td>0.1</td>
<td>32.3</td>
<td>82.3</td>
<td>97.6</td>
<td>90.9</td>
</tr>
<tr>
<td>\textit{S. cremoris} HP</td>
<td></td>
<td>118.5</td>
<td>92.9</td>
<td>93.4</td>
<td>107.9</td>
<td>93.9</td>
<td>93.5</td>
<td>93.5</td>
</tr>
<tr>
<td>\textit{S. lactis} ml 3</td>
<td></td>
<td>103.4</td>
<td>120.8</td>
<td>126.5</td>
<td>103.9</td>
<td>100.8</td>
<td>0.3</td>
<td>89.5</td>
</tr>
</tbody>
</table>

Adsorption mixtures containing 1.0 mg. of cell wall and 3 to 11 x 10\textsuperscript{3} p.f.u. in 3.0 ml. of adsorption medium were shaken at 30° for 15 min. and titrated for residual p.f.u. Cell walls were omitted from 'controls'. Phages adsorbed by each type of cell wall are shown in boxes and these are average values from two experiments; other results refer to single experiments.
titre occurred when phage was incubated with trypsin alone at a concentration of 100 μg/ml. A 1% suspension of the crude cell wall in 0.05 M-tris + HCl buffer (pH 8.0) containing 50 μg/ml of trypsin was incubated at 37° for 18 hr; after centrifuging, the supernatant fluid was dialysed against 2 changes of deionized water (100 vols) at 4° and the non-diffusible material freeze-dried. When 100 mg. of this material was filtered through a column of Sephadex G-200 equilibrated with 0.1 M-KCl, the phage-inactivating activity was obtained in the first of two protein-containing peaks (Fig. 3). Comparison of the elution volume of the phage-inactivating material with those of proteins of known molecular weight indicated that it had a molecular weight in the range 200,000 to 400,000. As this material contained approximately equal amounts of lipid and protein it seems probable that it was a lipoprotein derived either from the cell wall or the plasma membrane.

![Gel-filtration diagram](image-url)

**Fig. 3.** Gel-filtration of the supernatant obtained by digesting the cell wall-plasma membrane fraction of *S. lactis* ML 3 with trypsin. 100 mg. of the supernatant was filtered through a 90 x 2.5 cm. column of Sephadex G-200 equilibrated with 0.1 M-KCl and eluted with the same solvent at a flow rate of 20 ml/hr. at 4°. ○, phage inactivation; □, column calibration (because of its carbohydrate content, γ globulin behaves as a protein with a mol. wt of about 210,000 (Andrews 1965)).

Crude cell wall preparations contained between 6% and 10% lipid and therefore appeared to contain a considerable amount of either cell wall lipoprotein or membrane material. Hence, strain ML 3 appeared to possess two types of phage receptors—one for phage *hp* located in the cell wall and another for phage *ml 3*, of uncertain location, but containing lipoprotein. Moreover, as the activity of the lipoprotein-containing phage *ml 3* receptor was not destroyed by trypsin, it seemed possible that lipoproteins were also present in phage receptors in the cells walls of some strains but that these were not released by trypsin.
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Effect of lipid solvents on the adsorption of phage by washed streptococci

Extracting cells with lipid solvents should decrease the activity of phage receptors which contained lipoprotein and we therefore examined the effect of n-butanol or alcohol and ether on the adsorption of phage by several strains of streptococci (Table 4).

Table 4. Effect of extracting streptococci with lipid-solvents on phage adsorption

<table>
<thead>
<tr>
<th>Streptococcus</th>
<th>Solvent*</th>
<th>Phage</th>
<th>Input phage</th>
<th>Residual phage</th>
<th>Adsorption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control cells</td>
<td>Extracted cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| *Washed streptococci were extracted with (A) ethyl alcohol followed by diethyl ether or (B) n-butanol, as described in the text.*

Adsorption mixtures containing approximately $3 \times 10^8$ cocci/ml. and phage (as shown) were shaken in 3 ml. of GL broth at 30°C for 15 min.

