AN ACTINOPHAGE FOR STREPTOMYCES GRISEUS[1,2]

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Insufficient differentiation is frequently made between the production by a microorganism of an autolytic principle, or an agent which dissolves the cell of the organism producing it, and a phage or transferable principle, which is capable of dissolving not only the cells of the culture producing it but also those of other cultures of the same or other organisms upon transfer. This has often led to confusion in the interpretation of certain stages in the life cycle of the organism or of some of its metabolic processes. Although much light has been thrown in recent years on the nature and mode of action of phages of bacteria, the production of such agents by other microorganisms, notably fungi and actinomycetes, is still insufficiently understood. The significance of the ability of a phage or a viruslike agent to attack filamentous microorganisms in the practical utilization of such organisms for the production of various chemical agents has recently been emphasized in the discovery of a phage which has the capacity to attack streptomycin-producing strains of *Streptomyces griseus*.

The term "bacteriophage" is usually applied to the virus or phage of bacteria, and the term "mycophage" to that of fungi. By analogy, the term "actinophage" may be used to designate the phage of actinomycetes. The origin of the phage, whether it is carried in the culture or brought in from outside like any other contamination, its nature and activity, and its similarity to bacterial phages form some of the most important problems in the elucidation of this natural phenomenon.

HISTORICAL

The first recorded observations of the lysis of an actinomyces culture and of the significance of this reaction in the life cycle of the organism and in the production of new strains were made by Dmitrieff (1934) and by Dmitrieff and Soutéeff (1936). A culture of an organism called by the authors *Actinomyces bovis*, and evidently belonging, according to modern concepts, to the genus *Streptomyces*, was found to undergo lysis in various media. When the culture was grown on agar media, the production of lysis was found to be associated only with the formation of a certain type of colony. The organism produced as a result of lysis two types of daughter colonies: one was similar to the mother colony and possessed continued capacity for lysis; the other did not lyse and was morphologically different from the first type. The lysing colonies possessed

strong proteolytic properties and apparently did not form any aerial mycelium; the nonlysing colonies were less proteolytic and formed a chalky aerial mycelium, which changed the reaction of litmus milk to alkaline. In broth cultures, lysis took place in 2 to 3 weeks; it was associated with the living organism and was of the nature of a nonenzymatic but nontransmissible lytic factor.

Wieringa and Wiebols (1936) and Wiebols and Wieringa (1936) reported that various actinomycetes isolated from infected potatoes underwent lysis in culture. This phenomenon was believed to be due to the production of specific transmissible phages. Various organisms yielded phages which were active also upon other organisms; thus A. bovis produced a phage which was active upon A. scabies and A. farcinicus. These investigators were thus the first to emphasize the formation by actinomycetes of filterable and transmissible agents comparable to bacteriophages and polyvalent in nature. Their probable role in the control of potato scab in the soil has been suggested.

In view of the taxonomic relationship between the actinomycetes and mycobacteria, it may also be of interest to recall that Steenken (1935) observed lysis among the latter. This did not, however, appear to be a result of phage action. A virulent culture of Mycobacterium yielded a nonvirulent strain (R variant) which began to lyse after 3 or 4 months.

Krassilnikov (1938) made a detailed study of the course of autolysis of different actinomycetes isolated from the soil. A well-developed colony on an agar plate gradually became slimy, flat, and transparent. When transferred to a fresh medium, the colony either failed to develop or produced a much-delayed growth. Autolysis did not occur over the whole surface of the colony, but took place in sectors or spots; frequently it began in the center and spread toward the periphery. This phenomenon appeared to be very general among parasitic organisms and occurred less commonly among the saprophytes.

Krassilnikov and Koreniako (1939) emphasized the resemblance of the process of autolysis among actinomycetes to the Twort phenomenon, or phage production by bacteria. They reported that the lytic factor of actinomycetes, contrary to the observations of Wieringa and Wiebols, was highly specific, since it had no action on other species or even on other strains of the same species of Actinomyces. Lysis took place when growth of the organism was delayed for one reason or another or at the time of aging of the culture. Since different cultures underwent lysis with varying degrees of rapidity, it was assumed that the quantitative production of the lytic factor or its mode of action was distinct with different organisms. At high temperatures (60 to 70 C), lysis occurred in a few minutes. The lytic agent was resistant to 80 C for 1 hour, but was destroyed at 100 C in 5 minutes. Not only living but also dead cells were affected, thus showing a difference in action from that of true phage.

