Phage-Associated Lysins Affecting Group N and Group D Streptococci

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SUMMARY

Phage lysins were prepared from high titre phage lysates of 3 strains of *Streptococcus lactis*, one of which was purified 500-fold by treatment with cold acetone, chromatography on Amberlite CG 50 ion-exchange resin, and fractional precipitation from potassium phosphate buffers. The lysins were activated by monovalent cations and were similar in physical properties. Viable streptococci of groups N and D were lysed, but not those in groups A, B and C. Different strains of group N streptococci were lysed at different rates, the rate of lysis being characteristic for individual phage lysins. A lytic enzyme, prepared from purified phage particles by freezing and thawing or by ultrasonic treatment, showed the same pH optimum and specificity as the corresponding phage lysin. Streptococci were lysed-from-without in the presence of high multiplicities of phage. The type of lysin produced in phage-infected culture was genetically determined by the phage. Lysis of streptococci by phage lysin was inhibited by an unidentified substance present in crude phage lysates. The lysis of different strains of streptococci by particulate phage, phage-tail enzyme and phage lysin occurred at the same relative rates. This is interpreted as evidence for the localization of the phage-lysin substrate within the phage receptor site. Possible reasons for the different specificities of phage lysins from streptococci of groups N and C are discussed.

INTRODUCTION

It has long been known that phage lysates contain a lytic agent which can act on the host bacterium, but is distinct from particulate phage (Bronfenbrenner & Muckenfuss, 1927). Evans (1934) demonstrated that a strain of group A streptococcus normally resistant to a group C phage, was lysed when grown in mixed culture with the phage-infected group C strain. This so-called 'nascent' phage reaction was shown by Maxted (1957) to be due to a lytic agent present in crude phage lysates of this particular strain of group C streptococcus; the lytic agent was active against group A, C and E streptococci. Naylor & Czulak (1956) suggested that the 'nascent' phage phenomenon shown by group N streptococci (the lactic acid streptococci) was caused by a lysin. These factors are similar to the 'facteur bactériostatique des lysates bactériophagique' of *Streptococcus lactis* reported by Wahl & Josse-Goichot (1950). The occurrence of phage-associated lysins (in the sequel phage-lysins) active against group D streptococci (Bleiweis & Zimmerman, 1961) and other bacterial genera such as *Bacillus megaterium* (Murphy, 1957), *Escherichia coli* (Brown, 1956; Adams & Park, 1956; Koch & Jordan, 1957) and *Staphylococcus aureus* (Ralston, Baer, Lieberman & Krueger, 1955) is well established.

The presence of phage-bound enzyme which attacked the host cell-wall was first observed with coliphage T2 by Weidel (1951) and has since been described for phages of *B. megaterium* (Murphy, 1960) and *Micrococcus lysodeikticus* (Brumfitt, 1960).

The present communication describes methods for the isolation of lysins from phages and phage lysates of some lactic acid streptococci, and some of the properties of these phage-lysins.

METHODS

Organisms

The following strains of streptococci were used (National Collection of Dairying Organisms, NCDO catalogue numbers are given in brackets):

Group N: *Streptococcus cremoris*: E8 (1196), HP (607), US3 (1197), z8 (1199), R6 (764), KH (1198), TR (1200), 924 (924), 803 (803), 972 (972); *S. lactis*: ML3 (763), C10 (509), 712 (712); *S. diacetylactis*: DRC1 (1007).

Group A: *S. pyogenes*: 3/3A, Richards (923), Pope (1716).

Group B: *S. agalactiae*: A178 (1351); NCTC 8187 (1336); 8080.

Group C: *S. dysgalactiae*: C4 (1357); K64 (939); S51.

Group D: *S. bovis*: Pearl 11 (597); *S. zymogenes*: Mahoney (586); *S. durans*: 98/D (596); *S. liquefaciens*: Elv/2025 (588); *S. faecalis*: 775 (581).

Bacteriophage strains: homologous to their appropriate strains of group N Streptococci: ml3, c10, 712.