Table 5. Isolation of phage ml 3 receptor substance from broken cells of S. lactis ML 3

<table>
<thead>
<tr>
<th>Cell fractionation procedure</th>
<th>Volume (ml.)</th>
<th>Dry weight (mg./ml.)</th>
<th>Phage receptor activity (units/ml. $\times 10^{-3}$)</th>
<th>Specific activity (units/mg. $\times 10^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broken cells centrifuged 10,000 rev./min. $\times 30$ min.</td>
<td>1420</td>
<td>—</td>
<td>4.29</td>
<td>6090</td>
</tr>
<tr>
<td>Supernatant (cytoplasm, plasma membrane and cell wall fragments)</td>
<td>1370</td>
<td>—</td>
<td>0.33</td>
<td>481</td>
</tr>
<tr>
<td>Centrifuged 21,000 rev./min. $\times 2$ hr</td>
<td>52</td>
<td>20.3</td>
<td>110.0</td>
<td>5720</td>
</tr>
<tr>
<td>Supernatant fluid (cytoplasm)</td>
<td>55</td>
<td>8.4*</td>
<td>180.0</td>
<td>990</td>
</tr>
<tr>
<td>Centrifuged 21,000 rev./min. $\times 2$ hr</td>
<td>35% 35%</td>
<td>—</td>
<td>0.16†</td>
<td>515</td>
</tr>
<tr>
<td>membrane from 1st cycle</td>
<td>58</td>
<td>13.7*</td>
<td>95.0</td>
<td>5500</td>
</tr>
<tr>
<td>membrane from 2nd cycle</td>
<td>58</td>
<td>10.2*</td>
<td>42.5</td>
<td>2470</td>
</tr>
<tr>
<td>membrane from 3rd cycle</td>
<td>55</td>
<td>8.4*</td>
<td>18.0</td>
<td>990</td>
</tr>
<tr>
<td>Dialysed and centrifuged 21,000 rev./min. $\times 2$ hr; combined supernatants</td>
<td>2340</td>
<td>—</td>
<td>0.22†</td>
<td>515</td>
</tr>
<tr>
<td>Combined washings of 3rd membrane fraction</td>
<td>275</td>
<td>—</td>
<td>0.16†</td>
<td>44</td>
</tr>
<tr>
<td>Purified membranes</td>
<td>20</td>
<td>2.2.5</td>
<td>39.1</td>
<td>782</td>
</tr>
</tbody>
</table>

* Calculated from dialysed membranes. † Average values.

Cells harvested from 40 l. of a tryptone-soya broth culture were washed twice with Ringer-phosphate solution and disintegrated in the Braun cell homogenizer and the plasma membrane purified as described in ‘Methods’.
This reduced the extent of adsorption of phage \textit{ml} 3 to \textit{S. lactis ML} 3 from 97\% to 26\% but had no effect on the adsorption of phage \textit{hp}. Similarly, treatment with \textit{n}-butanol completely eliminated the adsorption of the homologous phage \textit{d 9} to \textit{S. cremoris} strains \textit{D 9} and \textit{TR} but had little effect on the adsorption of phage \textit{tr}. Extraction of three other strains, \textit{S. lactis 712}, \textit{S. cremoris HP} and \textit{S. cremoris 799} had no significant effect on their ability to adsorb their homologous phages but reduced the adsorption of phage \textit{m2 si} to \textit{S. lactis M2st} by 44\%. These results indicate that phage \textit{d 9} receptor substance (and possibly that of phage \textit{m2 s} like that of phage \textit{ml} 3) contained lipid but that the receptors of phages, \textit{hp}, \textit{799} and \textit{712} did not.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Inactivation of phage \textit{ml} 3 by cell fractions of \textit{S. lactis ML} 3. Phage was incubated with the plasma membrane (\textcircled{O}), cell wall (\textbullet) or cytoplasmic fraction (\textsquare) in 0.5 ml. of GL broth at 30\° for 30 min.}
\end{figure}

\textbf{Location of phage \textit{ml} 3 receptors}

\textit{Streptococcus lactis} ML 3 was disintegrated in the Braun cell homogenizer and cell wall and plasma membrane fractions purified as described in 'Methods'. Although the cell wall did not adsorb phage \textit{ml} 3 and the cytoplasmic fraction (obtained by centrifuging the broken cocci at 21,000 rev./min. for 2 hr) possessed very low phage-inactivating activity, the phage was inactivated by very small amounts of the plasma membrane (Fig. 4). One \textmu g. of the most active preparation of purified membranes inactivated
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about 10^6 p.f.u., but before purification the specific activity of the membrane preparations were approximately 5 times higher than that of the purified material (Table 5). The considerable loss in phage-receptor activity during purification of the membrane was much greater than the amount of activity released into solution during the purification procedure (Table 5). As activity was also lost on freeze-drying, which also caused changes in the physical properties of the membranes (see ‘Methods’), membrane suspensions were normally stored at −20°C.