Katznelson (1940) isolated from manure composts a thermophilic culture of an actinomyces which underwent rapid lysis at 50 C when grown on starch ammonium sulfate agar media; no transmissible lytic agent could be demonstrated. Schatz and Waksman (1945), studying the production of streptomycin by different strains of S. griseus obtained from colonies of a given culture, observed

that colonies devoid of aerial mycelium produced no streptomycin. Such colonies gave rise to cultures which underwent much more rapid lysis than the normal cultures producing aerial mycelium. In the practical production of streptomycin it is generally observed that under submerged conditions of growth maximum formation or accumulation of the antibiotic corresponds to the beginning of lysis; advanced lysis usually results in a rapid destruction or inactivation of the streptomycin already produced.

Although these meager series of observations seemed to point definitely to the capacity of some actinomycetes to produce phagelike agents under certain conditions of culture, they threw very little light upon the nature and activities of these agents. They were not even sufficiently differentiated from lytic reactions due to enzymelike mechanisms.

The problem of phage production by actinomycetes entered a new phase with the discovery that the streptomycin-producing strains of *Streptomyces griseus* are subject to attack by a virus or a phagelike agent. This reaction appeared to be quite distinct from the lytic phenomenon mentioned above.

Saudek and Colingsworth (1947) were the first to report the production by *S. griseus* of a transmissible lytic agent which had all the properties of phage. The phage developed in the presence of young cultures of *S. griseus*. These workers used the plaque method with a phage-sensitive strain of *S. griseus* for measuring the concentration of the phage. Streptomycin production was partly or completely prevented by the phage. Cultures resistant to the phage could easily be isolated.

This problem was independently investigated by Woodruff (1947). When a submerged culture of *S. griseus* was placed in a stationary condition, with plugs removed from the flask, and exposed to laboratory air for 24 hours, the freshly formed pellicle showed evidence of plaque formation. The same phenomenon was observed in a factory 500 miles away. Multiplication of the phage took place upon each transfer of a filtered culture into a fresh culture of *S. griseus*. After six transfers, each phage particle increased to 75×10^{20} particles. The phage was active against all streptomycin-producing strains of *S. griseus* but not upon the non-streptomycin-producing strains. The culture produced phage-resistant strains readily. These retained the capacity of producing streptomycin but were not free from phage. The actinophage had properties similar to bacterial phages, such as those of *Escherichia coli*, as shown both by cultural characteristics and by appearance in photographs made by means of an electron microscope.

It has thus been established beyond doubt that at least certain species of *Streptomyces* can be attacked by a true phage. In view of the possible importance of this phenomenon in streptomycin production, and also in order to throw light upon its significance in the life cycle of the organisms producing the phage and in the taxonomy of actinomycetes as a whole, a detailed study was undertaken of the production, nature, and activity of this phage. Certain of the more immediate problems were at first investigated. These included the sensitivity of various strains of *S. griseus* to actinophage, the effect of actinophage upon the

growth and streptomycin production by *S. griseus* in static and in submerged culture, multiplication of active phage under different conditions of culture of *S. griseus*, effect of temperature upon phage activity, and the action of *S. griseus* phage upon non-streptomycin-producing strains of this organism and upon other actinomycetes.

EXPERIMENTAL METHODS AND RESULTS

Cultures used. A number of strains of *S. griseus* were used. These included several original isolations of streptomycin-producing cultures and a number of active and inactive strains obtained from them by colony selection. In addition, other strains of *S. griseus* not producing any streptomycin and other actinomycetes taken from the culture collection or freshly isolated from various substrates were also investigated.

The more important cultures are listed here.

(1) Streptomycin-producing strains of *S. griseus:*
   S. griseus 3463, the original streptomycin-producing culture 18–16.
   S. griseus 3480, an original culture isolated independently.
   S. griseus 3481, another original isolate.
   S. griseus nos. 4 and 9, strains isolated from culture 3463.
   S. griseus 3475, a strain isolated from culture no. 4.
   S. griseus 3523 and 3524, streptomycin-producing cultures comparable to nos. 4 and 9.
   S. griseus 3475-2PR, a phage-resistant culture obtained from 3475.
(2) Non-streptomycin-producing cultures of *S. griseus:*
   S. griseus 3478, a culture producing grisein.
   S. griseus 3326, the original culture of *A. griseus* isolated in this laboratory in 1915 and kept on artificial media since then.
   S. griseus 3326a, the same culture as above, which was deposited with the Central-bureau in Holland in 1920 and recently received from that collection.
   S. griseus 3522, culture isolated by Bucherer and also received from Holland.
   S. griseus 3495, culture isolated from no. 4; it does not produce streptomycin, but forms another still unidentified antibiotic.
(3) Other cultures:
   Streptomyces bikiniensis, a streptomycin-producing organism distinct morphologic-ally and culturally from *S. griseus* and isolated from a Bikini soil (Johnstone and Waksman, 1947).
   Streptomyces violaceus-ruber, a culture isolated from the soil and kept for many years in the collection.