Media and propagation. Glucose Lemco (GL) broth consisting of (g./l.): Evans peptone, 10; Oxoid Lab-Lemco, 10; glucose, 10; NaCl, 5; water to 1000 ml.; adjusted pH to 7.2; glucose Lemco (GL) agar was prepared from GL broth by adding 1.5% agar. Strains of groups A, B, C and D streptococci were incubated at 37°; strains of group N at 30°. Growth was estimated by readings of extinction with a Hilger Biochem Absorptiometer at 580 mμ (E_{580}): and E_{580} of 1.0 corresponded to about 9×10^8 cocci cm⁻². Viable streptococci were enumerated by the method of Miles & Misra (1938). Phage counts were made by adding 20–200 phage particles (in 1.0 ml.) to 3.0 ml. of $\frac{1}{2}$ strength GL agar containing 0.025% (w/v) CaCl₂ and 0.1 ml. of a 24 hr culture of the homologous streptococcus at 45°, and poured on GL agar. The efficiency of plating was about 0.1.

Preparation of cell-wall suspensions. Cocci were harvested from GL broth, washed twice in distilled water and broken by shaking with ballotini beads (Chance no. 12) in a Mickle disintegrator in the cold room. After shaking for 2 hr the cell walls were freed from cytoplasmic impurities by treatment with trypsin, pepsin and ribonuclease (Cummins & Harris, 1956).

Assay of phage-lysin. Cocci grown for 18 hr in GL broth were washed in $\frac{1}{4}$ strength Ringer's solution, resuspended in 0.1 M-K-phosphate buffer (pH 6.7) and adjusted to give an E_{580} of 0.8 when diluted fivefold; 1.0 ml. of the standardized suspension pre-warmed to 37° was added to 4.0 ml. of enzyme preparation in the same buffer also at 37°; lysis was followed by measuring the decrease in extinction. It was found that lysis followed first-order reaction kinetics, the velocity constant K being given by the relationship:

$$K = \frac{2.3}{t} \log 10 \frac{a}{a-x}$$

where $a = E_{580}$ at 0 min., and $x = E_{580}$ at t min. One unit of activity was defined as the amount of enzyme required to give $K = 1$ under the conditions defined above. Figure 1 shows that there was a linear relationship between concentration and activity of the *ml 3* enzyme.

Purification of phage-lysins

Preparation of phage-lysates of streptococci. 20 l. of GL broth, pre-warmed to 30°, were given a 10% (v/v) inoculum of a 12 hr culture of appropriate streptococcus and incubated at 30°. When the extinction had risen to 0.6 (in about 3 hr) the cocci were harvested and resuspended in 100 ml. of $\frac{1}{4}$ strength Ringer's solution. This suspension was added to 3.5 l. double-strength GL broth at 30° which contained sufficient phage to give a phage:coccus ratio of about 6. The infected culture was stirred at 30° and maintained at pH 6.5. After 2 hr about 90% of the cocci were lysed. A few milligrams of DNAase were added to the viscous lysate, and after another 5 min. at 30° the lysate was centrifuged for 20 min. at 2000g to remove unlysed cocci and some cell debris. The supernatant fluid was chilled to 0° and all subsequent steps were done in a cold room at 4°.

Removal of phage by precipitation with acetone. Acetone at -20° was added to 1 l. batches of coccal lysate to give 40% (v/v) acetone; the temperature was kept at 0° by immersing the flask in an ice + salt mixture, which was left overnight at 4° and then filtered through Whatman no. 40 paper. The original phage titre of 10^{12} /ml. was decreased to 10^9 /ml. in the filtrate by this procedure.

Chromatography on Amberlite CG 50 resin. The clear filtrate from the previous step was passed through a 50 × 2 cm. column of Amberlite CG 50 ion-exchange resin equilibrated with a 60 + 40 by vol. mixture of 0.002 M-K-phosphate (pH 5.5) + acetone. The resin was washed with about 10 bed-volumes of 0.002 M-K-phosphate (pH 5.5) and then eluted with 0.2 M-K-phosphate (pH 7.3), 10 ml. fractions being collected and assayed. The active fractions were pooled, shell-frozen and finally freeze-dried.

Fractional precipitation from potassium phosphate buffers. The freeze-dried powder was shaken with 30 ml. distilled water at 37°. After about 1 min. the clear red-brown supernatant fluid was decanted from a small quantity of insoluble material and left overnight at 4°. A light-yellow precipitate (P1) was removed by centrifugation, washed with ice-cold 1.5 M-K-phosphate (pH 6.0) and dissolved in 0.1 M-K-phosphate (pH 6.7). This solution (S1) on standing at 4° overnight gave a second precipitate (P2) of inactive material which was removed by centrifugation. The colourless supernatant solution (S2) contained the enzyme.

