Chemical Composition of a Bacteriophage for Streptococcus diacetilactis

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Abstract

Electron photomicrographs revealed that the tadpole-shaped phage particle had a head width and length of 60 μm and a tail length of about 170 μm. The dried phage contained 4.45% P and 12.8% of the protein was N.

Bacteriophages prepared in broth medium were recovered by differential centrifugation and purified in a cesium chloride density gradient. An amino acid analysis revealed the following mole percentages for these acids: lysine, 8.1; histidine, 1.7; arginine, 3.8; aspartic acid plus asparagine, 10.0; threonine, 7.5; serine, 6.8; glutamic acid plus glutamine, 12.0; proline, 3.4; glycine, 9.0; alanine, 9.5; valine, 8.9; methionine, 1.1; isoleucine, 7.9; tyrosine, 2.2 and phenylalanine, 2.8.

The nucleic acid of the phage was double-stranded DNA with a thermal melting point (Tm) of 84.3°C; from this, an average composition of 35.7% guanine plus cytosine was calculated.

The morphology and other properties of lactic streptococcal bacteriophages have been studied by several workers (9–13, 25). To date, however, no one has characterized the nucleic acid or protein components of these viruses. Such studies of the T series of phages for Escherichia coli have been made (18), and the present communication reports the application and modification of previously described procedures in characterizing a bacteriophage for Streptococcus diacetilactis.

Electron microscope studies of the lactic streptococcal bacteriophages have been reported by Parmelee et al. (26), Sandine et al. (28), and Williamson and Bertaud (31). These reports indicate that not all lactic streptococcal phages are morphologically similar; for example, the phages studied by the former two groups of workers were 180 to 280 μm in total length; they were tadpole-shaped with a head diameter of 60 to 90 μm and a tail length of 120 to 190 μm. However, photographs published by Williamson and Bertaud (31) revealed a lactic phage that had a head diameter of 70 to 80 μm and a curved tail 560 to 610 μm in length.

The protein coats of the T phages have been analyzed for amino acid content by Fraser and Jerrel (18), Fitch and Susman (17), and Polson and Wyckoff (27). Also, DNA of these viruses has been isolated, purified, and analyzed by several workers (8, 14, 29). The heat denaturation of DNA, accomplished by the method of DeLey and Schell (15) also has been used to characterize the nucleic acid of phages. Heating is accomplished to determine the thermal melting point (Tm), the temperature at which the double-stranded DNA separated into a single-stranded random coil; the Tm value then is used to calculate the average guanine plus cytosine (G + C) content of the DNA. A new method of plotting the thermal denaturation data has been described by Knittel et al. (20, 21). This method makes use of normal-probability graph paper for determination of the Tm. Plot of the thermal melting data in this manner results in a straight line and the 50 percentile point on the graph indicates the Tm value.

Experimental Procedure

The lactic streptococcal bacteriophage studied was isolated from Cottage cheese whey obtained from a commercial dairy. The host for the bacteriophage, a strain of S. diacetilactis, was obtained from a commercial mixed-strain lactic starter by serial dilution plating with lactic agar (16). The designations given the host organism and the bacteriophage in our laboratory were AB3M9 and AC1L16, respectively.

Phage lysates were prepared by infecting the host organism in log growth phase with the phage at a multiplicity of infection (the ratio of number of plaque-forming units to the number of bacteria) of approximately 0.01. The infected cultures were incubated at room temper-
Preparation of bacterial deoxyribonucleic acid. The method of Marmur (23) was the basic procedure used for isolation of DNA from the host organism, though it was necessary to modify the procedure to obtain DNA from the lactic streptococci. Eighteen liters of sterile lactic broth were inoculated with 50 ml of an 18-hr culture of \textit{S. diacetilactis} AB3M9, followed by incubation of the culture at 30°C for 24 hr. The cells were harvested using the continuous flow centrifugation attachment for the Sorvall RC2 refrigerated centrifuge operating at a rotor speed of 10,000 rpm. Adjustment of the flow rate by pinch clamps was made to insure that the supernatant was clear. The cells were resuspended in 0.15 M sodium chloride prepared in 0.1 M ethylenediaminetetraacetic acid (EDTA) buffer at pH 8.0. The cells were then resedimented in the SS-34 rotor of the Sorvall RC2 operating at 4,500 rpm.