Phage used. A phage preparation, originally obtained from Merck and Company and designated as M, was used in all these investigations.

Assay methods. In preliminary experiments, 0.1-ml portions of *S. griseus* culture filtrate containing the phage were added to 10-ml portions of nutrient agar; these were poured into petri plates and allowed to solidify. Aqueous spore suspensions of different strains of *S. griseus* were streaked on the surface of the plates and incubated at 28 C for 48 hours. The growth of strains nos. 4 and 9 was completely inhibited. Strain 3475 showed a few small colonies on the plate. As these appeared to be resistant to the action of the phage, they were picked from the plates and inoculated upon fresh agar slants. Several cultures were

obtained from the resistant colonies. One was selected for further study and designated as 3475-2PR.

To assay the phage preparations quantitatively, 10-ml portions of nutrient agar were poured into a series of five petri plates. These were streaked with spore suspensions of different strains of *S. griseus* and incubated for 24 hours. The diluted phage preparations were then poured over the plates. Upon further incubation at 28 C, the surface growth of the actinomyces streaks showed numerous plaques (figure 1). These were difficult to count. The results obtained
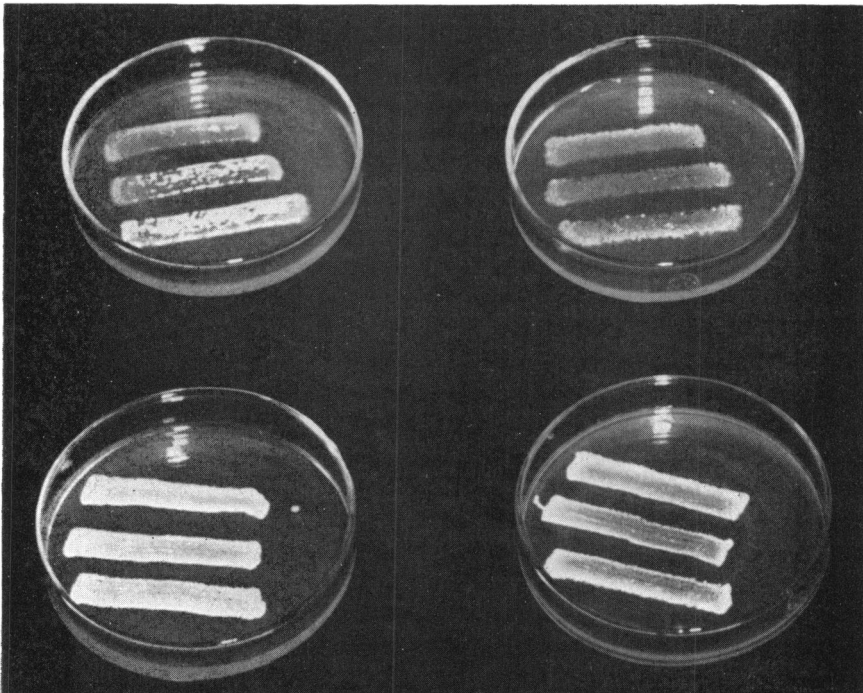

FIG. 1. EFFECT OF ACTINOPHAGE UPON THE DEVELOPMENT OF DIFFERENT STRAINS OF S. GRISEUS

Top: Phage-treated; bottom: controls. Left pair: 3475-2PR; right pair: 3475.

were only approximate and need not be reported here, since they were qualitative rather than quantitative.

When the phage was added simultaneously with the inoculum to the fresh medium, growth of the organism was completely prevented; but when the phage was added to cultures which had already been well sporulated, no phage multiplication occurred. These results prove emphatically that the actinophage acts best upon young cultures, as already emphasized by others (Woodruff, 1947). Various streptomycin-producing strains of *S. griseus* appeared to respond differently to the action of the phage, some being less affected than others. When colonies were picked from the agar streak that had been infected with

phage, they produced cultures that were especially resistant to the action of the phage, although they were still capable of supporting considerable phage growth.

