# A BACTERIAL VIRUS FOR ACTINOMYCES GRISEUS[1]

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Attack by bacterial viruses on members of the genus *Actinomyces* has been reported in only a few instances, but bacteriophages which attack *Eumycetes* are prevalent. Many industrial processes which employ bacteria are subject to bacteriophage infestations. The isolation of bacteriophage from soil and sewage may be easily accomplished, but soil cannot be considered an abundant source of actinophage. Isolations of actinomycetes are usually made from fertile soils; however, evidence of phage action is seldom noted. A transmissible and filterable lytic agent, which attacks actinomycetes, was reported by Wieringa and Wiebols (1936). This particular phenomenon may be explained as being due to the action of a polyvalent actinophage which initiated lysis not only of the parent culture but also of several other species. There are other reports of lysis of *Actinomyces* for which actinophages could not be demonstrated. However, the methods used would fail to demonstrate the actinophage for *Actinomyces griseus* (Dmitrieff and Soutéeff, 1936; Katznelson, 1940).

Accompanying the recent large-scale industrial utilization of actinomycetes for the production of antibiotic substances, study of the group has been intensified (Schatz, Bugie, and Waksman, 1944; Porter, 1946). The accumulative generations of growth of the cultures, constantly subject to chance contamination through faulty air filtration or insufficiently sterile laboratory and plant equipment and through errors in techniques, have made it highly probable that actinophages would be rediscovered for actinomycetes. In fact, a recent report indicated that an actinophage has been isolated from the streptomycin fermentation (Saudek and Colingsworth, 1947).

## EXPERIMENTAL WORK

We have observed an actinophage in laboratory cultures of *A. griseus* which were exposed to laboratory air for a 24-hour period. Moreover, outbreaks have occurred in a streptomycin production plant, located about 500 miles distant from the research laboratory. First recognition of the actinophage occurred in laboratory shake flasks. *A. griseus* cultures, which had developed under submerged conditions for 24 hours from a 10 per cent vegetative inoculum, were changed to stationary incubation conditions and the cotton plugs removed. Thin pellicles developed which showed evidence of plaque formation similar to that usually associated with bacteriophage development. The cultures were

[1] Throughout this paper the designation *Actinomyces* has been used to conform with the fifth edition of *Bergey's Manual of Determinative Bacteriology*. In each case, the organism referred to may be classified under the terminology proposed by Waksman and Henrici (1943) as *Streptomyces*.

filtered through ultrafine fritted glass filters, and the filtrates proved to be free from bacterial or actinomycete contamination. The filtrate, when added to a newly inoculated submerged culture of *A. griseus*, prevented initiation of growth.