The harvested cells were resuspended in the pH 8.0 saline-EDTA buffer to a volume of 25 ml. Crystalline lysozyme (4 mg) was added to the cells and the mixture was held in a 37°C water bath for four hours. Two milliliters of a 25% sodium lauryl sulfate solution were added and the mixture, now viscous, was cooled in an ice bath and then transferred to a glass-stoppered 250-ml Erlenmeyer flask. The sodium ion concentration was adjusted to 1.0 M by addition of 5.0 M sodium perchlorate. A volume of 24:1 chloroform:isooamyl alcohol mixture was then added to deproteinize the DNA preparation. The mixture was shaken on a reciprocal shaker (160 strokes/minute) at room temperature for 30 minutes. The resulting mixture of protein and nucleic acid was centrifuged at 12,000 rpm for 5 minutes. The top layer was removed with a pipette, and the nucleic acid was precipitated by addition of two volumes of ice-cold 95% ethanol. The nucleic acid then was recovered from the alcohol-water interface on a glass stirring rod. The collected DNA was finally dissolved in saline-citrate solution (0.015 M NaCl and 0.0015 M sodium citrate) adjusted as before to the SSC concentration.

The extracted nucleic acid was then treated with ribonuclease (0.2% in 0.15 M NaCl, pH 5.0, Calbiochem, Los Angeles, California) for 30 minutes at a final DNA concentration of 50 μg/milliliter. The nucleic acid solution was treated again with the chloroform:isooamyl alcohol, precipitated and redissolved in 9 ml of dilute saline citrate. One milliliter of 5.0 M sodium acetate was added and the solution stirred. The nucleic acid was precipitated by the addition of 5 ml of isopropyl alcohol, drop-wise, to the mixing solution. The DNA was washed, first in 70% ethanol and then in 95% ethanol, and finally dissolved in SSC solution at pH 7.0.

Preparation of bacteriophage DNA. Three liters of phage lysate of bacteriophage AC1L1 16M grown on \textit{S. diacetilactis} AB3M9 were prepared in modified lactic broth. Bacterial debris was removed by continuous flow centrifugation. Bacterial DNA was destroyed by addition of DNase (0.04 μg per milliliter of lysate) with incubation at 37°C for one hour after the Mg⁺ concentration was adjusted to 0.01 M with a solution of MgSO₄. After incubation the lysate was centrifuged for 90 min with the 21 rotor in the Beckman Model L-2 ultracentrifuge at 17,500 rpm. The supernatant was discarded, and the pellets were allowed to suspend naturally by holding in 1.10 ml of SSC in the centrifuge tubes at 4°C overnight. The resuspended phage samples were pooled.

The procedure for the preparation of purified phage DNA was a modified method of Anderson (4). Equal volumes of phage suspension and buffer-saturated phenol (the phenol was distilled just prior to use and showed no trace of yellow color) were gently mixed for five minutes. The buffer used for saturation of the phenol was 0.15 M NaCl, 0.15 M Na₂HPO₄, and 0.02 M sodium citrate, adjusted to pH 8.0. The phage-phenol mixture was centrifuged at 4,500 rpm for five minutes. The aqueous layer was drawn off with the aid of a capillary pipette and extracted twice more with equal volumes of buffer-saturated phenol. Following the phenol extractions, five deproteinizations were performed with the chloroform:isooamyl alcohol (24:1) procedure described above. The DNA solution and the chloroform:isooamyl alcohol were mixed in equal volumes, shaken gently for five minutes, centrifuged at 4,500 rpm for five minutes, and the aqueous layer then removed.

After deproteinization, the DNA preparation was dialyzed in one-half-inch cellophane dialysis tubing for 48 hours against five changes of SSC.
The concentration of DNA was determined using 260 and 280 nm absorbancy readings with the nomograph of Adams (1) prepared from other data (30).