The following method was finally adapted for assaying the concentration of phage in a given preparation. A 5-day-old shaken culture of a streptomycin-producing strain of *S. griseus* (no. 3463 being used mostly for this purpose) was filtered aseptically through paper and used as the source of culture material for inoculation of plates. The phage preparation, designated as M-1, was obtained by inoculating the M phage into young cultures of *S. griseus*, which were allowed to incubate for 24 to 72 hours and then were passed through a Seitz filter. A
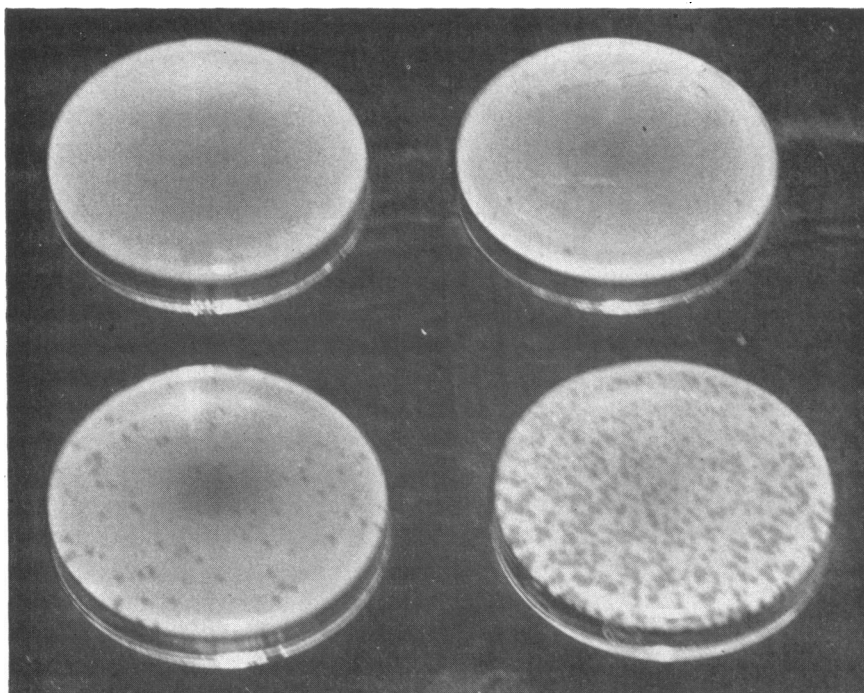

FIG. 2. PLAQUE FORMATION BY ACTINOPHAGE ON PLATES OF S. GRISEUS

Top: left to right, control and phage diluted 1:100,000; bottom: left to right, phage diluted 1:10,000 and 1:1,000.

series of dilutions of phage, ranging from $1:10^6$ to $1:10^{12}$, were added to 10-ml portions of sterile nutrient agar, which had been inoculated with 0.1-ml portions of the paper-filtered *S. griseus* culture. The agar portions were poured into plates, mixed thoroughly, and incubated at 28 C for 2 days. The plaque counts were then made, as shown in figure 2. The M-1 phage preparation contained 4.2×10^{10} particles per ml.

This method of assay gave accurate and reproducible results. The first phage preparation, M-1, was kept in the refrigerator and used as a standard. This procedure was now standardized, especially in regard to the effect of size of inoculum and of temperature of incubation. Three different amounts of filtered

7-day-old shaken culture of no. 3463 were added to nutrient agar to give final 10, 1, and 0.1 per cent concentrations. They were inoculated with different dilutions of M-1 phage and incubated at 28 C for 48 hours. The following results were obtained:

Culture inoculum per 100 ml of agar ml	Plaque counts $\times 10^7$
10.0	391
1.0	698
0.1	756

These results show that a lower inoculum gave higher counts; a 1 per cent inoculum was, therefore, adopted for all subsequent work.

In studying the effect of temperature of incubation upon plaque development, two temperatures were used, 28 C and 37 C. Normal plaque development took place at the lower temperature. No plaques appeared on the plates incubated at 37 C. When these plates were subsequently placed at room temperature for an additional 24 hours, plaques were rapidly produced with 1 per cent inoculum. None of the plates inoculated with 10 per cent of culture material produced any plaques, which points to the fact that not only is a temperature of 37 C unfavorable for phage multiplication, but at that temperature an excess inoculum exerts a destructive, or at least an adsorptive, effect upon the phage.

