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The Action of Fumagillin on a Bacteriophage of *Staphylococcus aureus*

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SUMMARY: Concentrations of fumagillin which allowed host growth prevented multiplication of phage 209 on *Staphylococcus aureus* 209. Fumagillin had no action on free phage and little on the adsorption of phage to its host, but when added 15 min. or less after the start of the latent period it prevented phage multiplication. Fumagillin present during adsorption was diluted out during the latent period; the longer fumagillin was present the smaller was the phage yield. Prolonged exposure of the host + phage mixture in Ringer's solution to a concentration of fumagillin too low to prevent phage multiplication lengthened the latent period and decreased the yield of phage, whether fumagillin was present during the burst or not. Under these conditions, when sufficient fumagillin was added to produce a phage-inhibitory concentration and later diluted out, the yield of phage was constant when the dilution was made in the period 0–20 min.

Asheshov, Strelitz & Hall (1949) reported the isolation of an *Aspergillus* species which produced two antibiotics active against bacteriophages, one being specific for 'streptophages', the other for some staphylococcal phages. These antibiotics did not act on free phage, or affect adsorption of phage to host. Even when added immediately after infection, the antibiotics did not always prevent a burst. When added 10–15 min. before the start of the second cycle of growth they prevented the second burst. The antibiotic active against staphylococcal phages was later shown (Asheshov, Strelitz & Hall, 1952) to be identical with the antiphage antibiotic, fumagillin, isolated by Eble & Hanson (1951) from a strain of *Aspergillus fumigatus*. As part of a programme of research into virus chemotherapy, the work of Asheshov *et al.* (1949) on the mode of action of fumagillin has been extended, using the system *Staphylococcus aureus* strain 209 + phage 209. The results are reported here.

EXPERIMENTAL

Host. *Staphylococcus aureus* strain 209 was used as the bacterial host.

Phage. Stock phage 209 of titre 6.8×10^7 particles/ml. was prepared as a bacteriologically sterile suspension in 50 % glycerol. It was stored at 5° as a 1/100 dilution in Ringer's solution.

Media. Lab-Lemco broth was used throughout as growth medium. Ringer's solution was used when a fluid was required which did not support host growth.

Fumagillin. This was obtained through the courtesy of Dr R. Coghill, Abbotts Laboratories Inc., U.S.A. It was made up as a 0.1 % (w/v) suspension in 10 % (v/v) acetone in water and stored at –20°.

Titration of phage. For one-step growth experiments: to 1 ml. phage

suspension in a test tube, 1 ml. of a 24 hr. culture of *Staphylococcus aureus* 209 and 3 ml. nutrient (tryptic digest) agar were added. After the contents had been mixed, the tubes were sloped and incubated at 37° overnight before reading. In all other experiments the mixture was poured on 15 ml. nutrient agar base.

Methods. These were essentially those of Mills (1953). In all one-step growth experiments, host cells previously suspended for 2 hr. in Ringer's solution (Benzer, 1952) were used for adsorption. Times were always measured from the moment of dilution in broth. All work was carried out at 37°. In one-step growth experiments phage liberation occurred over a long period; because of this, measurements of phage yield were made before liberation had ended (usually at 120 min.). Yields were only compared under conditions where the latent period was constant, and as sampling and counting phage was always complete in less than 5 min., it is believed that little error was introduced.

RESULTS

All points on graphs are the mean of three counts. The latent period of phage 209 on *Staphylococcus aureus* 209 was about 60 min. (Fig. 3). The yield of phage in one-step growth experiments was usually four to six times phage input. No estimate was made of the percentage adsorption.

Active concentration of fumagillin. In a serial dilution test (Chantrill *et al.* 1952) fumagillin at 16 µg./ml. allowed host growth but at 0.6 µg./ml. prevented phage growth. In one-step growth experiments it prevented phage growth at 0.5 µg./ml. but not at 0.25 µg./ml. Except where otherwise stated, 1 µg./ml. fumagillin was used for this work.

Action of fumagillin on host growth. Fumagillin at 1 µg./ml. had no observable effect on host growth during the first 4 hr. as measured by poured plate colony counts. After this time, growth was slightly retarded (Fig. 1).

Action on free phage. When phage 209 was exposed to 2 µg. fumagillin/ml. in broth at 37° for 3 hr., the titre remained constant at 1.5×10^5 particles/ml.