Table 1 shows that a 500-fold purification of *ml 3* phage-lysin was achieved by the above procedure. Attempts at further purification on Sephadex G 100, DEAE cellulose and carboxymethylcellulose led to considerable losses of activity. The purified enzyme was relatively unstable: storage for 6 days at 4° resulted in 50% loss of activity. The freeze-dried preparation retained much of its activity when stored for 18 months in a desiccator at 4° and has been used routinely to prepare cell-free extracts of group N streptococci.

A shorter method of purification was used in some experiments. Coccal lysates were freed from phage by centrifugation for 2 hr at 100,000g and the phage-lysin in the supernatant fluid precipitated by adding solid ammonium sulphate.

The *ml 3* phage-lysin precipitated at between 50 and 75% saturation; the *c 10* enzyme was precipitated at 25–50% saturation. These precipitates were dissolved in 0.1 M-K-phosphate (pH 6.7) and de-salted by passage through columns of Sephadex G25. The phage-lysins prepared by the two methods appeared to have the same properties.

Table 1. *Purification of ml 3 phage-lysin*

Step	Volume (ml.)	Units/ml.	Total units	mg./protein/ml.
Crude phage lysate	3,400	14.5	49,200	10.0
Eluate from Amberlite CG 50 column	455	47.1	21,400	2.1
Precipitate P1 from 2 M-potassium phosphate	7.5	1,400	10,500	2.34
Supernatant fluid S2	7.5	875	6,550	1.20

Step	Specific activity	Purification	Yield (%)	Total phage
Crude phage lysate	1.45	—	—	9.5×10^{14}
Eluate from Amberlite CG 50 column	22.4	15.4	43.5	8.1×10^{11}
Precipitate P1 from 2 M-potassium phosphate	600	414	21.4	3.3×10^9
Supernatant fluid S2	730	503	13.3	1.0×10^9

Table 2. *Isolation of a lytic enzyme from ml 3 phage particles*

Step	Units/ml.	Volume (ml.)	Total units	Phage/ml.	Total phage
Washed phage particles	2.56	2.5	6.4	3.6×10^{11}	9×10^{11}
Frozen-thawed phage					
Before centrifugation	0.64	10	6.4	—	—
Supernatant fluid	0.224	10	2.24	2.5×10^9	0.25×10^{11}
Deposit	2.21	2.5	5.5	1.33×10^{11}	3.34×10^{11}
Phage-treated with ultra-sonic vibrations					
Before centrifugation	0.63	10	6.3	—	—
Supernatant fluid	0.36	10	3.56	4×10^9	0.4×10^{11}
Deposit	1.21	2.5	3.02	8.8×10^{10}	2.2×10^{11}

Amount of enzyme in original phage suspension: 6.4 units
Amount of enzyme released into solution: 5.8 units
Amount of enzyme remaining in phage particles: 3.25 units
Total amount of enzyme recovered 10.5 units
That is a recovery of 157 %

Preparation of a lytic enzyme from purified ml 3 phage particles. Phage *ml 3* particles were freed from cell debris and phage-lysin by centrifugation (three cycles of 8000g for 30 min. followed by 100,000g for 2 hr). A lytic enzyme was then released from a concentrated suspension of the particles in 0.1 M-K-phosphate (pH 6.7) by the freezing and thawing method of Weidel & Primosigh (1957). After freezing and thawing 20 times, about 70% of the phage was non-infective although no increase in viscosity was observed. After centrifugation at 100,000g for 2 hr the deposit, which presumably contained phage ghosts as well as infective particles, was ultrasonically treated at 0° in a Dawe Soniprobe Type 1130A (Dawe Instruments Ltd., London). Six exposures for 30 sec. at position 6 were separated by

cooling periods of 2 min.; insoluble material was deposited by centrifugation (100,000g). The amount of enzyme released into the supernatant by these methods is given in Table 2.

RESULTS

Physical properties of ml 3 and c 10 phage-lysins

Effect of pH value. The effect of pH value on the activity of the two phage-lysins was determined in 0.1 M-K-phosphate. Both enzymes showed a broad peak of activity in the range pH 6–7.5 with an optimum at pH 6.5–6.8.