Effect of the phage on growth and streptomycin production by S. griseus. In a preliminary experiment on the effect of phage upon stationary cultures grown in standard medium for streptomycin production,[3] it was found that when the phage was placed in drops upon 2- and 3-day-old pellicles and allowed to incubate further at 28 C, many clear patches were produced in the pellicles, especially in the younger ones. Further investigation indicated that the study of the effect of phage upon growth of *S. griseus* and upon streptomycin production could best be conducted in submerged cultures. This is brought out in table 1. When the phage was added at the time of inoculation of the cultures, very little streptomycin was produced at the earlier periods of incubation, namely, after 3 and 4 days. When the cultures were allowed to incubate further, active streptomycin production occurred, as shown by the 5- and 6-day readings. This is a result, no doubt, of the development of resistant strains in the culture upon continued incubation.

A comparative study of the effect of phage on streptomycin production by strain 3475 of *S. griseus* and by the phage-resistant culture 3475-2PR, isolated from the foregoing strain, under submerged and stationary conditions of growth, tends to confirm the observation above. This is brought out in table 2. After 3 and 4 days' incubation in shaken cultures, no streptomycin was produced by the original strain in the presence of phage; however, after 8 days streptomycin production occurred, the activity of the low phage inoculum equaling that of the controls. The resistant strain, on the other hand, gave good streptomycin

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[3] This medium contained 1 per cent glucose, 0.5 per cent each of peptone, meat extract, and NaCl in tap water.

activity during the early incubation period, the presence of phage exerting only a slight depressive effect upon the total activity. Similar results were obtained in stationary cultures, the recovery of the streptomycin-producing capacity in the phage-containing cultures being much slower, however. Under these conditions, the phage-resistant strain did not produce so much streptomycin as the original culture.

TABLE 1

Influence of phage on streptomycin production in submerged culture

PHAGE ADDED*	INCUBATION, DAYS			
	3	4†	5	6†
	Streptomycin produced, μg/ml			
0	21	100	99	133
+	5	8	78	56

* One ml of phage, containing 4×10^{10} particles per ml, was added to 60-ml portions of glucose peptone meat extract NaCl medium in 250-ml Erlenmeyer flasks inoculated with spore suspensions of *S. griseus*.

† Cultures were kept static for 24 hours, then placed in a shaking machine.

TABLE 2

Effect of addition of phage upon growth and streptomycin production by original S. griseus and by a phage-resistant strain†

PHAGE ADDED		STREPTOMYCIN, μg/ML, PRODUCED AFTER DAYS					
After incubation	Phage per ml,. × 10⁷	Submerged cultures			Stationary cultures		
		3	4	8	8	10	24
Strain 3475							
hours							
0	0	32	111	192	140	188	189
Start	0.7	<5	<5	208	<5	<5	113
Start	70	<5	<5	128	<5	<5	93
24	70	<5	<5	122	<5	<5	24
Resistant strain 3475-2PR							
0	0	124	132	172	16	23	79
Start	70	117	108	140	19	50	36

More detailed results of further experiments on phage multiplication and the effect of phage upon streptomycin production under submerged and stationary conditions of culture are reported in tables 3 and 4. When the phage was added to the culture simultaneously with the inoculum, it multiplied rapidly and at first completely prevented streptomycin production; on further incubation, streptomycin production set in rapidly, and later tended to approach that of the control. The only possible interpretation is that the development of a phage-

resistant strain of *S. griseus* occurred in the culture. When the phage was added to the submerged cultures 16 hours after inoculation, its rate of multiplication was much more rapid, because of the greater amount of available mycelium, with a corresponding reduction in streptomycin production. Here again, streptomycin production set in rapidly later, as a result of the development of resistant strains. Similar results were obtained when the phage was added to the

TABLE 3

Effect of phage upon the production of streptomycin by S. griseus in submerged culture

PHAGE* ADDED AFTER	INCUBATION, DAYS					
	3		5		7	
	Phage × 10⁸	Sm†	Phage × 10⁸	Sm	Phage × 10⁸	Sm
		μg/ml		μg/ml		μg/ml
Control...............	0	94	0	168	0	258
0 hours...............	1	<5	100	<5	208	192
16 hours..............	37	<5	75	5	92	172
2 days................	177	22	222	18	382	36
4 days................	—	—	24	122	45	272
6 days................	—	—	—	—	53	300

* One-tenth ml of phage preparation, containing 40 × 10⁸ particles, was added to each flask containing 60 ml of medium; this is equivalent to 0.67 × 10⁸ phage particles per 1 ml of medium. All results are reported per 1 ml of culture.

