CHARACTERISTICS OF STREPTOMYCES GRISEUS STRAINS RESISTANT TO PHAGE

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In 1947 phage for Streptomyces griseus appeared in several commercial fermentations (Reilly, Harris and Waksman, 1947; Saudek and Colingsworth, 1947; Smith, Kuhn, and Miesel, 1947; Woodruff, Nunheimer, and Lee, 1947). There has never been an outbreak of phage at this laboratory although a number of workers have been studying the growth of S. griseus or developing new strains. Nevertheless, in 1949, Hoehn isolated from soil of this area several phages active against S. griseus. Chang (1953) worked on the characterization of these phages and their host specificity. Rice (1953) found that neither aerial nor submerged spores were susceptible to the phage. It has been the purpose of this work to develop and study strains of S. griseus resistant, but not lysogenic, to phage.

A resistant culture is one which is not visibly lysed by the phage in question either on agar or in broth. It does not produce phage when grown in pure culture or when grown with a sensitive host. The factor of primary interest is that these resistant cultures are not lysed by the phage although it is adsorbed from the medium. The adsorption of phage on resistant streptococci has been reported before (Henry and Henry, 1946). This work and ours are contrary to the generalization that phage is adsorbed only by hosts capable of supporting its multiplication (Anderson, 1949). It has been reported recently that resistant strains of Escherichia coli do not adsorb phage (Lieb, 1953).

MATERIALS AND METHODS

In most of the present work S. griseus no. 4 which produces streptomycin was used. It was obtained from Dr. Waksman in 1947 and designated as strain 1947, sensitive parent. The phage used was W2a which was isolated by Hoehn from garden soil with the above culture as host. Some work was done also with S. griseus strain 1945, a nonstreptomycin producing strain also obtained from Dr. Waksman. It was used as host for phage W-1 which was isolated from Lake Mendota beach sand. Other phages used were Hoehn's W-3 isolated from garden soil with the 1945 strain as host and W-5 from Virginia clay with a 1949 strain of S. griseus as host. Two phages from other sources were used also: Phage B from Bristol Laboratories, Inc., with the 1947 strain as host and phage C-131 from Eli Lilly and Company with their C-131 strain of S. griseus as host. M366P1, a phage resistant strain of S. griseus obtained from Abbott Laboratories, was also used in certain experiments.

All cultures were stored in soil stocks and spores from 4-7 day slants on potato-glucose agar used for inoculum. Phage filtrates adjusted to pH 7 were kept at 4 C.

The nature of the growth of the organism makes it difficult to obtain uniform cells since the growth cycle starts with spore germination and develops mycelium with many branching filaments. The life cycle in nutrient medium requires several days for formation and subsequent fragmentation of conidiospores, and even longer for autolysis of the remaining mycelium. Because the spores and older mycelium are not susceptible to phage, studies on adsorption and lysis must be made on the young, 3-18 hr mycelium. The mycelium used in the first series of experiments was grown in B16 broth: cerelose 0.25 per cent, peptone 0.1 per cent, yeast extract 0.3 per cent, and tap water. These cultures were incubated before and during the experiments in 50 ml broth in 250 ml Erlenmeyer flasks on a shaker at about 300 rpm at 30 C. For some short-timed experiments where only the adsorption was studied and more frequent readings were desired, a simplified "adsorption medium" was used in standing 8 inch test tubes. This was 0.1 per cent peptone water with 0.005
m Ca++. It had been found that the phage was relatively stable in the peptone water and that the addition of this molarity of calcium allowed normal adsorption, penetration, and multiplication to occur. Cells washed in peptone water and incubated in the “adsorption medium” were used in order to show a definite total adsorption in short-timed experiments. After removal of cells, free phage was determined by plating with young vegetative cells of strain 1947 as host, and counting plaques at 24 hours.

In the second series of experiments the mycelium was grown in modified Waksman’s broth: beef extract 0.5 per cent, peptone 0.5 per cent, NaCl 0.3 per cent, celereolose 1.0 per cent, and tap water. The cultures were grown with shaking in 7 ml of this medium in 18 mm test tubes for 12–14 hours. The cells were then centrifuged, washed three times with urea water (0.0001 M urea), and used as inoculum for adsorption studies in the basal synthetic medium: 0.0001 M urea, 0.005 M Ca++, and water double-distilled in glass. The phage is stable in this solution, and normal adsorption, penetration, and multiplication occur with the sensitive parent. Free phage was determined as above.