Cations. Various authors (e.g. Doughty & Hayashi, 1962; Ghuyssens & Strominger, 1963) have emphasized the importance of cations for the activity of enzymes which act on bacterial cell walls. Both *ml 3* and *c 10* phage-lysins were activated by Na^+ , Li^+ , and NH_4^+ . Figure 2 shows the influence of Na^+ concentration on the activity of *c 10* phage-lysin. There was a sharp optimum at ionic strength 0.15; higher concentrations were inhibitory.

Fig. 1

Fig. 2

Fig. 1. *ml 3* phage-lysin activity measured as a function of protein concentration. Activity was measured with vacuum dried cells of *Streptococcus cremoris* E8.

Fig. 2. The influence of ionic strength on the activity of *c 10* phage-lysin against *Streptococcus cremoris* KII in NaCl solutions buffered with 0.005 M-phosphate, (pH 6.7).

Divalent cations Ca^{2+} and Mg^{2+} also activated the phage-lysins, but were less effective than monovalent cations although not inhibitory at higher ionic strengths.

Influence of reducing agents. The *ml 3* phage-lysin was relatively unstable; losses of the order of 50 % occurred on dialysis at 4° or on standing in the refrigerator for a few days. Other phage-lysins have been shown to be susceptible to oxidation or to require reducing agents for maximum activity (Maxted, 1957; Bleiweis & Zimmerman, 1961; Doughty & Hayashi, 1962). However, none of the group N phage-lysins was affected significantly by cysteine, ascorbic acid or thioglycollic (mercapto-acetic) acid in the concentration range 10^{-1} – 10^{-4} M, nor was it possible to reactivate aged preparations with reducing agents.

Heat activation and inactivation. Both of the phage-lysins had high temperature coefficients ($Q_{10} = 4.9$) and possessed almost identical energies of activation: 28.0 kcal. mole⁻¹ for the *ml 3* and *c 10* phage-lysins, respectively. At temperatures above 37°, rapid heat inactivation occurred. Figure 3 shows the effect of pH value on the heat inactivation of the two phage-lysins. Although *c 10* phage-lysin was more resistant to heat than the *ml 3* phage-lysin, the inactivation of both had the same dependency on pH value.

Fig. 3

Fig. 4

Fig. 3. The effect of pH on the heat inactivation of *ml 3* and *c 10* phage-lysins. The lysins (in 0.1 M-phosphate) were held at 45.5° for 3 min. and then rapidly cooled to 0°. Residual activity was measured with *Streptococcus cremoris* KH for *c 10* lysin, and *S. cremoris* E8 (vacuum dried) for *ml 3* lysin. ○ = *ml 3* phage-lysin; △ = phage-lysin.

Fig. 4. Lysis of *Streptococcus cremoris* KH by *c 10* phage-lysin—the effect of ageing of the cocci on the rate and extent of lysis. ○ — ○, 96 hr culture; ● — ●, 12 hr culture; △ — △, 12 hr culture aged 7 days at 0° in 0.1 M-phosphate pH 6.7; ▲ — ▲, 12 hr culture aged 5 days at 20° in 0.1 M-phosphate pH 6.7.

Effect of age of streptococci on their susceptibility to phage-lysin

Doughty & Hayashi (1962) reported that ageing of group A streptococci led to a progressive resistance towards lysis by the group C phage-lysins. A similar effect has been found in the lysis of group N streptococci; the effect of age on the lysis of *Streptococcus cremoris* KH by the *c 10* phage-lysin is illustrated in Fig. 4. Cocci from 4-day cultures were much less readily lysed than those from a log-phase culture (12 hr). The storage of cocci at 4° resulted in no loss of susceptibility, whereas those kept at room temperature became significantly more resistant.

Lysis of viable streptococci and the specificity of phage-lysins

Phage-lysin from *ml 3* was tested against a representative selection of streptococci of groups A, B, C, D and N. The conditions of assay were as given under

'Methods' except that a much wider range of phage-lysin concentration was used (up to 105 units). The first column in Table 3 represents the activity of the phage-lysin towards each streptococcal strain; the second column gives the activity expressed as a ratio of that towards a reference strain, *Streptococcus cremoris* E8 (see below).

