STUDIES ON STREPTOMYCES PHAGE

I. Growth Characteristics of the Streptomyces griseus Host-Phage System

C. M. Gilmour, E. C. Noller, and B. Watkins

Department of Bacteriology and Hygiene, Oregon State College, Corvallis, Oregon

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Within recent years, phages capable of infecting various Streptomyces species have been reported (Saudek and Colingsworth, 1947; Reilly et al., 1947; Koerber et al., 1950; Perlman et al., 1951; and Alexander and McCoy, 1956). Hoehn (1949) and subsequently Gilmour and Butthala (1950) demonstrated that streptomyces phage is indigenous to soil, the natural habitat of the host.

The streptomyces host-phage system stands unique among microbial viruses. Of primary significance is the branching, moldlike growth habit of the host. Almost immediately following spore germination (which may form one or more germination tubes) branching of the newly germinated spore occurs. Upon infection with a virulent phage the lytic aspect of the phage-host interaction may be observed by either turbidimetric or microscopic methods. Both approaches are utilized in the present study in demonstrating the lytic feature of the phage infection cycle. The more quantitative aspect, however, of the phage-host interaction lies in following phage growth by means of the one-step growth procedure: adsorption, latent period, and burst size. In this regard it is important to stress the point that a germinated streptomyces spore whether showing one or four germ tubes will plate out as a single colony. Thus the streptomyces cell input value required for accurate burst size values does not represent the true unicellular state. At the present time, this imposes certain restrictions on the extent to which one can apply calculations based on the accurate determination of initial infective centers. It is well, therefore, to recognize that the adsorption, latent period, and burst size data given in the present paper relate to phage infection of a newly germinated spore; the closest facsimile to the unicellular condition.

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EXPERIMENTAL METHODS

Culture growth. A strain (3475 Waksman) of Streptomyces griseus was used for all growth experiments. Spores for inocula were obtained from bottle slants of a modified nutrient agar medium (agar, 1.5 per cent; peptone, 0.5 per cent; beef extract, 0.3 per cent; yeast extract, 0.01 per cent; and glucose, 0.5 per cent. After 96 hr incubation at 30 C, growth and sporulation were at a maximum. Viable spore counts were standardized against turbidity by running plate counts on spore dilutions of known optical density.

Lysis. The lytic condition, characteristic of the system under study, was observed by turbidimetric and electron microscopic procedures. For simplicity in making direct turbidimetric readings, spores were grown for 6 hr in glass tubes containing glucose nutrient broth. Unless otherwise stated, the growth tubes were incubated at 30 C, on a rotary type shaker at 240 rotations per min. Turbidity was determined with a Beckman model B spectrophotometer at a wave length of 640 mμ. Lysis of the host cells was followed by noting changes in the optical density readings after the introduction of phage. At various intervals, samples were removed and smears prepared. The smears were stained with aqueous crystal violet and photomicrographs made using Kodak Plus-X sheet film.

Specimens for electron microscopy were drawn from cell-phage mixtures and centrifuged preparations. These were suspended in distilled water and deposited on collodion-filmed screens. After air drying, the screens were shadowed with 30 A of chromium from an angle of 20°. Original magnification was 16,800 X.