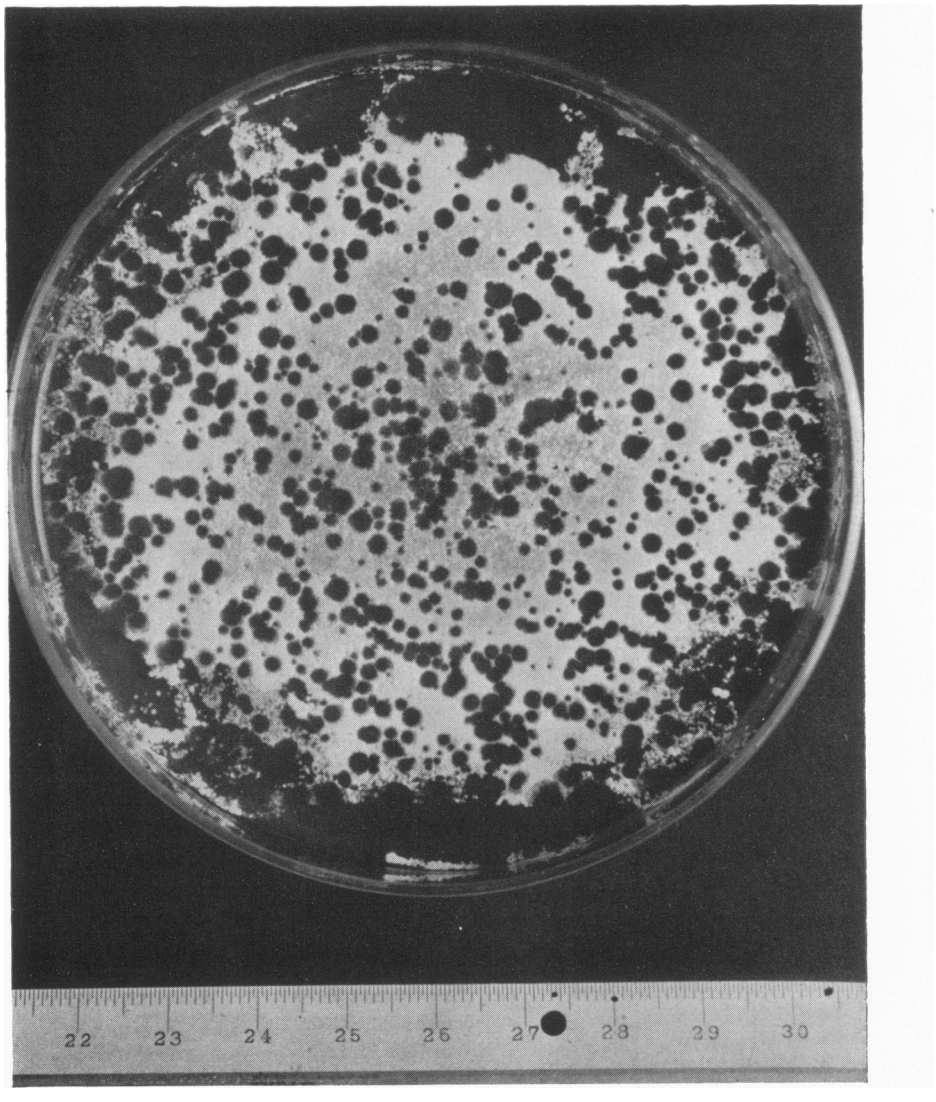


FIG. 1. THE FORMATION OF PLAQUES ON A PETRI DISH CULTURE

Various dilutions of the filtrate were placed on an agar medium with *A. griseus* spores. The typical "moth-eaten" cultures, characteristic of bacteriophage contamination, developed within 24 hours. Plaques did not spread during additional incubation. Within 48 hours the *A. griseus* growth between the plaques had sporulated and counts of the plaques could be made with ease

(figure 1). The filtrate from the culture in which the first evidence of actinophage was noted contained 55,000,000 plaque-forming units per ml. A few resistant cultures of *A. griseus* developed when exposed to high concentrations of the actinophage.

Actinophage infestations of *A. griseus* in a streptomycin production plant have occurred. In each case simultaneous bacterial contamination or other factors indicated an outside source of the actinophage. No evidence has been found that the actinophage was derived from stock cultures of *A. griseus*.

*Multiplication of actinophage.* The lytic agent was carried through several cultures of *A. griseus* in series and initiated lysis in each instance. To prove transmissibility of the agent, 0.01 ml of a bacteria-free filtrate was transferred to 50 ml of *A. griseus* culture. After 24 hours of submerged growth, the lysed culture was filtered and 0.01 ml of filtrate added to a new culture. The transfers, with filtrations between each, were continued for a total of six cultures.

TABLE 1

*Multiplication of actinophage*

| TRANSFER | ACTINOPHAGE PER ML | MULTIPLICATION FACTOR | |
|---|---|---|---|
| | | Individual transfers | Accumulative |
| Phage inoculum | 20,000,000,000 | | |
| 1st transfer | 32,800,000,000 | 8,200 | 8,200 |
| 2nd transfer | 100,000,000,000 | 16,000 | $131 \times 10^6$ |
| 3rd transfer | 36,000,000,000 | 1,800 | $236 \times 10^9$ |
| 4th transfer | 48,000,000,000 | 6,600 | $156 \times 10^{13}$ |
| 5th transfer | 64,000,000,000 | 6,600 | $103 \times 10^{17}$ |
| 6th transfer | 9,600,000,000 | 735 | $75 \times 10^{20}$ |
| Control *A. griseus* | <10 | | |

Filtrates from each flask were saved and plated by the plaque method for the determination of numbers of actinophage. These determinations (table 1) prove that the agent is transmissible and multiplies after each transfer. For each plaque-forming particle added to the first *A. griseus* culture in the series, a total of $75 \times 10^{20}$ particles had been produced on completion of the sixth transfer.

*Actinophage-susceptible strains of A. griseus.* Most bacteriophages are specific in activity against a single strain of a species. Actinophage was first isolated from cultures of *A. griseus* no. 9, from the collection of the New Jersey Agricultural Experiment Station, and was subsequently found in fermentations with other strains, of different streptomycin-producing capacities, from the collection. Likewise, three ultraviolet mutants of *A. griseus*, morphologically distinct from the parent, were susceptible. Centraalbureau vor Schimmelculture cultures labeled *A. griseus* Waksman and Curtis and *A. griseus* Bucherer were resistant to the action of the actinophage. However, no streptomycin was produced by these strains. Six additional species of *Actinomyces* were not affected by the actinophage.