Specificity of phage inactivation by the plasma membrane of Streptococcus lactis ML 3

The phage receptor activity of the plasma membrane of strain ML 3 was found to be extremely specific. Thus, out of seven phages tested, only phage ml 3 was inactivated by the membrane (Fig. 5). Neither phage γzz, which was homologous for strain ML 3 and adsorbed by its cell wall, nor phage d 9, which appeared to adsorb to a membrane type of receptor, was inactivated. The phage receptor activity of phage ml 3 resistant strains of S. lactis ML 3 was also examined. Although about 90 % of the resistant mutants obtained from a phage ml 3 lysate of strain ML 3 adsorbed the phage, two mutant strains (ML 3/5 and ML 3/15) did not. The membranes isolated from these mutants possessed very little or no phage receptor activity (Fig. 6) indicating that phage receptors were absent from or non-functional in the membranes of these resistant mutants. Strains ML 3/5 and ML 3/15 were not resistant to phage γzz, however, and adsorbed this phage to the same extent as the parent strain, thus demonstrating the independence of the genetic control of the phage ml 3 and γzz phage receptor substances.

The chemical composition of the membranes of the phage resistant strain ML 3/5 was similar to that of the phage-sensitive parent strain, ML 3 (Table 6). Both contained rather more lipid (about 40 %) than reported for the membranes of other species of Gram-positive bacteria (Kodicek, 1962; Salton & Freer, 1965), about 50 % protein and from 3 to 6 % RNA. The plasma membrane of S. cremoris 799 contained less lipid (30 %) than those of S. lactis ML 3 and ML 3/5. The lipids of the membranes of these strains were examined by thin-layer chromatography. They contained large amounts of phospholipids and fatty acids, small amounts of triglycerides but little or no monoor di-glycerides, sterols or sterol esters. Hence the types of lipids in the membranes of lactic streptococci appear to be similar to those reported for other bacterial membranes (Weibull, 1957; Kodicek, 1962).

Table 6. Chemical composition of the plasma membranes of
S. lactis ML 3 and ML 3/5 and S. cremoris 799

<table>
<thead>
<tr>
<th></th>
<th>S. lactis ML 3</th>
<th>S. lactis ML 3/5</th>
<th>S. cremoris 799</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>48.2</td>
<td>50.8</td>
<td>55.6</td>
</tr>
<tr>
<td>Lipid</td>
<td>36.0</td>
<td>42.9</td>
<td>30.0</td>
</tr>
<tr>
<td>RNA</td>
<td>2.9</td>
<td>5.8</td>
<td>4.7</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.9</td>
<td>0.4</td>
<td>N.D.</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.6</td>
<td>0.4</td>
<td>N.D.</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>0</td>
<td>Trace</td>
<td>0</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

N.D.: present but not determined quantitatively
Fig. 5. Specificity of the phage inactivating activity of the plasma membrane of *S. lactis* ML 3.
The phages were incubated with 25 to 500 μg. of the membrane in 0.5 ml. of GL broth at 30° for 30 min. Phages: O, ml 3; ●, kk; □, ms 52; ■, kp; △, 712; ▲, e 8; ▽, d 9.

Fig. 6. Absence of phage receptor activity in plasma membranes of phage-resistant mutants of *S. lactis* ML 3. O, Membrane of phage-sensitive strain ML 3, 100 μg.; ●, membrane of phage-resistant mutant ML 3/5, 500 μg.; □, membrane of phage-resistant mutant, ML 3/15, 500 μg.
Cation requirements for ml 3 phage receptor activity

Like other phages, ml 3 does not adsorb to washed cocci in the absence of mono- or divalent cations and like other streptococcal phages (Reiter, 1949; Shew, 1949) requires divalent cations for multiplication but not for adsorption. We therefore examined the effect of Na\(^+\) and Ca\(^{2+}\) ions on the reversible inactivation of phage ml 3 by the plasma membrane of \textit{S. lactis} ML 3. The phage-inactivating activity of the membrane was approximately the same in 0.1 M-NaCl + 0.01 % gelatin as in GL broth but no inactivation occurred in deionized water (Fig. 7). The addition of 0.01 M-CaCl\(_2\) did not affect the amount of phage inactivated by the membrane, indicating that Ca\(^{2+}\) was not required for this stage of the infective process.