Table 3. *The specificity of ml 3 phage-lysin towards strains of streptococci in Groups A, B, C, D and N*

Group	Strain	Activity/ml.*	Relative activity/ml.†
	<i>S. cremoris</i> E8	76.5	19.1
N	<i>S. cremoris</i> E8 (vacuum dried)	401	100
N	<i>S. cremoris</i> HP	8.95	3.98
N	<i>S. cremoris</i> US3	25.8	6.27
N	<i>S. cremoris</i> Z8	5.6	1.35
N	<i>S. cremoris</i> R6	35.6	8.85
N	<i>S. lactis</i> ML3	6.05	1.50
N	<i>S. lactis</i> C10	1.52	0.38
N	<i>S. lactis</i> 712	18.3	4.55
N	<i>S. diacetylactis</i> DRC1	9.45	2.36
A	<i>S. pyogenes</i> 3/3A	0	0
A	<i>S. pyogenes</i> Richards	0	0
A	<i>S. pyogenes</i> Pope	0	0
B	<i>S. agalactiae</i> A178	0	0
B	<i>S. agalactiae</i> 8187	0	0
B	<i>S. agalactiae</i> 8080	0	0
C	<i>S. dysgalactiae</i> C4	0	0
C	<i>S. dysgalactiae</i> K64	0	0
C	<i>S. dysgalactiae</i> S52	0	0
D	<i>S. bovis</i> Pearl 11	0.39	0.10
D	<i>S. zymogenes</i> Mahoney	1.09	0.27
D	<i>S. durans</i> 98/D	0.24	0.06
D	<i>S. liquefaciens</i> Elv/2025	1.13	0.28
D	<i>S. faecalis</i> 775	0.94	0.23

* The activity was measured as described under 'Methods'.

† The relative activity is defined as the ratio:

$$\frac{\text{Activity towards a strain}}{\text{Activity towards the reference strain (vacuum dried E8)}}$$

Table 4. *A comparison of the specificities of ml 3 and c10 phage-lysins*

Strain	ml 3 phage-lysin		c 10 phage-lysin	
	Activity/ml.*	Relative activity/ml.†	Activity/ml.*	Relative activity/ml.†
<i>S. cremoris</i> KH	4.33	1.00	50.3	1.00
<i>S. cremoris</i> 924	6.41	1.48	5.75	0.114
<i>S. cremoris</i> E8	13.1	3.22	4.35	0.087
<i>S. cremoris</i> TR	5.79	1.32	10.35	0.206
<i>S. lactis</i> M2S1	4.95	1.14	6.32	0.126

* The activity was measured as described under 'Methods'.

† Relative activity is defined as the ratio:

$$\frac{\text{Activity towards a strain}}{\text{Activity towards the reference strain (KH)}}$$

Strains of groups A, B and C streptococci were unaffected by *ml 3* phage-lysin, but the groups D and N strains were all lysed. Strains within a group varied in their susceptibility to the phage lysin. *Streptococcus cremoris* E8 was the most readily lysed and, dried over CaCl_2 in a desiccator at 4° , was a useful reference organism for use in assaying *ml 3* phage-lysin. *Micrococcus lysodeikticus* was not lysed by *ml 3* phage-lysin.

When several strains of group N streptococci were examined for their sensitivity to the *c 10* phage-lysin, the pattern of susceptibility to the *c 10* phage-lysin was found to be different from that to the *ml 3* phage-lysin (Table 4). The differences are most apparent when activities are expressed relative to the activity towards one particular strain (in this case *Streptococcus cremoris* KH). Strain KH was the most susceptible strain for the *c 10* phage-lysin and the least affected by the *ml 3* phage-lysin, proving that the two phage host systems produced two different phage-lysins. Although these have many physical properties in common, they can be differentiated by their kind of specificity patterns and also in their differing solubilities in ammonium sulphate solutions (see Methods).

The effect of phage-lysin and lysozyme on viable streptococci and cell walls

Viable cocci of lactic streptococci were lysed at very different rates by the *ml 3* phage-lysin (Table 3). This variation was also found with isolated cell walls of different streptococcal strains. Figure 5 shows that cell walls of the very sensitive strain of *Streptococcus cremoris* E8 were lysed much more rapidly than those of the relatively resistant *S. lactis* c10. Cell walls and viable cocci have the same relative susceptibilities to phage-lysin: this indicated that cell-wall structure was more important than other factors (e.g. osmotic fragility) in determining the inter-strain variations in sensitivity to the phage-lysin.