RESULTS

Development of cultures resistant to phage. Two methods of developing resistant strains were attempted with S. griseus strain 1947 against W2a phage. The first consisted of spreading 0.1 ml of spore suspension (10^4 per ml) and 0.1 ml phage filtrate (10^9 particles per ml) on plates and incubating several days. The first growth was all lysed, but colonies could be picked from the secondary growth and tested for resistance. Less than 50 per cent of the isolates were resistant, probably because there was insufficient contact between the phage and susceptible cells.

The second method was devised so that the cells to be treated would be very young, contact would be assured, and a larger population dealt with. The spores (10^6 per ml) were germinated in B16 broth in flasks on the shaker. Two hours later the phage (10^9 per ml) was added to the above culture. After 8 hours, when there had been at least two cycles of adsorption and release of phage and consequently an extremely high titer of phage, the entire culture was plated and the isolates tested for resistance. From the original 80 million spores, 336 apparently resistant colonies developed, giving an average of 4.2 resistant colonies per million of spores. Fifty-eight of these colonies were tested for resistance, and about one-third were found to be resistant by critical definition. Therefore, approximately one resistant strain per million original spores was found, a logical rate to expect from preexistent chance mutation if resistance is a phenomenon of bacterial mutation.

Proof of resistance. Of the resistant strains developed by the first method the more heavily sporulated and more typical S. griseus colonies were chosen to test for resistance. All colonies resulting from the second method were picked. The colonies were transferred to agar slants and subsequently tested for resistance in broth and on agar. Triplicate broth cultures were inoculated as follows: (a) with the test strain alone, (b) with the test strain and phage, and (c) with the test strain and the parent sensitive strain. The (a) and (c) cultures were plated out with sensitive host spores at 12, 24, 48, and 96 hours to look for evidence of phage by plaque formation. The (b) culture was compared for visual lysis to a sensitive strain with added phage and was also plated out at the above hours to detect any multiplication of the phage.

The agar plate tests were: (a) plating the resistant strains alone to detect plaques, (b) plating with sensitive strain 1947 spores to detect any phage by lysis of the sensitive host, and (c) plating with phage to see if added phage would cause lysis.

Strains that were not lysed in broth or on agar by a high titer of phage were considered to be resistant and not sensitive. If the same strains gave no evidence of phage when grown alone or with a sensitive strain, they were called resistant and not lysogenic. These strains were retested after 10 transfers and at the end of a year and were still resistant.

Adsorption of W2a phage on the resistant strains. The percentage of adsorption of phage on its host and the length of the latent period vary not only with the strain, but also with the medium, especially the calcium concentration, pH, aeration, and age of culture. For the first set of experiments our procedure was standardized by using the parent strain 1947, and then comparing its capacity to adsorb the phage to that of the resistant strains at optimum conditions for the parent. The most complete adsorption (90 per
cent) with the sensitive parent and a 1.5 hour latent period was obtained in B16 medium on the shaker with 12 hour mycelium at 10^4 per ml concentration of phage and cells. It was found that equally good adsorption could be obtained by using 0.1 per cent peptone water and 0.005 M Ca^{++} in standing 8 inch test tubes with a 10 ml volume of cell-phage mixture. The molarity of Ca^{++} was varied from 1 M to 5 X 10^{-7} M. The most adsorption on both the sensitive parent and the resistant strains occurred at 0.005 M (table 1). This procedure, although it lengthened the latent period of the sensitive system from 1.5 to 2.5 hours, was found valuable for frequent readings during the early, rapid adsorption period (30 min). Maximal adsorption in peptone-adsorption medium with the resistant cultures was never noted before 3 or 4 hours. For example, at 3.5 hours 82 per cent adsorption of W2a phage on strain RI-37 was noted. A test of 7 other resistant strains by this method showed about 50 per cent adsorption but no release through 4 hours, whereas the sensitive parent adsorbed 90 per cent in 30 min, and release started after a 2.5 hour latent period (figure 1). It will be noted also that release from the sensitive parent involved multiplication as shown by rise of titer.