![Graph](image)

Fig. 7. Effect of cations on inactivation of phage ml 3 by the plasma membrane of \textit{S. lactis} ML 3. Mixtures of phage and membrane in 0.5 ml. of O, GL broth; \(\triangle\), deionized water; \(\square\), 0.1 M-NaCl + 0.01 % gelatin, or \(\bullet\), 0.1 M-NaCl + 0.01 M-CaCl\(_2\) + 0.01 % gelatin, were incubated at 30° for 30 min.

DISCUSSION

At multiplicities of infection between 0.1 and 1.0 p.f.u./coccus the adsorption of phage to lactic streptococci was found to be a fairly specific process. Nevertheless, most strains of coccus adsorbed one or more heterologous strains of phage. This situation is not confined to the lactic streptococci, many strains of staphylococcus, for example, adsorb heterologous strains of phage (Morse, 1965). The inability of an irreversibly adsorbed phage to multiply may be due to the failure of the phage to complete later stages in its maturation cycle or, in the case of a temperate phage, because of lysogenic immunity. However, we have been unable to show that phage \textit{hp} is temperate for \textit{S. lactis} ML 3, in spite of its irreversible adsorption to that strain, and we know of no
case in which lysogeny has been rigorously demonstrated in group N streptococci. Both homologous and heterologous phages adsorbed to the cell walls of some strains. The observed specificity of the adsorption of phages to streptococci is consistent with the existence of several types of phage receptor, each having a characteristic specificity. Thus phages \( tr \) and \( 799 \) were adsorbed by the cell walls of \( S. cremoris \) strains \( TR \), \( 799 \) and \( D \), indicating that both attach to a common receptor, present in all three strains. Phages \( 699, \ kh \) and \( k \) were all adsorbed by cell walls of \( S. cremoris \) strains \( 699, \ KH \) and \( K \), which, therefore, appeared to possess another type of receptor. Similarly, cell walls of \( S. lactis \) \( ML \). 3 and \( S. cremoris \) \( hp \) were found to possess a receptor specific for phage \( hp \). Doubtless many other types of phage receptor exist in the cell walls of other lactic streptococci and although we did not find more than one type of receptor in any one cell wall, only a limited number of strains were examined and others might well possess several types. We have recently purified phage-receptor substances from the cell walls of some lactic streptococci and their properties will be described in a later paper.

Phage \( ml \) 3 attached only reversibly to the cell wall but was irreversibly inactivated by the plasma membrane of \( S. lactis \) \( ML \). 3. As only phage \( ml \) 3 was inactivated, the membrane also determined the specificity of adsorption to the host cell. Several strains, e.g. \( ML \) 3, \( D \) 9 and \( TR \), appeared to contain both cell-wall and plasma-membrane types of phage receptor. This appears to be the first report of the location of phage receptor material in a bacterial plasma membrane although there is some evidence that a similar situation exists in the closely related group D streptococci. Hence, phage was found by Timperley, Horne & Stewart-Tull (1966) to adsorb to heat-killed cells but not to the cell wall of \( Streptococcus faecalis \) and the phage-inactivating particles obtained by the action of lysozyme on \( Streptococcus faecium \) by Vidaver & Brock (1966) contained both cell wall and membrane material. As the phage-receptor activity of the particles from \( S. faecium \) was sensitive to heat and treatment with mild acids or alkali, to 6 M-urea and proteolytic enzymes (differing in this last respect from the \( S. lactis \) \( ML \). 3 phage receptor substance), Vidaver & Brock (1966) concluded that protein was an integral part of the phage receptor. Phage P 3 was adsorbed by cell wall preparations which Vidaver & Brock (1966) obtained by the differential centrifugation of mechanically disintegrated cells of \( S. faecium \). However, we have found that although it was relatively easy to obtain membranes of group N streptococci free of cell wall components by differential centrifugation in sucrose solutions, it was very much more difficult to obtain cell walls free of membrane material. Even after three cycles of differential centrifugation followed by extensive washing with buffers and water, 6 out of 7 preparations of the cell wall of \( S. lactis \) \( ML \). 3 were heavily contaminated with membrane material, the removal of which required either digestion with proteinases or extraction with detergent. Our (unpublished) studies on the action of muramidases on the cell wall + plasma membrane fraction of disintegrated cells of \( S. lactis \) \( ML \). 3 indicate the presence of some type of attachment between the cell wall and the plasma membrane, which may explain why spheroplasts rather than protoplasts are produced when group N streptococci are digested with muramidases (Smith & Shattock, 1964). Our initial failure to appreciate the intimate association between the cell wall and plasma membrane of strain \( ML \). 3 resulted in misdirected attempts to characterise a cell-wall-protein phage receptor and delayed considerably the location of the receptor in the plasma membrane. A similar type of association between the cell wall and plasma
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The membrane of *S. faecium* could account for the occurrence of cell wall material in the phage receptor particles obtained from spheroplasts by Vivader & Brock (1966).