† Sm = streptomycin.

TABLE 4

Effect of phage upon the production of streptomycin by S. griseus in stationary culture

PHAGE* ADDED AFTER	INCUBATION, DAYS					
	9		13		17	
	Phage × 10⁸	Sm	Phage × 10⁸	Sm	Phage × 10⁸	Sm
		μg/ml		μg/ml		μg/ml
Control...............	0	306	0	252	0	273
0 hours...............	19	<5	31	16	24	178
16 hours..............	27	<5	38	6	33	79
2 days................	47	26	588	36	605	94
6 days................	29	300	38	219	73	300
12 days...............	—	—	0.9	185	0.4	300

* Same as table 3.

48-hour-old cultures, the effect being magnified, as shown by the more rapid rate of phage development. The small amount of streptomycin formed at the time the phage was added did not increase until the seventh day, when the ability to form streptomycin was apparently recovered. When the phage was added to the 4- and 6-day-old cultures, at a time when growth had reached a maximum, there was a very limited amount of phage multiplication, and little effect was exerted on the streptomycin that had already been produced in the medium.

The results obtained under stationary conditions fully confirmed the results on the submerged cultures, namely, that phage multiplication was at a maximum when added to the 2-day-old cultures, that the addition of phage at the time of inoculation or soon afterward represses streptomycin production, that this is

TABLE 5

Effect of phage upon the growth, phage multiplication, and streptomycin production by different actinomycetes in stationary cultures

ORGANISM	PHAGE ADDED*	9 DAYS		13 DAYS	
		Phage per ml × 10⁷	Sm	Phage per ml × 10⁷	Sm
			µg/ml		µg/ml
Streptomycin-producing strains of *S. griseus*					
No. 3463	0	—	—	0	21
	+	—	—	200	5
No. 3475	0	0	30	0	180
	+	>50	<5	370	<5
No. 3480	0	0	31	0	189
	+	10	<5	30	28
No. 3481	0	0	73	0	174
	+	50	<5	260	13
No. 4	0	0	43	0	201
	+	30	<5	160	<5
3475-2PR	0	>0.01	40	40	129
	+	>50	16	370	75
S. griseus 3478	0	0	<5	0	<5
	+	0	<5	0	<5
S. griseus 3326a	0	—	—	0	<5
	+	—	—	<0.2	<5
S. bikiniensis	0	0	<5	0	30
	+	3	30	7	33

* Each 60-ml flask of culture received at start 0.1 ml of M-1 phage, amounting to 7 × 10⁷ particles per 1 ml of medium.

followed by the development of resistant strains which result in a considerably delayed formation of the antibiotic, and that, when added to older cultures, some phage development occurs with little effect upon the streptomycin present in the culture.

Effect of phage M-1 upon different strains of S. griseus and upon other actinomycetes. A detailed study of the effect of phage upon different cultures of actinomycetes, comprising different species and strains, brought out the fact (tables 5 and

6) that phage M-1 affected all the streptomycin-producing strains of *S. griseus*; it inhibited streptomycin production, and it multiplied at the expense of the growth of the organism. It had little effect upon the growth of other organisms. The two non-streptomycin-producing strains of *S. griseus* as well as some of the other actinomycetes tended to destroy or adsorb the phage, the mechanism of

TABLE 6

Phage multiplication in shaken cultures of various actinomycetes and its effect upon the production of antibiotics

ORGANISM	PHAGE* ADDED AFTER HOURS OF INCUBATION	TOTAL INCUBATION, DAYS				
		2	4		6	
		Phage per ml × 10^7	Phage per ml × 10^7	Antibiotic activity	Phage per ml × 10^7	Antibiotic activity
				S units/ml		*S units/ml*
S. griseus no. 4......	Control†	0	0‡	66	0	90
S. griseus no. 4......	Start	22	650	35	930	48
S. griseus no. 4......	24	9,500	7,000	96	4,600	135
S. griseus no. 4......	48	—	166	120	90	90
S. griseus 3478.......	Control	0	0	—	—	14
S. griseus 3478.......	Start	8	1.3	—	—	15
S. bikiniensis........	Control	0	0	29	—	18
S. bikiniensis........	Start	0.05	0.13	24	0	30
S. lavendulae........	Control	—	0	15	—	<10
S. lavendulae........	Start	—	8.8	<10	—	<10
S. violaceus-ruber....	Control	—	—	—	0	—
S. violaceus-ruber....	Start	—	—	—	0.00002	—
Nocardia asteroides..	Control	—	—	—	0	—
Nocardia asteroides..	Start	—	—	—	9.4	—
Micromonospora sp...	Control	—	—	—	0	—
Micromonospora sp...	Start	—	—	—	9.1	—

* 70 × 10⁶ phage particles added per ml of culture.