Thermal denaturation studies. The general procedure outlined by DeLey and Schell (15) was used to obtain the absorbancy data used for determination of the thermal melting (Tm) values of the DNA preparations. The purified DNA was dissolved in SSC at pH 7.0 and diluted with SSC to give an absorbancy reading between 0.20 and 0.25 at 260 nm. Three milliliters of the solution were placed in a glass-stoppered Beckman standard silica cuvette. Three milliliters of the SSC solution were placed in a second cuvette which was used as a blank. The two cuvettes were placed in a cuvette holder and then in the sample chamber of a Beckman DU spectrophotometer that was part of a Gilford Model 2000 multiple absorbance recording system. The Gilford system was equipped with a cuvette positioner, a linear thermosensor, an auxiliary offset control, and an automatic blank compensator.

The sample chamber was enclosed on two sides by thermospacers connected to a circulating, thermostatically controlled Haake water bath. The bath circulated heated propylene glycol through the thermospacers. A second set of thermospacers circulated tap water which protected the optical system of the spectrophotometer from excessive heat.

The DNA sample was equilibrated at 25°C and the absorbancy was recorded. The temperature of the chamber was raised to 70°C and the samples allowed to equilibrate for about 20 minutes before further temperature increase. After equilibrium at 70°C, the temperature was raised by manual adjustment of the Haake thermostat at a rate of about 0.25 degree per minute. The changes in absorbancy and temperature were recorded.

The temperature was increased until a plateau in absorbancy was reached. At this point a manual reading of the absorbancy was taken. Data were taken from the curves so that the absorbancy readings could be corrected for thermal expansion by multiplying the absorbancy by the ratio: Volume of water sample at T°C ÷ volume of sample at 25°C.

Phage purification for amino acid analysis. Twenty-two liters of phage ACIL116M grown on S. diacetilactis AB3M9 in one-liter quantities of the modified lactic broth were prepared. The lysates were clarified by centrifugation, using the GSA rotor of the RC2 Sorvall centrifuge operating for 20 minutes at 6,000 rpm. The clarified lysates were filtered successively through 1.2 and 0.45 μm pore diameter size membrane filters for removal of bacterial debris. The filtrate was then centrifuged with the 21 rotor of the Beckman Model L2 ultracentrifuge running at 17,500 rpm for 90 min. The pellets were resuspended by standing overnight in 3 ml of Tris-saline buffer (0.05 M NaCl, 0.01 M Tris, and 0.001 M MgCl₂ at pH 7.3). The resuspended pellets were then centrifuged at low speed to pellet debris. The supernatant was centrifuged for two hours at 19,000 rpm in the 30 rotor of the Model L2. The pellets were allowed to resuspend by standing overnight in 3 ml of the Tris-saline buffer. The resultant suspension was centrifuged at low speed for removal of debris and the supernatant filtered through a 0.8 μm diameter pore size membrane filter for removal of as much debris as possible.

A cesium chloride density gradient was used for phage purification. The method was essentially that of Ganesan and Lederberg (19). The cesium chloride, 99% (K and K Laboratories, Plainview, New York), was prepared as a saturated solution in the Tris-saline buffer. Two milliliters of saturated cesium chloride and 3 ml of phage suspension were added to a 5-ml nitrocellulose centrifuge tube, followed by centrifugation at 29,000 rpm for 20 to 24 hours at 4°C in the SW 39 swinging bucket rotor of the Beckman Model L ultracentrifuge.

The band of phage (Fig. 1) was visible in the gradient and a Beckman tube slicer was used to remove the band. The phages were removed from the gradient and were dialyzed against two changes of Tris-saline buffer for 48 hours.

Amino acid analysis of phage. Preparation
of phage protein for amino acid analysis was performed using the methods of Fitch and Sussman (17). The purified phage in Tris-saline buffer had a bluish opalescence. An equal volume of 10% trichloroacetic acid (TCA) was added to the suspension of phage that contained about 4 mg of protein, as determined by the Lowry method (22). The mixture was heated to 90°C for 15 minutes, cooled, and centrifuged for 30 minutes at 5,000 rpm.