The activity of the phage *ml* 3 receptor was markedly reduced by extracting cells of *S. lactis* ML 3 with lipid solvents and this also destroyed the phage *d 9* receptors of *S. cremoris* D 9, suggesting that this phage receptor also occurred in the plasma membrane. However, the destruction of a phage receptor by lipid-solvents is in itself no proof that the receptor occurs in the plasma membrane, as the receptors for coliphages T2 and T6 are cell wall lipoproteins (Weidel *et al.* 1954) and those of T3, T4 and T7 are cell wall lipopolysaccharides (Jesaitis & Goebel, 1952). Hence, the inability of alcohol- and ether-extracted cells of *Corynebacterium diphtheriae* to adsorb phage B (Kerszman, 1966) may have been due to an effect of lipid-containing substances in the cell wall rather than in the plasma membrane.

The phage-inactivating activity of membranes of *Streptococcus lactis* ML 3 appears to be considerably higher than that reported by Morse (1965) for the cell wall of *Staphylococcus aureus*. Morse (1965) found that 1 µg of staphylococcal wall inactivated about 10^4 p.f.u. whereas in our experiments 1 µg of the membrane of *S. lactis* ML 3 inactivated about 10^6 p.f.u. and the specific activity of unpurified membrane preparations was approximately 5 times greater. As the efficiency of plating of phage *ml* 3 is only about 0.05 to 0.10 (Oram, 1965) the number of particles inactivated by the membrane was probably even greater than our results indicate.

We have considered the possibility that the *ml* 3 phage receptor was not an integral part of the plasma membrane but became loosely associated with, or adsorbed to, the membrane during the disintegration of the cocci. However, this would not explain the loss of phage-receptor activity during the purification of the membranes, as these losses greatly exceeded the amount of activity released into solution during the purification procedure. It therefore seems probable that the observed loss of activity was due to the destruction of some labile component of the phage receptor. Recently a lipoprotein, containing only traces of carbohydrate and no nucleic acid constituents, was obtained from the membrane of strain ML 3 and possessed the phage receptor material of this organism (Oram, unpublished results).

It is generally accepted, by analogy with the coliphages, that adsorption of phage is followed by the penetration of the host cell wall by the tail, or tail-core, of the phage particle. However, in the case of phage *ml* 3, penetration of the cell wall must presumably precede attachment of the phage to the plasma membrane. Like many other phages, phage *ml* 3 possesses a muralytic enzyme (Oram & Reiter, 1965) which hydrolyses the linkages between N-acetyl muramic acid and N-acetylglucosamine residues in the cell wall glycosaminopeptide (Oram, 1965). The phage-bound enzyme has now been shown to lyse-from-without strains of lactic streptococci which do not adsorb phage *ml* 3 irreversibly. Hence, although adsorption of phage *ml* 3 to cell walls is reversible with respect to phage, the phage may produce holes in the cell wall which presumably expose the phage receptors in the membrane. It is of interest that Bradley & Kay (1960) found that the tail of phage *ml* 3 (obtained from us but unfortunately termed 3 ML by them) measured 1000 × 90 Å and had no sheath, base plate or other tail appendage. Base plates and tail fibres are known to be involved in the adsorption of coliphages T2 and T4 to *Escherichia coli* (Simon & Anderson, 1967); and staphylococcal phages, which also adsorb to the host cell wall (Hotchin, Dawson & Elford, 1952) possess a knob at the base of the tail (Hotchin, 1954; Bradley & Kay, 1960). The tail
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of phage ml 3 was found by Bradley & Kay (1960) to have a cross-sectional diameter of 90 Å, slightly greater than the 70 Å diameter of the core of the sheathed tail of coliphage T2 (Brenner et al. 1959) which penetrates the outer layers of the cell wall but not the plasma membrane of E. coli (Simon & Anderson, 1967). Hence the sheath-less tail of phage ml 3 appears to be functionally equivalent to the tail-core of the T-even coliphages, in that each penetrates the cell wall as far as the plasma membrane.

We gratefully thank Mrs W. Cole for her excellent technical assistance.

REFERENCES


Adsorption of phage to group N streptococci


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