*Effect of culture age.* The actinophage multiplies at the expense of submerged cultures of *A. griseus* of various ages. Complete lysis has been noted only with an inoculum consisting of spores of *A. griseus*. Six hours after inoculation the cultures incubated with actinophage show a faint turbidity due to germinated spores. Shortly thereafter, the cultures lyse completely, and only occasionally does a resistant colony grow out. With submerged vegetative inoculum, actinophage multiplication can be proved by the determination of numbers by the plaque method, but lysis is not complete. With 5 to 10 per cent by volume of submerged inoculum, there is little difference in degree of turbidity and streptomycin production in 24-hour-old control cultures and in cultures infected with actinophage. Usually, the infected cultures fragment at an earlier time than control cultures. Since streptomycin accumulation ceases about the time of fragmentation, yields are lower in infected cultures. The majority of *A. griseus* cells which remain in the infected cultures following fragmentation are resistant

TABLE 2

*Sensitivity of bacteriophages to chemicals in the absence of cells*

| AGENT* | VIRICIDAL DILUTION | |
|---|---|---|
| | *E. coli* bacteriophage | *A. griseus* actinophage |
| Acriflavine | <0.004 mg/ml | 0.004 mg/ml |
| *Actinomyces* 34 | 1:250 | 1:32 |
| *Actinomyces* 11 | 1:65 | <1:2 |
| *Bacterium* 24 | 1:250 | <1:2 |

* Bacterial virus exposed to agent 16 hours at 37 C in nutrient broth substrate.

to the action of the actinophage. Of 13 production lots of *A. griseus* which fragmented early, 11 were found to contain actinophage.

*Actinophage-resistant strains of A. griseus.* Several resistant cultures have been selected following exposure of *A. griseus* to the actinophage. Approximately half of the isolates are equal to the parent in streptomycin production. Many appear to be lysogenic. One culture, which produced high yields of streptomycin in the presence or absence of added actinophage, always had two or three plaques of lysis in agar slant cultures. Filtrates of a series of four submerged culture transfers, in series, all contained approximately 100 plaque-forming actinophage particles per ml for a sensitive strain of *A. griseus*. The actinophage was capable of multiplying to a slight extent on the resistant isolate. Such cultures are dangerous for routine use in the production of streptomycin, since conditions are favorable for the multiplication of any actinophage variants which gain the ability to attack the resistant culture.

*Sensitivity of actinophage to heat.* The susceptibility of the actinophage to heat was determined. Filtrates of a lysed culture of *A. griseus* grown on a glucose "N-Z-amine" meat extract medium were used as a source of actinophage. No evidence of destruction occurred from heating a filtrate containing 2,880,-

000,000 particles per ml at 80 C for 15 minutes in a water bath. At 85 C, 0.02 per cent of the actinophage particles survived, and at 90 C, 0.00002 per cent survived. Only 0.5 per cent of 500,000,000 *A. griseus* spores per ml of water remained viable after heating at 60 C for 15 minutes.

*Sensitivity of actinophage to chemicals.* Several compounds have been shown to destroy *Escherichia coli* bacteriophage during a 16-hour incubation at 24 C in nutrient broth, in the absence of bacterial cells. Acriflavine, a filtrate from an unidentified bacterium, and filtrates from two actinomycetes have been most active. The agents were tested against the actinophage under similar conditions. The latter virus was more resistant than the *E. coli* bacteriophage (table 2).

*A. griseus* was inhibited by acriflavine in concentrations which were destructive to the actinophage. The filtrate of *Actinomyces* 34, which had no

TABLE 3

*Sensitivity of bacteriophages to chemicals in the presence of cells*

| AGENT | *E. coli* PER ML* | | MG *A. griseus* CULTURE† | |
|---|---|---|---|---|
| | Control | + Phage | Control | + Phage |
| None | $3 \times 10^{11}$ | $2 \times 10^{3}$ | 50 | 7 |
| 1:40 Act. no. 34 | $2 \times 10^{11}$ | $3 \times 10^{8}$ | 43 | 8 |
| 0:001 mg/ml Acriflavine | $2 \times 10^{11}$ | $5 \times 10^{6}$ | 4 | 7 |
| 0:0001 mg/ml Acriflavine | $1 \times 10^{11}$ | $2 \times 10^{3}$ | 1 | 3 |

* Four hours' incubation.

† Twenty-four hours' incubation from spore inoculum, 30 ml volume.

inhibitory effect on growth of *A. griseus*, did not retard lysis of *A. griseus* by the actinophage (table 3).

*Morphology of actinophage.*[2] Preparations made from cover slip impressions of plaques for electron microscope observation demonstrated the particulate nature of the lytic agent and its close resemblance to strains of *E. coli* bacteriophage (Luria and Anderson, 1942). The chromium shadowing technique indicated a surprising diversity of structure of the actinophage particles (figures 2 and 3). Practically all particles had a long, relatively thick but bent tail of approximately 0.015 by 0.15 microns. Whereas the majority of the heads appeared symmetrically spherical, 0.05 microns in diameter, many were composed of two distinct bodies and a few appeared to be similar to tetrads.

