The Life-Cycle of an Actinophage for *Streptomyces venezuelae*

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SUMMARY

The life-cycle of actinophage MSP2 in host *Streptomyces venezuelae* s13 was divisible into distinct stages by manipulating the cultural conditions and by the use of certain chemical compounds. Two types of attachment were observed: reversible and irreversible. Reversible attachment occurred instantaneously and was relatively independent of temperature and phage concentration. The controlling variables in extent of adsorption were the amount of host mycelium and ionic content of the medium. Irreversible attachment was time dependent and temperature dependent. Addition of acriflavine allowed phage attachment and injection to occur, but suppressed some stage of intracellular growth or maturation. Acriflavine prevented formation of viable phage, but phage components were demonstrated immunologically and electron-microscopically. Chilling of phage-infected cultures allowed lysis to proceed, with release of preformed phages; but no new phages were formed. These results indicate that the life-cycle of actinophage MSP2 is experimentally separable into attachment, injection, growth, maturation and lysis.

INTRODUCTION

Previous studies with actinophages MSP2 (Jones, 1963), MSP8 (Kolstad & Bradley, 1964) and MVP7 (Bradley, Lee & Jones, 1963) established procedures for routine propagation, concentration and purification of several litres of lysates containing 10^{11} plaque-forming units (p.f.u.)/ml. The specific action on phage growth of factors such as temperature of incubation and ionic content of the medium were not studied during the earlier works. It seemed probable that the life-cycle of actinophages, like coliphages (Stent, 1963), could be separated into five stages: attachment, injection, growth, maturation, lysis. It was reasonable to expect the composition of the medium and metabolic inhibitors to affect these stages differentially. This report shows that the life-cycle of actinophage MSP2 can be resolved into distinct stages by manipulation of the cultural conditions and by the use of certain chemical compounds.

METHODS

Stock cultures of *Streptomyces venezuelae* s13 were maintained at 30° on tomato-paste oatmeal agar (Gottlieb, 1961) by serial single colony subculture. Spore preparations were made by suspending in peptone yeast-extract broth (Bradley, 1959)

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the surface growth from 7- to 10-day stock cultures and homogenizing it with a tissue grinder. Vegetative mycelium of *S. venezuelae* was cultured in flasks of peptone yeast-extract broth inoculated with spore suspension to give 10^6 to 10^7 spores/ml. The flasks were shaken at 30° for 18 hr.

For studies of the attachment of phage to host, young vegetative mycelium was collected by centrifugation, washed twice with appropriate salt solution and suspended to give an extinction of 0.2 at $420\text{ m}\mu$ (E_{420}) in the Bausch & Lomb Spectronic 20; this corresponded to about 10^8 colony-forming units/ml. Purified actinophage MSP2 was added to give 10^7 p.f.u./ml. Reversible attachment was determined by comparing the number of p.f.u. in the whole reaction mixture with the number in the supernatant fluid after centrifuging down the mycelium with attached phage. For irreversible attachment, appropriately diluted antiserum was added to the reaction mixture to neutralize the phage particles which were either unattached or only reversibly adsorbed to host mycelium. Hyperimmune antiserum was prepared by repeatedly injecting rabbits (Adams, 1959) with purified actinophage MSP2. The antiserum was absorbed with washed host mycelium. An alternative assay of irreversible attachment was the number of p.f.u. remaining attached to the mycelium after repeated washing with 0.15 M-NaCl. Viable actinophage particles were counted by the conventional soft-agar overlay technique (Jones & Bradley, 1962).

For growth studies, young vegetative mycelium was harvested by centrifugation, suspended in a solution 0.004 M in $\text{Ca}(\text{NO}_3)_2$ and 0.0015 M in NaCl. Purified actinophage MSP2 was added to give a multiplicity of about 10 phage particles/host colony-forming unit. After 30 min., the infected mycelium was collected, washed with 0.15 M-NaCl to remove free and reversibly attached phage, suspended in peptone yeast-extract broth and incubated with agitation. At intervals for 3–5 hr, samples were assayed for plaque-forming particles.

