Lactic Streptococcal Phage-Associated Lysin. I. Lysis of Heterologous Lactic Streptococci by a Phage-Induced Lysin

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Abstract

This investigation concerns the study of a lactic phage-associated lysin released when Streptococcus lactis strain C10 cells were lysed by their homologous e10 phage. The lysin appeared in the medium when the cells lysed. A spreading halo surrounding the clear phage plaques was observed after prolonged incubation of double agar layer plates. Both living and sensitized C10 cells were lysed by the e10-lysin. All heterologous lactic streptococcal strains studied thus far were lysed by the e10-lysin. A lysozyme-sensitive strain of Sarcina lutea was very resistant to e10-lysin. Micrococcus lysodeikticus substrate cells (Difco) were lysed rapidly during the initial reaction period, but lysis diminished rapidly soon thereafter. The role of e10-lysin in the nascent phenomenon is discussed.

Phage-associated lysins have been discovered and studied in several host-phage systems (5-9, 11). However, relatively little information is available concerning lactic phage-associated lysins. Naylor and Czulak (9) studied a lactic streptococcal phage lysin capable of lysing lactic streptococcal strains even when the culture was resistant to the phage. A nascent phenomenon, attributable to the phage-induced lysin, was described. Whitehead et al. (12) and Collins (3) studied a nascent phage phenomenon but did not suggest that a lytic agent was involved. They attributed the nascent effect to some inhibitory action of the phage on the supposedly resistant lactic streptococcal strains. Since most lactic streptococci are resistant to the action of lysozyme (2), even under carefully controlled conditions, the discovery of a lytic agent of biological origin capable of cellular lysis would be of value in studying the structure or chemical composition of lactic streptococci. The object of this investigation was to determine the nature of lysin production by a lactic host-phage system, to determine its specificity for different bacteria, and to examine its nascent characteristics.

Materials and Methods

Stock cultures. Lactic streptococcal strains 712, W2, and HP were supplied by E. B. Collins (Department of Food Science and Technology, University of California, Davis). Strains MIJ, C10, C2, and their homologous phage were obtained from R. E. Hargrove (Dairy Products Lab., Eastern Util. Develop. Div., USDA). Stock cultures were maintained on trypticase soy agar (BBL) and in litmus milk (BBL). Phage stocks were maintained in litmus milk and in trypticase soy broth supplemented with 0.02% L-cystine (4) and 0.0005 M CaCl₂ (10). This medium will be subsequently referred to as TSCa.

Phage assays. Phage assays were determined by the double agar layer technique (1), using trypticase soy agar containing 0.02% added L-cystine, subsequently designated TSA. A 4-5-hr broth culture of C10 bacteria was used for the seed layer. All cultures were repropagated for three consecutive days in TSCa prior to their use.

Preparation of heat-killed substrate cells. Substrate cells were grown in trypticase soy broth supplemented with 0.02% L-cystine TScy. Heat-killed substrate cells were prepared by harvesting 5-6-hr TScy cultures, washing them twice in 0.067 M Na₂HPO₄/KH₂PO₄ phosphate Sorenson buffer at pH 6.5, then resuspending the cells in the same buffer. The cell suspensions were standardized to contain approximately 10⁸ cells/mliliter. The cell suspensions were heat-killed at 65°C for 1 hr, then stored at 4°C.

Assay of e10-lys in activity. Lytic activity was measured by turbidimetric determination of cellular lysis, using a Klett-Summerson photoelectric colorimeter (11). The reaction mixture was composed of substrate cells, buffer, and a suitable dilution of e10-lys in, so that the total volume was 5 ml. Standard chemically cleaned Klett tubes were used to take lysis measurements. For each test, all samples received a uniform amount of cells unless otherwise stated.
All c10-lysin assays were run at 37 C and at pH 6.5 unless otherwise indicated. Changes in turbidity were converted to changes in bacterial count by use of a standard curve prepared from direct microscopic counts of S. lactis strain C10 cells. From the data, the course of lysis could be represented by plotting the logarithm of the unlysed bacteria against time under the experimental conditions. Estimates of lysis activity were based upon determination of the initial velocity constant, K, calculated from the equation: 

\[ K = \frac{2.3}{t} \log \frac{B_0}{B_t} \]

where \( B_0 \) represents the initial concentration of cells per milliliter and \( B_t \) the cells per milliliter lysed in any given time, t. The K values are only valid for comparisons within individual experiments.

**Production of c10-lysin.** The production of c10-lysin was determined in 1/2 strength TSCa C10 cultures at 30 C when the initial phage/bacterium ratio was approximately 1/100. Samples of the cell/phage mixture were removed periodically and chilled in an ice bath. Total and free phage titers were determined on millipore filtered and unfiltered samples. Lysin activity was determined on centrifuged lysate samples. Suitable dilutions of the supernatant layers were mixed with approximately 2 \( \times 10^8 \) C10 cells/milliliter at 37 C. The velocity constants, K/min, were calculated as described in the previous paragraph.