Table 1. *The action of fumagillin (1 µg./ml.) on the adsorption of phage 209 on Staphylococcus aureus 209 in Ringer's solution at 37°*

Fumagillin		Count (plaques/tube)	
During adsorption	During phage growth	10 min.	120 min.
—	—	12, 11, 12	77, 63, 64
—	+	7, 7, 6	12, 12, 4
+	—	17, 13, 11	42, 43, 37
+	+	8, 6, 13	7, 12, 9

Action on adsorption. When fumagillin was present during adsorption and diluted out at the start of the latent period, a burst occurred but the yield of phage was a little smaller (Table 1); hence any action fumagillin had on adsorption could only be small.

Fig. 1

Fig. 2

Fig. 1. The action of fumagillin ($1 \mu\text{g./ml.}$) on the growth of *Staphylococcus aureus* 209 in broth at 37° . $\times-\times$, -fumagillin; $\square-\square$, +fumagillin. Growth was estimated by poured-plate colony counts.

Fig. 2. The effect of fumagillin ($1 \mu\text{g./ml.}$) on the yield of phage 209 on *Staphylococcus aureus* 209 in one-step growth experiments. Squares, fumagillin initially present at $1 \mu\text{g./ml.}$, diluted to $0.1 \mu\text{g./ml.}$ at different times; crosses, fumagillin added to $1 \mu\text{g./ml.}$ at different times. Dotted lines, preliminary exposure of host and phage to $0.1 \mu\text{g./ml.}$ fumagillin for 90 min. before transfer to broth. Full lines, no preliminary exposure to fumagillin. Counts were made by sloping 1 ml. samples and counting plaques after incubation overnight at 37° .

Fig. 3

Fig. 4

Fig. 3. The effect of adding fumagillin ($1 \mu\text{g./ml.}$) at 30 min. on the latent period of phage 209 on *Staphylococcus aureus* 209 in broth. $\times-\times$, -fumagillin; $\square-\square$, +fumagillin. Counts were made by sloping 1 ml. samples and counting plaques after incubation overnight at 37° .

Fig. 4. The effect of diluting fumagillin from $1 \mu\text{g./ml.}$ to lower concentrations on the latent period of phage 209 on *Staphylococcus aureus* 209 in broth. $\times---\times$, control-fumagillin; $\circ-\circ$, fumagillin diluted to $0.002 \mu\text{g./ml.}$ at 0 min.; $\triangle-\triangle$, fumagillin diluted to $0.1 \mu\text{g./ml.}$ at 0 min.; $\nabla-\nabla$, fumagillin diluted to $0.1 \mu\text{g./ml.}$ at 40 min. Except in the control, fumagillin was present during adsorption at a concentration of $1 \mu\text{g./ml.}$ Counts were made by sloping 1 ml. samples and counting plaques after incubation overnight at 37° .

Action on intracellular phage

Time of action. Adsorption was carried out in the absence of fumagillin which was added at various times after dilution into broth. There was no burst when fumagillin was added at 15 min., but a small burst when it was added at 20 min. (Fig. 2). This behaviour differed from that reported by Asheshov *et al.* (1949), who found that fumagillin did not prevent a burst even if added when phage and host were mixed. When fumagillin was added at 30 min., the latent period was not affected (Fig. 3).

Fig. 5

Fig. 5. Effect of preliminary exposure to fumagillin (0.1 $\mu\text{g./ml.}$) on the latent period of 209 phage on *Staphylococcus aureus* 209 in broth. $\times-\times$, control—fumagillin throughout; $\circ-\circ$, fumagillin absent during phage growth; $\square-\square$, fumagillin (0.1 $\mu\text{g./ml.}$) present during phage growth. After adsorption of phage to host, the mixture was held at 37° in Ringer's solution for $1\frac{1}{2}$ hr. in the presence or absence of fumagillin.

Fig. 6

Fig. 6. The action of fumagillin (0.1 $\mu\text{g./ml.}$) on the liberation of intracellular 209 phage from *Staphylococcus aureus* 209 by cyanide. $\times-\times$, control, — fumagillin—cyanide; $\circ-\circ$, + fumagillin — cyanide; $\square-\square$, — fumagillin + cyanide; $\triangle-\triangle$, + fumagillin + cyanide. KCN was added to a final concentration of 0.03M at the times shown. At 150 min. it was diluted 1:100 and 1 ml. samples were then counted. When KCN was omitted, samples were diluted 1:100 at the times shown, and 1 ml. samples counted immediately.