Egg-white lysozyme lyses group N streptococci only in the presence of EDTA (Brown, Sandine & Elliker, 1962) and has no effect on group A streptococcus cell-walls (Krause & McCarty, 1961). In Fig. 6 a comparison is made between the action of *ml 3* phage-lysin and lysozyme on viable cocci and cell walls of *Streptococcus cremoris* 972. Although lysozyme in the absence of EDTA was without apparent action on whole organisms, it was more effective in lysing cell walls than was *ml 3* phage-lysin at concentrations which gave rapid lysis of viable cocci. The similarity between the effects of EDTA on viable cocci and modifications of the cell surface during the preparation of cell walls on their sensitivity to lysozyme is being investigated.

The relationship between ml 3 phage-lysin and a lytic enzyme obtained from ml 3 phage particles

It is not known whether the lytic enzyme prepared from *ml 3* phage particles was obtained as discrete protein molecules or whether it was still attached to phage-tail subunits not sedimented by the centrifugation procedure. It is noticeable from Table 2, that in addition to lysis by the soluble phage-tail enzyme, *S. cremoris* E8 is also lysed by the infective particles of *ml 3* phage. This was due to a lysis-from-without phenomenon. During the isolation of the phage-tail enzyme, an increase in the total amount of lytic activity occurred. This might have been due to a variety

of factors. The irreversible adsorption of the phage to the bacterial surface might have resulted in a 'blocking' of the activity of the phage-bound tail enzyme but not of the soluble phage-tail enzyme. The release of more than one molecule of enzyme from each phage particle during the preparation of the phage-tail enzyme would be expected to give an increase in the effective enzyme concentration.

The pH optimum of the phage-tail enzyme was measured with dried *Streptococcus cremoris* E8 and found to be identical with that of *ml* 3 phage-lysin. A comparison of the specificities of the two enzymes is given in Table 5. From these results, the two enzymes appear to be identical.

Fig. 5

Fig. 6

Fig. 5. Lysis of viable cocci and cell walls by *ml* 3 phage-lysin. 10 mg. (dry weight) of cells or cell walls were suspended in 5 ml. of 0.1 M-phosphate buffer, pH 6.7 containing 1.0 unit of *ml* 3 phage lysin. ○ — ○, E8 cell walls; ● — ●, E8 viable cells; △ — △, c10 cell walls; ▲ — ▲, c10 viable cells.

Fig. 6. Lysis of viable cocci and cell walls of *Streptococcus cremoris* 972 by *ml* 3 lysin and lysozyme. 10 mg. (dry weight) of viable cocci or cell walls was suspended in 5.0 ml. 0.1 M-phosphate buffer, pH 6.7, containing either *ml* 3 phage-lysin (12 units) or lysozyme (2.5 mg.). ○ — ○, Viable cocci + phage-lysin; △ — △, viable cocci + lysozyme; ● — ●, cell walls + phage-lysin; ▲ — ▲, cell walls + lysozyme.

Phage control of lysin production

Since the two phage-lysins (*ml* 3, *c* 10) differed in certain respects, and since no lytic activity was detected in cell-free extracts of non-infected cocci an experiment was devised to illustrate the role of phage in lysin production. *Streptococcus lactis* ML3 is susceptible to two phages: *ml* 3, which forms large plaques and 712 which gives minute plaques. The same host was used to prepare phage-lysins by infection with either phage race. Whereas phage *ml* 3 produced large quantities of phage-

lysin, phage 712 gave lysates with little or no activity towards viable cocci. Treatment of cocci with acetone had been found to increase their sensitivity to *ml* 3 phage-lysin. When the 712 lysate was tested against acetone-treated cocci, a weak

Table 5. *A comparison of the specificities of ml 3 phage-lysin and the lytic enzyme from ml 3 phage particles*

Strain	Phage lysin		Enzyme from thawed and thawed phage	
	Activity units/ml. $\times 10$	Relative activity*	Activity units/ml. $\times 10^2$	Relative activity*
<i>S. cremoris</i> KH	1.86	1.0	2.62	1.0
<i>S. cremoris</i> E 8	3.16	1.70	4.38	1.68
<i>S. cremoris</i> 972	1.0	0.53	1.46	0.56
<i>S. lactis</i> ML 3	0.62	0.37	0.62	0.24
<i>S. lactis</i> C 10	0.53	0.28	0.88	0.33