**Preparation of crude c10-lysin.** Crude c10-lysin was prepared by infecting log phase S. lactis C10 cultures with phage at a phage-to-bacterium ratio of 1/100. The cultures were grown in 1/2 strength TSCa. The CaCl₂ was added just prior to inoculation with the C10 organisms. The phage-cell mixtures were shaken for 5 min and incubated at 30 C until lysis was complete (1-2.5 hr). The lysates were centrifuged at 7,000 rpm in a Servall RC-2 refrigerated centrifuge for 20 min at 4 C. Ammonium sulfate fractionations were made at 0-55% saturation and 55-75% saturation. The precipitate from the 55-75% fraction contained essentially all the c10-lysin activity. This precipitate was resuspended in Sorenson buffer (pH 6.5). Dialysis against the same buffer for 48 hr, followed by two successive 1-hr centrifugations at 45,000 rpm in a Beckman model L2 ultracentrifuge, reduced the phage titer to less than 10⁴ particles/milliliter.

**Production of c10-lysin at different stages of growth.** A 250-ml TSCa culture of S. lactis strain C10, contained in a sidearm flask to facilitate turbidity measurements, was prepared. Samples were removed at periodic intervals and were inoculated with c10 phage so that the phage/bacterium ratio was approximately the same at each sampling. Each sample was shaken and incubated at 30 C until lysis was complete, or no longer than 6 hr. The lysates were then centrifuged and assayed for c10-lysin activity.

**c10-Lysin activity on heat-killed and resting cells harvested at different stages of growth.** A C10 TSey culture was prepared in the manner described in the preceding section. Duplicate samples were taken at periodic intervals. The samples were harvested and washed twice in Sorenson buffer. One set of the duplicate samples was heat-treated at 65 C for 1 hr. The samples were then tested for susceptibility to c10-lysin activity.

**Lysozyme studies.** The activity of c10-lysin and lysozyme (Worthington Biochemical Corporation, Freehold, N.J.) was compared in reciprocal tests using C10 cells, a lysozyme-sensitive strain of Sarcina lutea, and Micrococcus lysodeikticus (lysozyme substrate, Difco). Sarcina cells were tested at a lysozyme concentration of 1 mg/milliliter, and M. lysodeikticus substrate cells at 0.002 mg/milliliter. S. lactis C10 cells were tested at both concentrations. Sarcina cells were grown on trypticase soy broth, harvested, and heat-treated at 65 C for 1 hr in a manner identical to the C10 cell preparations. Lysozyme action on C10 cells was also determined in the presence of 0.1 M versene (disodium salt of ethylenediaminetetraacetic acid) and 0.15 M NaCl at pH 8.0.

**Results and Discussion.**

The first indication of c10-lysin production was the appearance of a spreading halo surrounding the small central c10 phage plaques. The halos appeared two or three days following the normal 24-hr incubation period and continued to increase in size up to nine days. If the plates were exposed to chloroform vapors for one-half hour immediately following the normal 24-hr incubation period, the halos appeared sooner.

The c10-lysin was present in the lysates of c10 phage-infected cultures in amounts increasing until mass lysis occurred. Attempts to detect the lytic agent in uninfected cultures by sonication or autolysin induction were not successful. The appearance of c10-lysin in the lysates of infected C10 cultures is shown in Figure 1. LFW represents lysis from without of approximately 10⁴ live log phase C10 cells/milliliter. This curve shows that when the concentration of phage or lysin was high enough, cell lysis was immediate, as opposed to normal
phage lysis, wherein a latent period precedes lysis. These results are similar to some presented by Ralston et al. (11) for a staphylococcal phage lysin.

Table 1 indicates that cl0-lysin production was greater during the log phase. This was also true of phage production, which again points to the close relationship between cl0-lysin and cl0 phage production. Although all the samples were completely lysed within 2.5 hr, except the 24-hr sample, the amount of lysis production was greatly reduced as the incubation time increased. On the other hand, the susceptibility of both resting and heat-killed S. lactis C10 cells to cl0-lysin activity was not very different regardless of the stage of growth up to 24 hr. These data indicate that the cl0-lysin has an extracellular action independent of the metabolic state of the susceptible substrate cells. When log phase C10 cells were grown in TSCa, harvested, and heat-treated, cl0-lysin activity did not occur.

The presence of calcium in the reaction tube did not influence the rate of lysis when added to heat-treated cells grown in the absence of calcium. The cl0-lysin lysed both resting and heat-killed C10 cells in the presence or absence of cl0 phage. Investigators (8, 11) of other phage-associated lysins have shown that the host cells must be sensitized by heat, UV irradiation, acetone, or some other means before the lysin, in the absence of phage, will lyse the substrate cells. Treatment of C10 cells with acetone and chloroform renders the cells more susceptible to lysis but is not a prerequisite for cl0-lysin activity. Maxted (7) found that a Group A streptococcal phage-induced lysis was able to lyse live as well as heat-killed cells. The effect of heat on cl0-lysin activity is shown in Figure 2. It appears that the greater the heat treatment the greater the percentage of resistant cells. The action mechanism of cl0-lysin on nonsensitized C10 cells in the absence of significant numbers of phage (1 phage particle/10^9 bacteria) is not known.