One-step growth studies. The S. griseus phage used in the present study was isolated from soil and has been designated as 514-3 (Gilmour and Butthala, 1950). Quantitative aspects of the phage-cell growth system were studied by the one-step growth curve as outlined by Adams.
Protocol included the following steps: newly germinated spores were centrifuged, washed in phosphate buffer (pH 6.8), and resuspended in test defined and undefined media (cell concentration, $1 \times 10^7$ per ml); stock phage added to cell suspension (phage concentration, $1 \times 10^4$ per ml); adsorption allowed to continue for 10 min; cell-phage mixture centrifuged (5 min), supernatant poured off to remove free phage, and infected hyphae resuspended again in test media; immediate dilution of the cell-phage mixture, and removal of aliquots to first and second growth tubes for assay of phage titer by the standard plaque method. The total time lapse between introduction of phage, adsorption, centrifugation, and removal of samples was approximately 15 to 20 min. Since preliminary experiments had shown no bursts of free phage prior to 90 min, the given time lapse was considered adequate. In each experiment adsorption was determined by assay of input free phage and subsequent decrease in free phage titer. The initial growth tests were carried out using glucose nutrient broth. In other experiments a basal salt medium was prepared containing the following components: $K_2HPO_4$, 0.1 per cent; $CaCl_2$, 0.001 M; $(NH_4)_2HPO_4$, 0.2 per cent; and glucose, 0.5 per cent. Selected amino acid groups were added to the above basal salt medium in the following proportions: L-glutamic acid, DL-alanine, and L-aspartic acid, 0.25 mg per ml; L-arginine, DL-asparagine, L-lysine, DL-serine, and DL-phenylalanine, 0.10 mg per ml; L-cysteine, L-cystine, L-glycine, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-proline, L-tyrosine, L-threonine, L-tryptophan, and L-valine, 0.05 mg per ml. The latter medium has been referred to in the text as the complete chemically defined medium. The described chemically defined medium allowed screening for ion effects as well as the over-all influence of amino acid groups on phage synthesis. Other pertinent data are included under results.

**EXPERIMENTAL RESULTS**

**Cell lysis.** The light photomicrographs of noninfected and infected hyphae are shown in figure 1. The inset electron micrograph of a newly germinated spore of *S. griseus* was included to depict

![Figure 1. A, inset, electron micrograph of germinated spore of *Streptomyces griseus*. Light photomicrograph of noninfected hyphae; B, partially lysed hyphae; C, nearly complete lysis of hyphae. Magnification, 3500X (reduced 7 per cent in reproduction here).](http://jb.asm.org/.../2017 by guest)
Figure 2. Electron micrographs of some aspects of the phage infection cycle of *Streptomyces griseus*. A, infected hyphae and absorbed phage prior to complete lysis; B, hyphae undergoing lysis and the appearance of honeycombed areas adjacent to point of phage release; C, extensive honeycombed area paralleling cell lysis; D, mass of phage heads and free tails depicting perhaps a biosynthetic pattern; E and F, general morphology of phage 514-3. Magnification, 28,000X.
the type of cell material initially infected with phage. It is evident that continued growth of the germination tubes produces a branching filamentous mass typical of the streptomyces group (figure 1.4). Progressive lysis of the mycelium is depicted in B and C of figure 1. Here clumping or aggregation of the mycelial mass occurs followed by a gradual distintegration of the individual filaments.

The electron micrographs shown in figure 2 are intended to illustrate certain aspects of the infection cycle and to give the morphology of the 514-3 phage of Strepomyces under study. Part A shows infected hyphae and adsorbed phage prior to complete lysis; part B, hyphae undergoing lysis and the appearance of honeycombed areas; part C, extensive honeycombed area paralleling cell lysis; part D, a mass of phage heads and free tails depicting perhaps a biosynthetic arrangement or pattern. Parts E and F show the general morphology of phage 514-3. It is evident that the phage of Strepomyces resembles in over-all morphology some members of the Escherichia coli series (Williams and Fraser, 1953) and other actinophages (Saudek and Colingsworth, 1947; Woodruff et al., 1947; and Koerber et al., 1950). The average head diameter appears to be 95 to 100 μ; the tail length, 350 to 360 μ; and tail width, 10 to 15 μ.

Typical cell lysis as determined by optical density readings is shown in figure 3. As might be expected, the initial phage-spore (germinated) ratio determined the time of lysis of the host mycelium. Addition of phage to give a phage-spore ratio of 2 to 1 had little effect on the subsequent growth of Strepomyces. When this ratio was increased to 20:1, and 200:1, lysis occurred in 4.5 hr and 2.0 hr, respectively. Further increases in favor of the phage did not shorten the observed time of lysis. As will be shown, the observed lysis time of 2.0 hr approximates the first detectable rise in phage titer and thereby represents a terminal point in the infection cycle.