Strain	Phage lysin		Enzyme from sonicated phage	
	Activity units/ml. $\times 10$	Relative activity*	Activity units/ml. $\times 10^2$	Relative activity*
<i>S. cremoris</i> KH	2.48	1.0	1.86	1.0
<i>S. cremoris</i> 803	2.18	0.88	1.63	0.88
<i>S. lactis</i> 712	1.23	0.50	0.73	0.39
<i>S. lactis</i> C 10	0.63	0.25	0.41	0.22

* The relative activity is defined as the ratio:

$$\frac{\text{Activity towards a strain}}{\text{Activity towards the reference strain (KH)}}$$

Table 6. *The influence of phage type on the properties of phage-lysins produced in Streptococcus lactis ML 3: comparison of the specificities of ml 3 lysin, 712 lysin, and ML 3/712 lysin (ML 3 lysed by 712—see text)*

A. Lysis of viable cocci (activity/ml.)			
Strain	<i>ml</i> 3 lysin	712 lysin	<i>ML</i> 3/712 lysin
<i>S. lactis</i> ML 3	2.7×10^{-2}	0	0
<i>S. lactis</i> C 10	1.2×10^{-2}	0	0
<i>S. lactis</i> 712	6.5×10^{-2}	0	1.2×10^{-3}
<i>S. cremoris</i> KH	1.8×10^{-1}	0	0
<i>S. cremoris</i> E 8	2.7×10^{-1}	0	0
<i>S. cremoris</i> HP	1.2×10^{-2}	0	1.6×10^{-3}
<i>S. cremoris</i> 803	3.3×10^{-1}	0	0

B. Lysis of acetone treated cocci (activity/ml.)			
Strain	<i>ml</i> 3 lysin	712 lysin	<i>ML</i> 3/712 lysin
<i>S. lactis</i> ML 3	1.3×10^{-1}	7.8×10^{-3}	2.6×10^{-2}
<i>S. lactis</i> C 10	2.2×10^{-2}	0	0
<i>S. lactis</i> 712	2.3×10^{-1}	1.4×10^{-2}	4.6×10^{-2}
<i>S. cremoris</i> KH	3.0×10^{-1}	0	0
<i>S. cremoris</i> E 8	8.1×10^{-1}	3.8×10^{-2}	1.7×10^{-1}
<i>S. cremoris</i> HP	3.7×10^{-2}	1.1×10^{-2}	5.7×10^{-2}
<i>S. cremoris</i> 803	1.03	3.6×10^{-2}	1.1×10^{-1}

The *ml* 3 was a partially purified preparation (1 mg./ml.); the 712 and *ML* 3/712 lysins were crude phage lysates freed of phage by centrifugation.

lytic activity against some but not all strains was detected. In Table 6 a comparison is made between the lytic activity of lysates of the host *S. lactis* ML3 lysed by phage *ml 3* or phage 712, and of *S. lactis* 712 lysed by phage 712. It is evident that the lysin found in lysates of *S. lactis* ML3 lysed by phage 712 was very similar to that of lysates of *S. lactis* 712 lysed by phage 712, and not at all like the lysate obtained when *S. lactis* ML3 was lysed by its homologous phage, *ml 3*. This shows that the lysin produced in lysates of *S. lactis* ML3 was under the genetic control of the infecting phage.

Fig. 7. The lysis-from-without of *Streptococcus lactis* ML3 by *ml 3* phage. Cocci were suspended in 0.1 M- κ -phosphate (pH 6.7) at 37° and phage added to give the multiplicities; ○—○, 0 (control); △—△, 13.5; □—□, 27; ●—●, 54.

Effect of particulate phage on the lysis of viable streptococci lysis-from-without

In a preliminary account of this work (Reiter & Oram, 1963) evidence was given that the lysis of streptococci by *ml 3* phage-lysin could be inhibited by the homologous phage. These results appeared convincing because increasing multiplicities of phage infection caused a progressive inhibition of lysis. Furthermore, *ml 3* phage-lysin was not inhibited by heterologous phages.