In the second series of experiments phage adsorption and multiplication occurred when sensitive 1947 cells (figure 2) were used in the synthetic urea medium with approximately 10^4 phage particles per ml. (Phage adsorption and later release also resulted when a resistant culture was used. Strain 1947 or the resistant strains showed maximum adsorption at 1 hour.) Multiplication of phage, as indicated by lysis of the sensitive host, began after about 2 hours and approached a maximum titer at 4.5 hours. The resistant cul-

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Figure 1. Adsorption of phage on sensitive parent and representative resistant strains.

Figure 2. Adsorption and release of phage from sensitive and resistant strains in urea adsorption medium.
with the sensitive strain and 9 hours with the resistant strain.

It is interesting to note adsorption on M366P1, a "resistant" strain obtained from Abbott Laboratories. Our W2a phage is adsorbed on it in the same manner with release but no multiplication. Also of interest is the activity of other *Streptomyces* phages in our collection on these resistant strains. Specificity studies made by Chang (1953) divided the 6 phages into 3 pairs according to their activity on 9 strains of *S. griseus* and on 9 other species of *Streptomyces*. When these phages were tested with our resistant strains, it was found that phage B (Bristol) propagates on the sensitive parent and is adsorbed to and released from the resistant strains in the same manner as W2a. Phages W-5 and C-131 both adsorbed to and propagated on the parent sensitive and resistant strains, with a latent period of approximately one hour. W-1 and W-3 phages were similar in that adsorption was poor on either the parent sensitive or resistant strains. The significance of the above behavior is that our resistant strains to phage W2a are also resistant to a similar phage, B, but not to the other four phages tested.

*Several lines of evidence as to the fate of the phage after adsorption to the resistant host cell.* Adsorption of 90 per cent of the free phage has been demonstrated on young resistant mycelium. The release of this phage from the resistant cells was noted by following the titer of the free phage in B16 medium for 55 hours after the addition of phage (figure 3). The data show that the phage was attached to the resistant strains for approximately 8 hours, and that there followed a gradual release of phage from 8 through 55 hours. The age of the cultures during this period was actually 24 through 71 hours since 16 hour cells had been used for the experiment. As usual, the cultures began to segment between 30 and 33 hours (actual age). Release of the phage started just before this and continued at a steady rate. The titer of free phage rose to the original level but not significantly beyond the original of $2.4 \times 10^7$ phage particles per ml. At 71 hours the titers in experiments with 4 resistant strains were as follows: RI-36—$2.7 \times 10^7$; RI-37—$2.6 \times 10^7$; RI-38—$2.5 \times 10^7$; RI-45—$2.2 \times 10^7$. It is interesting that the release of the phage starts at the time that the mycelium begins to segment.

![Figure 3. Adsorption and release of phage W2a from sensitive parent and representative resistant strains.](image1)

![Figure 4. One-step growth curve. Cell and phage titer for adsorption was $10^8$ per ml. Each tube was diluted 1:100 at 35 min. During release the cell and phage titer was $10^8$ per ml.](image2)

One-step growth curves were run to test for the propagation of the phage on the resistant cells. These experiments were set up with peptone adsorption medium and a 35 minute adsorption period. Then the sensitive control with phage and the resistant strains with phage were diluted 1:100 to prevent further adsorption, and the titer of the free phage determined through a 4 hour period. The sensitive parent showed multiplication of the phage within one hour, but the resistant strains showed none through the 4 hours, thus indicating no propagation of the phage on the resistant strains (figure 4).

Another experiment to detect propagation was
run by serial dilution. In this work 1 ml phage (10⁷ per ml) was added to 100 ml of 12 hour mycelium of the resistant cultures. Every 48 hours the cultures were transferred by adding 1 ml of the old culture to 100 ml of new broth. The titer of the total phage was followed. By the fourth transfer all phage was lost by serial dilution, thus indicating no propagation.

The above work has shown that the phage is adsorbed to the resistant cells, and that in B16 medium there is eventually a release with no propagation. The purpose of the second set of experiments (with the synthetic urea medium) was to show whether the phage entered the resistant cell, after its adsorption, or remained attached to the exterior of the host.