The precipitate was washed three times with 5% TCA and finally suspended in a small amount of H₂O and dialyzed 26 hours against distilled H₂O. The dialyzed precipitate was lyophilized. The dried protein was added to an ignition tube (VirTis—5-ml ampule) and flushed repeatedly with nitrogen after addition of constant-boiling HCl (6). The tubes were then sealed and placed in an oven at 110°C for 24 hours. Aliquots of the resultant hydrolysate were chromatographed on a Beckman Spinco Model 120 amino acid analyzer.

Electron microscopy. Bacteriophages which had been purified in the cesium chloride density gradient were used for negative staining and examination. An RCA EM2 electron microscope was used. The negatively stained preparations were made by the method of Brenner and Horne (7).

Phosphorus determination. The determination of phosphorus in the purified lyophilized phage was accomplished by the method of Allen (3). A standard curve was prepared for the spectrophotometric procedure by dissolving 1.0967 g of KH₂PO₄ (dried in an air oven at 110°C for 24 hours) in water and diluting to 250 ml.

Nitrogen determination. The micro-Kjeldahl method of Ballentine [(5) p. 989–991] was used to determine the nitrogen content of the phage protein. A Lab Con Co Model A (Laboratory Construction Company, Kansas City, Missouri) micro-Kjeldahl apparatus was used. The phage protein was prepared by TCA precipitation as described previously.

Results

Electron microscopy. Figure 2 is an electron micrograph of Phage ACIL1 16M. It can be seen that the preparation is free of contaminating debris, emphasizing that the cesium chloride density gradient purification allows recovery of intact phage particles without accompanying bacterial debris. Also, the phage particles appear to be morphologically homogeneous. From the measurement of five phage particles it was estimated that the head was about 63 μm in length and about 58 μm in width; the tail was about 168 μm in length and 11 μm in width.

Photographic enlargement was estimated by placing a 200-mesh copper electron microscope specimen grid on the photographic plate. The magnification could be calculated readily by comparing the size of the grid in the enlargement with the actual grid size.

Deoxyribonucleic acid studies. The DNA of Phage ACIL1 16M was determined to be double-stranded on the basis of a sharp rise in absorbancy observed near 84°C during thermal melting. The melting profile reflecting absorbancy changes during thermal denaturation of the phage DNA is shown in Figure 3. To obtain the thermal melting (Tm) value, the absorbancy change data corrected for thermal expansion were plotted on normal probability graph paper as described by Knittel et al. (21). Table 1 illustrates the treatment of thermal denaturation data needed for plotting on normal probability graph paper and Figure 4 is the resulting normal probability plot. From this plot the Tm value for the phage DNA was 84.3°C. This compares well with the Tm from the melting profile given in Figure 3.

Figure 5 shows the normal probability plot of the thermal denaturation data for S. diacetilactis AB3M9 DNA. This organism was used as the host in preparation of the lysate of phage ACIL1 16M; the Tm was 81.3°C.
Marmur and Doty (24) related the Tm value of a DNA sample to the average guanine plus cytosine (G + C) composition by the formula: 

$$T_m = 69.3 + 0.42(\% \text{ G + C})$$

Using this formula, the per cent G + C of the DNA of Phage ACIL1 16M was 35.7, while that of the host organism was 28.5.

**Amino acid analysis.** Table 2 gives the amino acid composition of the protein obtained from Phage ACIL1 16M. The data are expressed in mole per cents of the total amino acids listed. Tryptophan, cysteine, and cystine were not determined, since the acid hydrolysis conditions destroyed these amino acids.

Table 3 gives the data obtained from the phage protein nitrogen and total phage phosphorus determinations. The nitrogen and phosphorus amounts are related to each other in the form of an N/P ratio; phage protein nitrogen does not include nitrogen found in the nucleic acid fraction.