† No phage was added to control cultures.

‡ One plaque appeared on one plate; there is some doubt as to whether this plaque was due to phage or was due to the growth of an *S. griseus* colony that was antagonistic to the test organism.

this reaction still being uncertain. The phage had no injurious effect either upon growth or upon streptomycin production of *S. bikiniensis*.

The foregoing results were confirmed by a number of other experiments, with minor variations. For example, no. 3495, a strain of *S. griseus* isolated from a streptomycin-producing culture, which does not form streptomycin but does form another antibiotic inactive against *Escherichia coli* but active against gram-positive bacteria, gave no phage multiplication but showed occasionally a

change in the nature of the antibiotic spectrum. *S. bikiniensis* allowed no phage multiplication, or actually brought about the disappearance of the phage, and showed at times increased streptomycin production in the presence of the phage. The latter reaction may have been due to nutritive effects of certain constituents of the phage preparation.

In a series of experiments upon phage multiplication in cultures of different strains of streptomycin-producing *S. griseus*, each of 10 such strains was inoculated into four 60-ml portions of broth in 250-ml Erlenmeyer flasks. To two flasks of each series, 0.1-ml portions of phage M-1 were added at the time of inoculation; two flasks were left as controls. Phage determinations were made after 2, 4, and 6 days of incubation at 28 C. The antibiotic potency of the cultures was determined by the usual cup technique against a streptomycin standard (table 7).

There was considerable variation among the different cultures both in the extent of phage multiplication and in the rapidity of recovery of streptomycin-producing potency. As in previous experiments, the phage-resistant culture 3475-2PR showed comparatively little effect of the phage upon streptomycin production.

Effect of temperature upon phage. In a preliminary experiment on the effect of temperature upon actinophage, several 5-ml portions of phage M-1, diluted to give 43×10^7 particles per ml, were placed in sterile test tubes and kept in a water bath at four different temperatures for 10 minutes. No significant destruction of the phage took place at 40 to 65 C; a definite reduction occurred at 75 to 80 C; and maximum destruction was reached at 100 C.

In a more carefully controlled experiment, similar dilutions of phage were made. They were placed in water baths and incubated at various temperatures for 10 minutes and for 1 hour (table 8). The phage was stable for 1 hour at 65 to 75 C. Appreciable reduction in the number of phage particles occurred in the tubes kept for 10 minutes at 85 to 90 C and a further decrease occurred upon continued incubation. Heating for 1 hour at 90 C was not sufficient, however, to destroy the phage completely.

To determine the effect of temperature with prolonged storage upon the survival of phage, several 10-ml portions of phage M-1 diluted 1:100 with sterile water were added to test tubes, stoppered with sterile rubber stoppers, and placed at four different temperatures. After several periods of incubation, phage determinations were made. The results show (table 9) that incubation for 3 days at 56.5 C brought about an appreciable decrease in phage concentration; after 12 days at this temperature more than 99 per cent of the phage was destroyed. At 37 C the decrease was much slower, incubation for 12 days giving about 58 per cent loss of phage concentration and nearly complete loss after 29 days. At 28 C there was a small decrease after 12 days' incubation and marked decrease after 29 days. There was no change in concentration of the phage at 6 C on continued incubation.

Further studies on the effect of temperature upon phage multiplication confirmed the previous results. The optimum was at 28 C. There was no increase

in phage content at 37 C, and at 56.5 C more than 97 per cent of the phage was destroyed in 1 day. The extent of phage multiplication depended largely upon the size of the inoculum. The greater the number of cells of S. *griseus* present in the culture, the greater was the amount of phage produced. At 56.5 C, the size

TABLE 7

Multiplication of phage in shaken cultures of streptomycin-producing strains of S. griseus