RESULTS

Reversible attachment

Attempts to obtain attachment of actinophage MSP2 to *Streptomyces venezuelae* mycelium in the actual culture fluid were unsuccessful; therefore young vegetative mycelium was grown in peptone yeast-extract broth, washed with salt solution and tested for ability to adsorb phage. Maximal attachment was obtained with solutions about 10^{-3} M in calcium, magnesium or manganese salts. Concentrations of NaCl exceeding 0.1 M inhibited attachment (Table 1). The attachment observed in inorganic salts medium with washed mycelium, with the free phage in the supernatant fluid used as the index of adsorption, was dependent upon host concentration but independent of phage concentration (Table 2). These results indicated that this adsorption was reversible. Reversibility was demonstrated directly by suspending the mycelium with attached phage in distilled water or in 0.1 M-NaCl (Table 3). Strains of *S. venezuelae* selected for resistance to actinophage MSP2 or to the closely related actinophage MSP8 retained the ability to adsorb MSP2. *Streptomyces rimosus*, a streptomycete naturally resistant to actinophage MSP2, had limited capacity to adsorb MSP2, whereas *Nocardia erythropolis* had none (Table 4).

Table 1. *Effect of cation content on attachment of actinophage MSP 2 to washed mycelium of Streptomyces venezuelae s13*

S. venezuelae s13 was grown overnight in peptone yeast-extract broth, harvested by centrifugation, washed with appropriate salt solution and suspended to the original volume. Actinophage MSP 2 was added and the reaction mixture shaken at 30° for 30 min. The whole mixture and the supernatant fluid after centrifugation were titrated.

Salt	Strength (M)	No. MSP 2 particles/ml.		Proportion of phage particles attached (%)
		Total	Free	
Ca(NO ₃) ₂ *	10 ⁻²	3.8 × 10 ⁶	2.8 × 10 ⁶	27
Ca(NO ₃) ₂ *	10 ⁻³	1.0 × 10 ⁶	6.0 × 10 ⁴	94
Ca(NO ₃) ₂ *	10 ⁻⁴	2.8 × 10 ⁶	2.2 × 10 ⁶	21
MgSO ₄ *	2 × 10 ⁻³	1.5 × 10 ⁶	1.7 × 10 ⁵	88
MnSO ₄ *	2 × 10 ⁻³	9.5 × 10 ⁵	7.0 × 10 ⁴	93
NaCl†	2 × 10 ⁻¹	1.2 × 10 ⁶	1.4 × 10 ⁶	0
NaCl†	2 × 10 ⁻³	1.3 × 10 ⁶	8.0 × 10 ⁴	94

* Salt solution also contained 2 × 10⁻³ M-NaCl.

† Salt solution also contained 2 × 10⁻³ M-Ca(NO₃)₂.

Table 2. *Effect of host and phage concentration on reversible attachment of actinophage MSP 2 to host (Streptomyces venezuelae s13) mycelium*

Washed mycelium of *S. venezuelae* s13 was suspended with phage MSP 2 in 4 × 10⁻³ M-Ca(NO₃)₂ + 10⁻³ M-NaCl and the reaction mixture was shaken at 30° for 30 min. Total phage and free phage in the supernatant fluid were determined.

Host concentration (E ₄₂₀)	Input phage titre	No. MSP 2 particles/ml.		Proportion of phage particles attached (%)
		Total	Free	
0.40	3.0 × 10 ⁸	2.8 × 10 ⁸	0.3 × 10 ⁸	89
0.40	3.0 × 10 ⁶	2.2 × 10 ⁶	0.2 × 10 ⁶	90
0.40	3.0 × 10 ⁴	2.9 × 10 ⁴	0.1 × 10 ⁴	97
0.28	1.0 × 10 ⁷	1.0 × 10 ⁷	0.3 × 10 ⁷	67
0.12	1.0 × 10 ⁷	1.0 × 10 ⁷	0.6 × 10 ⁷	40
0.03	1.0 × 10 ⁷	1.0 × 10 ⁷	0.9 × 10 ⁷	10

Table 3. *Desorption of actinophage MSP 2 from washed Streptomyces venezuelae s13 mycelium*

Washed mycelium was suspended in 10⁻³ M-Ca(NO₃)₂ + 10⁻³ M-NaCl; phage was added and the reaction mixture shaken at 30° for 30 min. Thereafter, the mycelium was sedimented by centrifugation and suspended in the indicated desorption media. The desorption mixture was shaken for 30 min. at 30°. Adsorbed phage was measured by difference between titres of the total reaction mixture and the supernatant fluid.