At that time we had not observed lysis-from-without by phage: this was not surprising as this phenomenon had been previously reported only for Gram-negative organisms. However, recent attempts to cause lysis-from-without were successful (Fig. 7). High multiplicities of *ml 3* phage lysed not only *S. lactis* ML3, but also a number of heterologous strains of group N streptococci. Lysis-from-without of these strains occurred at different rates, but the relative rates of lysis followed the

same specificity pattern as those obtained for *ml 3* phage-lysin and *ml 3* phage-tail enzyme.

Since these results were in direct contradiction to the inhibition of lysis by homologous phage, the earlier experiments were repeated. An *ml 3* phage lysate freed of phage by centrifugation (100,000*g* for 2 hr) lysed all the heterologous strains which were tested, but was inactive against *S. lactis* ML3. However, this strain was sensitive to a purified preparation of *ml 3* phage-lysin, indicating the presence of an inhibitor in the crude preparations. Since phage *ml 3* particles could be freed of the inhibitor by several washes with $\frac{1}{4}$ strength Ringer's solution, it now seems probable that our earlier phage preparations were contaminated with the inhibitor.

DISCUSSION

In common with a number of other phage-lysins the group N streptococcus phage-lysins were activated by certain cations and inhibited by reagents which inactivate SH groups. However, unlike the phage-lysins from a group C streptococcus (Doughty & Hayashi, 1962) and a group D streptococcus (Bleiweis & Zimmerman, 1961) the lysis of streptococci by the *ml 3* and *c 10* phage-lysins was not enhanced by reducing agents.

The specificity of the *ml 3* phage-lysin is quite different from that of the phage-lysin of a group C streptococcus (Maxted, 1957). Barkulis, Smith, Botralik & Heymann (1964) reported that a phage-lysin, prepared from a group C streptococcus differed from egg-white lysozyme in the nature of the susceptible chemical bond; whereas lysozyme acts as a muramidase, breaking the bond between the *N*-acetylmuramic acid (NAMA) and the *N*-acetylglucosamine (NAGA) residues in the mucopeptide polymers of the cell-wall, the group C streptococcal phage-lysin acted as a glucosaminidase, breaking the NAGA-NAMA linkage:

Our work (unpublished) shows that the *ml 3* phage-lysin is, like egg-white lysozyme, a muramidase. It seems possible that the specificity difference between the phage-lysins of the group C and group N streptococci may be due to differences in both the cell-wall structure and the site of enzyme action. The lysis of group D streptococci by the *ml 3* phage-lysin is not surprising in view of the serological similarities between the streptococci of groups D and N (Sharpe, 1952).

Whereas the streptococcal phage-lysins act on viable streptococci, the phage-lysins of *Bacillus megaterium* (Murphy, 1957) and of *Staphylococcus aureus* (Ralston, Baer, Liebermann & Krueger, 1957) were not active against viable bacteria unless particulate phage was also present. Ralston (1963) proposed that teichoic acids in the phage receptor sites mask regions in the staphylococcal wall which are sensitive to phage-lysin. Since the removal of teichoic acids with hot 5% trichloroacetic acid left the cocci sensitive to phage-lysin, she suggested that the adsorption of phage involved changes in the arrangement of teichoic acid, leading to the exposure of the lysin-sensitive sites. Teichoic acids are absent from the cell walls of the group N streptococci (Oram & Reiter, unpublished) and to our knowledge have not been

found in the cell walls of group A streptococci, lysis of which by group C streptococcus phage-lysin is also independent of phage sensitization (Krause, 1958).

The lysis-from-without of the group N streptococci by phage is similar to that by the phage-tail enzyme and by phage-lysin. The relative rates of lysis for different strains of streptococci are very similar in each case. It seems, therefore, that the three processes occur at the same site on the bacterial surface—the phage receptor. Both *ml 3* and *c 10* phages adsorb to a variety of streptococci within the group, without phage multiplication (Oram & Reiter, unpublished). The differences in the rate of lysis of a strain by the *ml 3* and *c 10* phage-lysins might well be due to attacks at different phage receptors. The proposal that the substrate of these phage-lysins lies within the phage receptor is supported by the inability of the phages to adsorb to mucopolysaccharide material released from the cell wall by phage-lysin. Lysozyme caused a similar loss in the integrity of the phage receptors of *Bacillus megaterium* KM (Salton, 1956) and of *Micrococcus lysodeikticus* (Brumfitt, 1960).

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