**Table 1**

<table>
<thead>
<tr>
<th>Inoculation time (hr)</th>
<th>Total bacteria added</th>
<th>Total phage</th>
<th>Velocity constant K/min</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>7.6 × 10^8</td>
<td>1.2 × 10^9</td>
<td>0.0835</td>
</tr>
<tr>
<td>4</td>
<td>1.5 × 10^10</td>
<td>2.4 × 10^9</td>
<td>0.0787</td>
</tr>
<tr>
<td>6</td>
<td>2.3 × 10^10</td>
<td>3.8 × 10^9</td>
<td>0.0481</td>
</tr>
<tr>
<td>7</td>
<td>3.1 × 10^10</td>
<td>5.7 × 10^9</td>
<td>0.0113</td>
</tr>
<tr>
<td>24</td>
<td>5.0 × 10^10</td>
<td>8.9 × 10^9</td>
<td>0.00001</td>
</tr>
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</table>

**Fig. 1.** Production of cl0-lysin and phage in 1/2 strength TSCa at 30°C when the initial phage bacterium ratio was approximately 1/100.

**Fig. 2.** Effect of cl0-lysin on S. lactis C10 following heat treatment for 1 hr at different temperatures.
The c10-lysin lysed all lactic streptococcal strains tested. In addition, an alpha and a beta hemolytic streptococcus were lysed as well as an enterococcus. The c10-lysin did not lyse a strain of E. coli, *Aerobacter* sp., or *Klebsiella* sp. Both living and heat-killed lactic streptococcal strains were lysed by c10-lysin. The lysis patterns of six strains, prepared identically, are shown in Figure 3. Table 2 shows the relative K/min values of c10-lysin on the same organisms following heat treatment at 65°C for 1 hr.

These results demonstrate the ability of c10-lysin to lyse heterologous lactic streptococcal strains. This explains the role lactic phage-induced lysins may play in the so-called nascent phenomenon. The work of Collins (3) showed that certain strains of lactic streptococci exhibit the nascent effect, whereas others do not. Strains W2 and HP were very susceptible, as they were in this study. This evidence supports the idea that phage-induced lysins such as c10-lysin are responsible for nascent lysis in mixed lactic streptococcal cultures.

Microscopic observation of lactic streptococcal cells undergoing lysis by c10-lysin reveals they become irregular in shape and that resistant cells become gram-negative. Work is being continued to identify the specific substrate of c10-lysin action.

A lysozyme-sensitive strain of *Saccharomyces lutea* was resistant to c10-lysin whether or not the cells were living or heat-killed. Lysozyme lysed resting and heat-killed C10 cells only slightly even in the presence of 0.1 M versene (disodium salt of ethylenediaminetetraacetic acid). Brown et al. (2) obtained similar results with versene and lysozyme when phosphate buffers were employed. *M. lysodeikticus* substrate was lysed rapidly by c10-lysin during the initial minutes of the reaction, but the rate of lysis decreased rapidly. These results are shown in Figure 4.

This study indicates that c10-lysin and perhaps other lysins from different lactic streptococcal host-phage systems may some day provide a means for lysis of lactic streptococci for investigations concerned with bacterial anatomy and genetics. This work also supports the results shown by Naylor and Czulak (9), that phage-lysins are responsible for nascent lysis of heterologous *S. lactis* cultures. Work is being continued in this laboratory toward the characterization of the c10 lytic agent. Attempts are being made to purify the c10-lysin and identify its specific substrate.

**Table 2**

<table>
<thead>
<tr>
<th>Strain</th>
<th>K/min</th>
</tr>
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<tbody>
<tr>
<td>W2</td>
<td>0.0976</td>
</tr>
<tr>
<td>712</td>
<td>0.0226</td>
</tr>
<tr>
<td>C2</td>
<td>0.0493</td>
</tr>
<tr>
<td>MLI</td>
<td>0.2008</td>
</tr>
<tr>
<td>HP</td>
<td>0.1118</td>
</tr>
</tbody>
</table>

**Fig. 3.** Effect of c10-lysin on live heterologous lactic streptococcal strains. C10/I represents a c10 phage-resistant mutant of *S. lactis* strain C10.

**Fig. 4.** Comparison of lysozyme and c10-lysin action on *S. lactis* C10 cells and *M. lysodeikticus*. Lysozyme concentration equals 0.002 mg/milliliter. A = lysozyme action on live *S. lactis* C10; B = lysozyme action on heat-killed C10 cells; C = action of c10-lysin on dried *M. lysodeikticus*; D = action of lysozyme on dried *M. lysodeikticus*. 
Acknowledgments
We wish to acknowledge the advice received from Drs. J. R. Suriano and S. Tokuda, as well as facilities provided by the Department of Medical Microbiology and some financial support from the Department of Animal and Dairy Husbandry. We also thank Drs. R. E. Hargrove and E. B. Collins for their kindness in providing cultures.

References
(8) Murphy, J. S. 1957. Phage Associated Enzyme of B. megaterium which Destroys the Bacterial Cell Wall. Virology, 4: 563.