It was first determined that concentrations of 0.5 M NaCl or greater added at zero time would completely inhibit adsorption and reproduction of W2a phage with the sensitive strain 1947. If the phage adsorbed to a resistant host remains on the exterior of that host, it should be possible to desorb the phage at will by addition of a suitable electrolyte in sufficient quantity to reverse the primary ionic adsorption step. With this in mind, 0.5 M NaCl was added at various times to the urea adsorption medium containing resistant host cells and 10⁴ particles of phage per ml. The results show that phage desorption occurs immediately upon addition of NaCl and is complete within 5 min (figure 5). If 0.5 M NaCl is added at zero time, no adsorption of W2a phage occurs with the resistant or sensitive host. As seen from the figure, NaCl can be added at various times between 0 times and 4 hours (before and after maximum adsorption had occurred but before any release according to previous data). No matter when the NaCl was added, the desorbed phage showed a titer equal to that of the phage originally added; this indicates complete removal of the phage and also no multiplication. Thus desorption can be made to occur at will, i.e., before a resistant host would normally release its phage, if no NaCl were added (approximately 13 hr).

The following experiment was run under similar conditions, except that antiseraum active against W2a phage was added to one set of tubes. The serum was added at 1–100 dilution after maximum adsorption of phage to the resistant host had occurred. NaCl was also added at a 0.35 per cent (0.06 M) level to all tubes and at this low level served merely as an electrolyte for antibody-phage attachment. The results show that phage is adsorbed to the resistant cells and that release of the phage occurs by 13 hours (figure 6). The antibody added to a duplicate

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![Figure 5](http://jb.asm.org/)  
*Figure 5. Effect of NaCl addition on release of phage adsorbed to a resistant strain.*

![Figure 6](http://jb.asm.org/)  
*Figure 6. Phenomenon of release of adsorbed phage from a resistant strain after removal of reef phage with homologous antiserum.*
tube inactivated all free phage. The antibody was removed after one hour by washing the cells in 0.1 per cent peptone water and resuspending in 0.0001 m urea. Free phage again appeared and seemed to reach a maximum release at about 13 hr as the addition of 0.5 m NaCl at that time did not increase the titer significantly. Even though the titer of the released phage never reached the original titer, definite release of adsorbed phage was shown.

DISCUSSION

The resistant strains studied were developed by treating germinating spores with 100 phage particles per spore at a young and very susceptible age. These isolates proved to be resistant and have remained so throughout our work. The phenomenon of interest was that they did adsorb and release phage. The original titer only was recovered. There was no propagation indicated when tested by one-step growth curve experiments; serial dilutions of growing cultures eliminated detectable phage at the arithmetic extinction point. The evidence we have points toward the hypothesis that the phage was held externally. This was shown by the fact that when NaCl was added to a resistant culture, after phage had been adsorbed to its cells, immediate and complete release of the adsorbed phage occurred. This might be considered similar to the work of Garen and Puck (1951) who described a reversible attachment of the T phages to Escherichia coli at suboptimal concentrations of the necessary ions. It is not possible to explain the immediate release of phage unless that phage is held externally. If NaCl was not added, release would occur in 8 hours, which may be due to aging of the cells and the change of the phage receptors on the cells. Just as old sensitive mycelium is insensitive to phage, so old resistant mycelium no longer has an affinity for phage.

The data obtained with antibody active against phage W2a show inactivation of the free phage. The titer of released phage after removal of antibody did not reach the titer of the adsorbed phage. This is probably due to the fact that the antibody was unpurified (active against cells and medium also) and could not be completely removed. This was evidenced by the fact that a 1:100 dilution of antibody added to strain 1947 and washed off in the same manner as with the resistant strains prevented multiplication of phage added later, by inactivating the added phage. Thus we have release but no propagation of phage from the resistant strains in nutrient or synthetic medium as the cells age, or at will with the addition of NaCl, showing adsorption to be an external attachment.

SUMMARY

Resistant strains of Streptomyces griseus were developed which retain indefinitely their resistant state toward the phage with which they were developed, but were not resistant to unrelated phages. These strains adsorb the phage to which they are resistant and release it without multiplication. It is postulated, from three lines of evidence, that the phage was carried externally on the resistant strains. Release of phage occurs upon aging of the resistant host, or can be made to occur at will by reversal of the primary adsorption step by ionic exchange. Addition of antiserum to a resistant strain with phage adsorbed to that strain inactivates all free phage. Upon removal of the antiserum, the free phage titer immediately begins to increase.

REFERENCES