STRAIN NO.	ADDITION OF PHAGE	DAYS OF INCUBATION			
		2		4	6
		Phage* per ml × 10⁷	Sm	Sm	Sm
			μg/ml	μg/ml	μg/ml
No. 3463	0	0	17	90	116
No. 3463	+	84	<5	<5	34
No. 3464	0	0	<5	28	20
No. 3464	+	70	<5	<5	18
No. 4	0	0	<5	15	12
No. 4	+	15	<5	<5	<5
No. 9	0	0	<5	>50	51
No. 9	+	64	<5	<5	51
No. 3475	0	0	<5	64	92
No. 3475	+	83	<5	<5	76
No. 3498	0	0	23	49	78
No. 3498	+	76	<5	<5	16
No. 3499	0	0	<5	43	90
No. 3499	+	20	<5	<5	<5
No. 3523	0	0	21	114	149
No. 3523	+	81	<5	<5	20
No. 3524	0	0	17	57	72
No. 3524	+	146	<5	7	32
No. 3475-2PR	0	<0.001	<5	98	104
No. 3475-2PR	+	8	<5	42	104

* The cultures treated with phage contained at start 4 × 10⁷ phage particles per ml.

of inoculum also had an effect upon the extent of phage destruction: the larger the inoculum, the lower was the rate of phage destruction. The nature of the medium in which the phage was suspended had a marked influence upon the rate of its destruction at 56.5 C. The phage suspended in water showed only about 40 per cent destruction in 1 day, whereas the phage placed in broth lost more than 99 per cent of its activity in the same time. After 3 days, the phage

diluted with broth was completely destroyed, whereas considerable phage was left in the aqueous suspension, although marked destruction had taken place.

Multiplication of actinophage in the presence of living and dead cells of S. griseus. Finally, studies were made upon the ability of the phage to multiply at the ex-

TABLE 8

Effect of temperature upon the stability of actinophage

(At start, 43 × 10⁷ phage particles per ml)

TEMPERATURE	PHAGE × 10⁷ PER ML, AFTER	
	10 minutes	1 hour
C		
Control	43	
65	45	45
75	—	45
85	0.07	0.0003
90	0.001	0*

* The actual count was 5 particles per ml.

TABLE 9

Stability of phage in aqueous suspension upon storage at several temperatures

TEMPERATURE OF STORAGE	PHAGE PARTICLES × 10⁷ PER ML, AFTER STORAGE*		
	3 days	12 days	29 days
C			
6	44	—	60
28	31	20	0.00005
37	37	15	0.0000009
56.5	18	0.001	0

* At start all preparations contained 36 × 10⁷ particles of phage per ml.

TABLE 10

Multiplication of actinophage in living and dead cultures of S. griseus

S. GRISEUS CULTURE	PHAGE × 10⁷		
	Start	1 day	2 days
Living........................	43	136	189
Dead........................	65	76	58

pense of living and dead cultures of *S. griseus.* Two 10-ml portions of a 40-hour-old shaken culture of a streptomycin-producing strain (no. 3475) were transferred aseptically to sterile test tubes. One tube was placed in a water bath at 75 C for 10 minutes, to kill the spores and mycelium of *S. griseus;* the second tube was not heated. To both tubes were added 0.1-ml portions of phage M-1; the tubes were incubated at 28 C and phage concentrations determined at the start and

after 1 and 2 days. The results presented in table 10 show that no multiplication of the phage took place in the presence of dead cells of *S. griseus*; in the presence of living cells, a fourfold increase in phage concentration occurred.

SUMMARY

The results obtained by Saudek and Colingsworth and by Woodruff on the production of phage by streptomycin-producing cultures of *Streptomyces griseus* have been fully confirmed.

Certain cultures of *S. griseus* are subject to attack by a virus which can be designated as "actinophage."

This phage attacks only the streptomycin-producing strains of *S. griseus*; it has no effect on other streptomycin-producing organisms.

In cultures of strains of *S. griseus* that do not produce streptomycin, the phage does not multiply and may actually be destroyed or adsorbed.

The actinophage of *S. griseus* multiplies only at the expense of living cell material and not upon the heat-killed material of this organism.

Phage-sensitive cultures of *S. griseus* give rise rapidly to strains which are resistant to the action of the phage.

The actinophage has an optimum temperature for multiplication at 28 C. It does not multiply at 37 C or above.

Actinophage can withstand a temperature of 75 C for 1 hour, but is completely destroyed at 100 C in 10 minutes.

The actinophage can be stored at 6 C without loss of activity, but storage at 28 C or at higher temperatures results in a loss of activity, the rate of loss being proportional to the temperature.

The nature of the medium in which the actinophage is suspended greatly influences the rate of its destruction.

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