### TABLE 1

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Absorbancy 260 m(\mu)</th>
<th>Corrected absorbancy 260 m(\mu)</th>
<th>Relative absorbancy 260 m(\mu)</th>
<th>Per cent increase in absorbancy</th>
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</thead>
<tbody>
<tr>
<td>25</td>
<td>0.191</td>
<td>0.191</td>
<td>1.000</td>
<td>0.00</td>
</tr>
<tr>
<td>82</td>
<td>0.209</td>
<td>0.215</td>
<td>1.025</td>
<td>25.00</td>
</tr>
<tr>
<td>84</td>
<td>0.220</td>
<td>0.226</td>
<td>1.184</td>
<td>43.33</td>
</tr>
<tr>
<td>85</td>
<td>0.230</td>
<td>0.233</td>
<td>1.250</td>
<td>63.33</td>
</tr>
<tr>
<td>86</td>
<td>0.240</td>
<td>0.247</td>
<td>1.295</td>
<td>78.33</td>
</tr>
<tr>
<td>87</td>
<td>0.248</td>
<td>0.256</td>
<td>1.340</td>
<td>93.33</td>
</tr>
<tr>
<td>88</td>
<td>0.252</td>
<td>0.260</td>
<td>1.365</td>
<td>100.00</td>
</tr>
</tbody>
</table>

\(^*\)Corrected absorbancy = \(A_t \times \frac{V_t}{V_{25C}}\) where \(A_t\) = observed absorbancy at temperature \(t\) and \(V_t\) = volume of water at temperature \(t\), divided by the volume of the same water sample at 25 C.

\(\frac{V_t}{V_{25C}}\) = volume of water at temperature \(t\), divided by the volume of the same water sample at 25 C.

\(^*\)Relative absorbancy = corrected absorbancy divided by the absorbancy at 25 C.

\(^*\)Per cent increase in absorbancy = per cent absorbancy increase of corrected absorbancy, using corrected absorbancy at 25 C as base and absorbancy increment between corrected absorbancy at 25 C and corrected absorbancy at 88 C as 100%.
TABLE 2
Amino acid composition of Phage ACIL1 16M

<table>
<thead>
<tr>
<th>Component</th>
<th>Mole %</th>
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<tbody>
<tr>
<td>Lysine</td>
<td>8.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.7</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.8</td>
</tr>
<tr>
<td>Aspartic Acid plus asparagine</td>
<td>10.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>7.5</td>
</tr>
<tr>
<td>Glutamic Acid plus glutamine</td>
<td>12.0</td>
</tr>
<tr>
<td>Proline</td>
<td>3.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>9.5</td>
</tr>
<tr>
<td>Valine</td>
<td>8.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.4</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*Values are given as the mole per cent of the total of the amino acids listed.

TABLE 3
Phage ACIL1 16M nitrogen and phosphorus content

<table>
<thead>
<tr>
<th>Element</th>
<th>µN/mg Phage protein</th>
<th>µP/mg Phage</th>
<th>N/P *</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>128.7</td>
<td>44.5</td>
<td>2.7</td>
</tr>
<tr>
<td>P</td>
<td></td>
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</tbody>
</table>

* N/P = µg N/mg phage protein + µg P/mg phage.

range, the phage DNA yielded values for increases in absorbancy that did not fall on the best fit line for the higher temperatures. Knittel et al. (21) observed the same phenomenon in studies on the DNA of lactic streptococcal strains. The explanation, based on cesium chloride density centrifugation data, was that there were molecules of DNA present in the sample that had a lower guanine plus cytosine content. Such molecules could represent satellite bands of DNA, perhaps episomal in nature. In this regard, Knittel et al. (21) were able to show that a skewed portion of the thermal melting curve could actually be resolved into a Gaussian distribution of DNA molecules which had a lower guanine plus cytosine content.

The amino acids listed in Table 2 are presented in the same manner as the data given by Fitch and Susman (17) for the T2, T4, T5, and T3 bacteriophages. These T phages are high in aspartic acid, glutamic acid, and alanine, just as was found here for Phage ACIL1 16M; the T phages are low in histidine and methionine, also true for Phage ACIL1 16M.

The nitrogen content of the phage protein was calculated as 12.8%. This is a low value, when one considers that a value between 14 and 16 is obtained for most proteins. One explanation for this low value is that the amount of dry weight protein analyzed was below 2.0 mg, and experience has shown that samples containing less than 2.0 mg will yield a slightly lower N content than those containing 2 to 10 mg of protein.

References
(1) Adams, E. DNA monograph. Undated publication distributed by Los Angeles, California, Corporation for Biochemical Research, 1p.

(6) Becker, R. B. 1966. Associate Professor of Chemistry, Science Research Institute, Oregon State University, Corvallis, Oregon. Personal communication.