**Growth studies.** The primary objective in this phase of the investigation was to study the growth process of the Strepomyces host-phage system in a more quantitative manner by an evaluation of adsorption, latent period, and relative yield. The observed adsorption levels determined in glucose nutrient broth and the complete, defined media have proved to be quite characteristic for phage 514-3 (table 1). Other adsorption data obtained for time intervals beyond the described 30-min period did not show any significant increases in the reported values. In general, a 30-min adsorption period in either media resulted in approximately 50 per cent adsorption of the input phage.

As was previously described, the given adsorption data were obtained by measurement of the decrease in free phage titer after centrifugation of the host cells. After removal of the unadsorbed phage and resuspension of the infected cells, a phage assay for number of infectious centers and number of surviving cells was also carried out. At a phage:spore input ratio of 0.1 and 10.0, the number of surviving hyphae was 25 and 60 per cent, respectively, of the initial spore count. In order to attain complete infection of the input hyphae count a phage:spore ratio of 100 or higher had to be used. In all probability, the variable spore germination picture where one or more germination tubes may or may not emerge, together with the over-all filamentous growth habit of the host, accounts for the rather

<table>
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<th>Time</th>
<th>Glucose Nutrient Broth</th>
<th>Complete Chemically Defined Medium</th>
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<td>30</td>
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Spores (3 × 10⁷ per ml) grown for 6 hr at 30 C; phage input 1 × 10⁶ per ml. Adsorption k values ranged from 1.2 to 1.6 × 10⁻⁵ ml per min.

![Figure 3. Growth of phage 514-3 as observed by lysis of Streptomyces griseus.](image)
high phage:spore input ratio required for complete killing of the host mycelium. Certainly the attachment of phage at one point on the young hyphae does not preclude the possibility of continued growth at the growing tip. This effect is shown in figure 3 by the growth patterns resulting after phage infection at various phage:spore ratios.

Following the afore-mentioned studies, preliminary one-step growth experiments were conducted. These disclosed that phage progeny was not released up to 90 min after adsorption. It was decided to carry out the final runs at a minimal adsorption time of 10 min and to use a final phage:spore concentration of $1 \times 10^4$ per ml and $1 \times 10^2$ per ml, respectively. The latter input ratio consistently gave less variable rise periods and a more discernible leveling off period. As shown in figure 4, the first detectable rise in phage titer with glucose nutrient broth as the host-phage medium occurred at 120 to 130 min after the close of the adsorption period. At the end of the rise period (30 min) an approximate 15 to 20 times increase in phage was normally obtained. In almost all these experiments 20 to 25 per cent adsorption of the input phage resulted with the

$\begin{array}{c|c|c|c|c}
\text{TABLE 2} \\
\text{Observed latent period and yield of phage in selected media} \\
\hline \\
\text{Latent Period} & \text{Yield per Spore}\ast \\
\hline
\text{min} & \\
\text{Glucose nutrient broth} & 120-125 & 75-100 \\
\text{Basal salt medium} & 120-125 & 10-20 \\
\text{Complete chemically defined medium} & 105-110 & 25-50 \\
\text{Complete chemically defined medium minus sulfur-amino acids} & 105-110 & 75-150 \\
\hline
\end{array}$

$\ast$ Range in yield represents low and high values obtained in 5 replicate experiments. In each run, phage:spore ratio was approximately 0.1 with a 10-min adsorption period.

10 min adsorption time. Appropriate calculations thereby showed average yields of from 75 to 100 phage particles per original germinated spore. When one, however, calls to mind the irregular nature of the initial spore germination state it seems unwise to regard such values as the final yield picture. Yet the reported data do provide a usable estimate of the end point of streptomycetes phage growth and thereby have proved quite useful in nutritional and phage biosynthetic studies presently being conducted in this laboratory.