Effect of diluting out. Fumagillin was present during adsorption and after transfer to broth. At various times during the latent period a dilution was made to a fumagillin concentration of 0.1 $\mu\text{g./ml.}$, and this concentration was not altered for the rest of the experiment. The later the suspension containing fumagillin was diluted the greater was the effect on phage yield (Fig. 2): the latent period was increased by about 25 min. irrespective of the time of dilution (Fig. 4); this increase was found to be due to the residual fumagillin. When the fumagillin was diluted to 0.002 $\mu\text{g./ml.}$ immediately after adsorption, the latent period was not affected (Fig. 4).

Effect of exposure to fumagillin at 0.1 µg./ml. After adsorption in the absence of fumagillin, the host + phage mixture was diluted in Ringer's solution containing fumagillin 0.1 $\mu\text{g./ml.}$ The mixture was held at 37° for 90 min. and

then diluted in broth. Under these conditions, the latent period was increased and the phage yield decreased (Fig. 5). The presence of 0.1 $\mu\text{g.}$ fumagillin/ml. during the actual burst had no further effect on the latent period, but resulted in a still smaller yield of phage (Fig. 5).

Preliminary exposure to 0.1 $\mu\text{g.}$ fumagillin/ml. in the above manner, did not affect the time of action of fumagillin (1 $\mu\text{g.}/\text{ml.}$) added at different times during the latent period (Fig. 2). When, however, fumagillin was added to 1 $\mu\text{g.}/\text{ml.}$ at the start of phage growth, and subsequently diluted to 0.1 $\mu\text{g.}/\text{ml.}$, for the period 0–20 min., the yield of phage was independent of time of dilution but decreased when the dilution was made at 30 min. (Fig. 2).

Effect of fumagillin on the appearance of intracellular phage. Fumagillin was present at a concentration of 1 $\mu\text{g.}/\text{ml.}$ during adsorption and was diluted to 0.1 $\mu\text{g.}/\text{ml.}$ on transfer to broth. At various times samples were taken and cyanide added to a final concentration of 0.03M to liberate any intracellular phage particles (Doermann, 1951). Each mixture was left for 150 min., diluted 1/100 and 1 ml. samples sloped. An increase in phage titre was found only when cyanide was added 15 min. before the end of the latent period (i.e. at 60 min.) or at times later than this. In the absence of fumagillin, intracellular phage was also detected 15 min. before the end of the normal latent period (i.e. at 45 min., Fig. 6). Under the conditions used, cyanide had no action on free phage 209.

DISCUSSION

Asheshov *et al.* (1949) found that their antibiotic had no action on free phage, on host growth or on the phage adsorption on its host. They also found that their antibiotic did not always prevent a burst even when added at the time of mixing phage and host. In the present work fumagillin prevented phage multiplication when added during the first 15 min. of phage growth. This discrepancy might be due to differences in a number of factors: experimental conditions, phage and host used, purity of the antibiotic, or even the antibiotic itself as it is not clear which of their two Asheshov *et al.* (1949) were using.

From the results reported above, fumagillin appears to have at least two different actions on the *Staphylococcus aureus* 209 + phage 209 system. At low concentrations fumagillin lengthens the latent period and decreases the yield of phage; at high concentrations fumagillin (when added early enough) has a true virustatic action, and (when added late enough to allow some phage growth) no action on the latent period. It seems reasonable to assume that when fumagillin is initially present at 1 $\mu\text{g.}/\text{ml.}$ and later diluted out, the decreased yield of phage with increased time of dilution reflects both the 'latent period effect', which is only slowly established, and the virustatic effect. The experiments in which fumagillin was always present at a concentration of at least 0.1 $\mu\text{g.}/\text{ml.}$ were attempts to study the virustatic effect by itself. The results are consistent with the hypothesis that fumagillin interferes with a stage in the development of vegetative phage. This stage occurs about 20 min. after the start of phage growth. As the first detectable intracellular

phage is not found till about 15 min. before the end of the latent period (i.e. at 45 min. in the absence of fumagillin and at 60 min. in 0.1 μ g. fumagillin/ml.) the action of fumagillin, unlike that of proflavine on T2 phage of *Escherichia coli* B (Foster, 1948), is not on phage maturation. Neither does it act on the stages immediately after infection, as does a phenanthridine on some phages of *Pseudomonas aeruginosa* (Mills, 1953; Dickinson, 1954). The work reported here does not allow any hypothesis about the nature of the stage in phage synthesis inhibited, but the specificity of action of fumagillin (Asheshov *et al.* 1949) suggests that the stage may be one peculiar to a few staphylococcal phages.

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