One or two preparations had a majority of particles with two tails (figure 3). The heads did not appear sufficiently dense to indicate that these particles were simply overlying actinophages, and it seems possible that the preparations represented plaques formed by morphological variants of actinophage which

[2] Electron microscope studies were made by Dr. James Hillier in the laboratories of the Radio Corporation of America, Princeton, New Jersey, with preparations supplied from our laboratory.

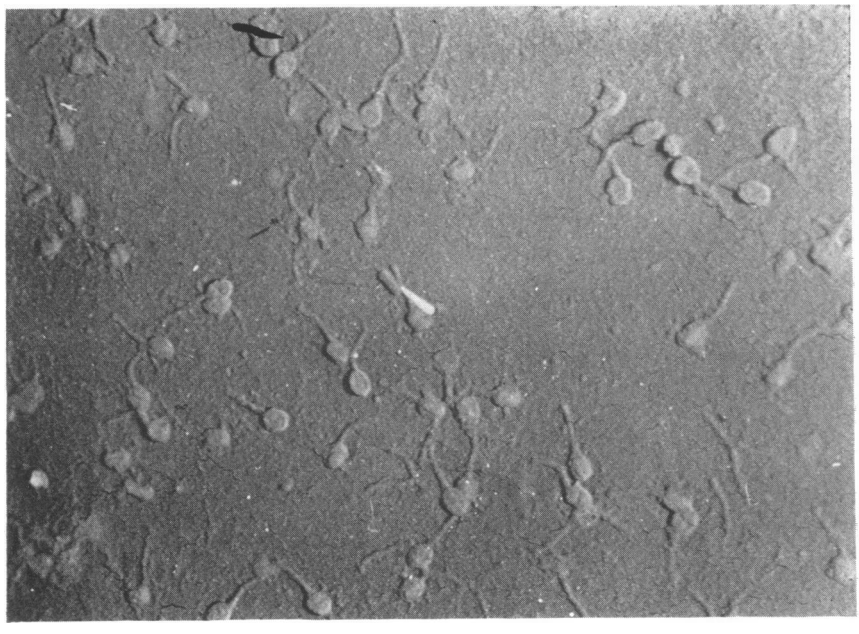

FIG. 2. ELECTRON MICROGRAPH SHOWING THE ACTINOPHAGE. × 37,500

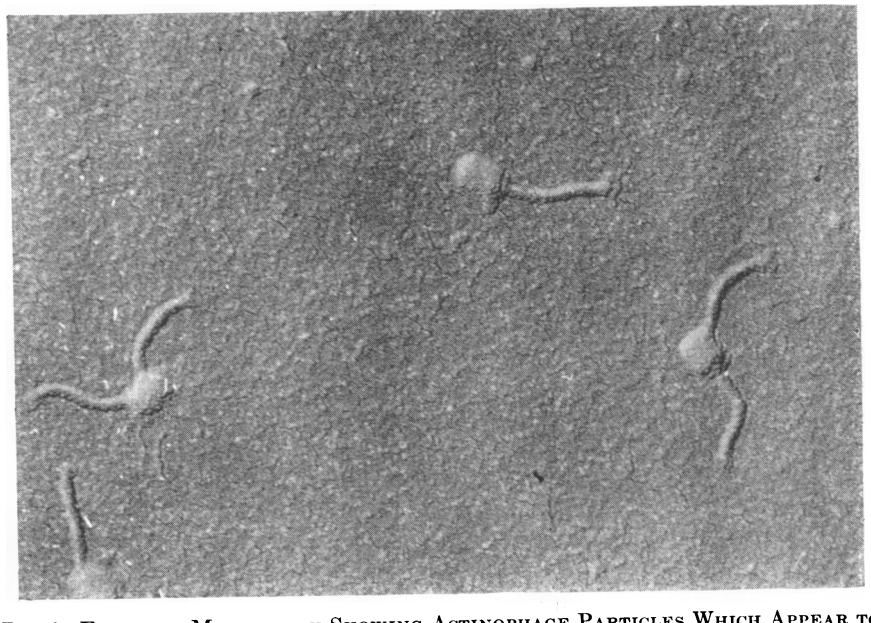

FIG. 3. ELECTRON MICROGRAPH SHOWING ACTINOPHAGE PARTICLES WHICH APPEAR TO HAVE TWO TAILS. × 95,000

retained infectivity. However, no proof can be offered at present for the origin of this unusual type. They were not present in the majority of the preparations.

SUMMARY

An actinophage has been isolated which infects strains of *Actinomyces griseus*. The virus is particulate, transmissible, and initiates lysis in young cells of *A. griseus*. It is more resistant to heat than are the spores of *A. griseus*, but is susceptible to certain viricidal agents which destroy *Escherichia coli* bacteriophage. Resistant cultures of *A. griseus* have been developed which may be lysogenic. Electron micrographs prove the particulate nature and demonstrate the morphology of the actinophage.

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