Other pertinent growth data are given in table 2. Interestingly enough, a low but significant yield of phage was obtained in the basal salt medium. It may well be that in the latter instance the greater part of the phage synthesis occurs at the expense of host endogenous material. In the defined medium supplemented with a wide range of amino acids, one-step growth experiments showed a significant reduction in latent period (105 to 110 min) and phage yield as compared to the growth time and yields obtained in glucose nutrient broth. Of perhaps greater importance was the greater phage yields obtained when cystine, cysteine, and methionine were removed from the defined medium. When these amino acids were removed singly, the effect centered on cysteine. Along with studies on required amino acids, the effect of inorganic ions was also investigated. In general, these studies supported other reports. Inactivation of phage 514-3 multiplication occurred with 0.3 per cent sodium chloride in nutrient broth and 0.2 per cent salt in

\begin{figure}[h]
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\includegraphics[width=0.5\textwidth]{Figure4.png}
\caption{One-step growth curve with phage 514-3 of Streptomyces griseus in glucose nutrient broth at 30 C. Input ratio of phage/spores = 0.1; adsorption time 10 min after which 20 per cent of the phage was adsorbed. Approximate phage yield per germinated spore = 90.}
\end{figure}
the defined medium. In either instance the salt inactivation was pronounced. The observation that other salts also show an inhibition of phage synthesis would tend to suggest that the salt effect can be attributed to the respective cation. In defined media preparations, where potassium phosphate was included, an identical inhibitory effect was observed. Calcium, however, when present at a concentration of 0.001 M effectively removed the afore-mentioned effect of the potassium ion. In like manner, no sodium ion inhibition was observed in the presence of calcium.

**DISCUSSION**

Evidence has been presented to serve as a morphological and growth description of *S. griseus* phage 514-3 (Gilmour and Buthala, 1950). With the exception of its greater size, the phage under study has an over-all structure quite similar to several bacteriophages and appears to bear a close resemblance to the type II actinophage of Koerber *et al.* (1950). Electron micrographs depict an infection cycle composed of the adsorption phase, hyphae lysis, and release of phage progeny characteristic of typical bacteriophage infection cycles. The observed honeycomb areas are particularly striking (figure 2B, C). As Wyckoff (1948) noted with several of the T series phages of *E. coli*, these concavities are of variable depth and have the approximate diameter of the isolated phage particles. In this regard the phage aggregates shown in figure 2D may be visualized as fitting the general honeycomb pattern shown in figure 2C and presumably represent a phase in the development of phage 514-3. Further studies are presently underway on this aspect of the problem.

The growth studies call attention to several points of primary interest. With the described media and at various input ratios, per cent adsorption of the phage under study rarely went beyond the 50 per cent level after a 60-min adsorption period. As might be expected, shorter adsorption periods gave correspondingly lower adsorption values. In this respect the 10-min adsorption time was utilized in the growth experiments since a much shorter rise period was obtained. The rather extended latent period obtained in broth and defined media proved to be quite constant throughout the entire study and appears to be similar to group A phage types reported by Alexander and McCoy (1956). As pointed out earlier, phage yield determinations given in the present study are based on the only reliable cell input count available, namely the initial spore count. Nevertheless, the yield data have proved quite consistent and useful in the evaluation of the various defined media used for phage propagation. The inhibitory effect of cysteine on phage reproduction remains unexplained. Related studies showed no appreciable difference in adsorption in the presence or absence of the sulfur amino acids. The fact that the latent period itself is not significantly retarded by cysteine would indicate that it does not repress intracellular multiplication. Joklik (1952) has suggested that cysteine interferes with the liberation of T1 and T2 rather than with their multiplication within the cell. Possibly this is the case with phage 514-3 of *S. griseus*.

The effect of the tested electrolytes on phage synthesis confirms other reports (Adams, 1950). It appears probable that the mechanism of action centers on a specific interference of adsorption by the cations, sodium and potassium. The observation that calcium reverses the sodium or potassium cation effect lends support to this contention.

**SUMMARY**

The morphological and growth characteristics of phage 514-3 of *Streptomyces griseus* have been studied. Light photomicrographs and electron micrographs depicted a host-phage growth system similar in many respects to phage infection cycles of the true bacteria. Cell lysis, adsorption, latent period, and yield were observed. Several media were tested for phage propagation. In this regard, phage growth was obtained in an inorganic medium as well as in a chemically defined medium supplemented with selected amino acids. Cysteine proved to be inhibitory to phage development. An observed inhibitory effect of sodium and potassium was removed by the addition of calcium.

**REFERENCES**