Attachment phase no. MSP 2 p.f.u./ml.		Desorption medium	Desorption phase no. MSP 2 p.f.u./ml.	
Total	Free		Total	Free
1.1 × 10 ⁶	9 × 10 ⁴	10 ⁻³ M-Ca(NO ₃) ₂ + 10 ⁻³ M-NaCl	9 × 10 ⁵	1 × 10 ⁴
1.2 × 10 ⁶	1 × 10 ⁵	10 ⁻³ M-NaCl	8 × 10 ⁵	7 × 10 ⁵
1.1 × 10 ⁶	7 × 10 ⁴	De-ionized water	6 × 10 ⁵	5 × 10 ⁵
1.1 × 10 ⁶	8 × 10 ⁴	10 ⁻² M-Ca(NO ₃) ₂	8 × 10 ⁵	3 × 10 ⁴

Irreversible attachment

In the previous experiments showing reversibility of attachment of phage to host, about 10 % of the adsorbed phage did not desorb, that is, a certain proportion were irreversibly attached to the host mycelium. The irreversibly adsorbed phage were not inactivated by antiserum directed against actinophage MSP2. Unlike the reversible phase, irreversible attachment was temperature dependent and time dependent (Table 5) and was not inhibited by 5 g. NaCl. (Table 6).

Table 4. *Specificity of attachment of actinophage MSP2 to mutants of Streptomyces venezuelae s 13 and to other actinomycetes*

Washed mycelia of the indicated actinomycetes were suspended in 4×10^{-3} M- $\text{Ca}(\text{NO}_3)_2 + 10^{-3}$ M-NaCl; phage was added; the mixtures were shaken at 30° for 30 min. Total and free phage were assayed.

Organism	Phenotype	No. MSP2 p.f.u./ml.		Proportion of phage particles attached (%)
		Total	Free	
<i>S. venezuelae</i> s 13	Sensitive to MSP2 and MSP8	1.1×10^6	0.1×10^6	99
<i>S. venezuelae</i> s 13 R2	Resistant to MSP2 and MSP8	1.2×10^6	1.8×10^6	85
<i>S. venezuelae</i> s 13 R8	Resistant to MSP2 and MSP8	1.4×10^6	0.8×10^6	94
<i>S. rimosus</i>	Resistant to MSP2 and MSP8	2.6×10^6	1.8×10^6	30
<i>Nocardia erythropolis</i>	Resistant to MSP2 and MSP8	4.7×10^6	4.7×10^6	0

Table 5. *Effect of time and temperature on attachment of actinophage MSP2 to washed mycelium of Streptomyces venezuelae s 13*

Washed mycelium of *S. venezuelae* s 13 was suspended in 4×10^{-3} M- $\text{Ca}(\text{NO}_3)_2 + 10^{-3}$ M-NaCl and equilibrated to the indicated temperature; phage was added to a final concentration of 1.6×10^6 p.f.u./ml. and the reaction mixture shaken for 30 min. Reversible attachment was measured as the difference between total and free phage. Irreversible attachment was defined as resistance to specific antiserum.

Temperature	Assay	Time (min.)		
		5	10	30
		No. attached phage particles (p.f.u.)		
5°	Reversible	1.6×10^6	1.4×10^6	1.6×10^6
30°	Reversible	1.4×10^6	1.3×10^6	1.3×10^6
56°	Reversible	1.5×10^6	1.5×10^6	1.3×10^6
5°	Irreversible	10^2	3×10^2	5×10^2
30°	Irreversible	3×10^4	6×10^4	2×10^6
56°	Irreversible	$< 10^2$	$< 10^2$	$< 10^2$

Vegetative growth

The multiplication curve of actinophage MSP2 was determined by measuring the p.f.u./ml. in serial samples of infected mycelia. The latent period was 75–90 min., the rise period was from 90 to 180 min., and the burst size varied between 500 and 5000 p.f.u. (Fig. 1). To determine intracellular growth, samples of an infected culture were disrupted by ultrasonic treatment. The brief ultrasonic treatment required to rupture the mycelium did not damage mature phage particles, but decreased the

Table 6. *Effect of NaCl concentration on attachment of actinophage MSP 2 to Streptomyces venezuelae s13*

Washed mycelium of *S. venezuelae* was suspended in solutions 0.004 M-Ca(NO₃)₂ and 0.1 M or 0.002 M in NaCl; phage was added. The reaction mixture was incubated at 30° for 30 min. Reversible attachment was determined as the difference between total and free phage. Phage not eluted by repeated washing with 0.15 M-NaCl were considered irreversibly attached.

NaCl (M)	No. MSP 2 p.f.u./ml.		
	Reversible assay		Irreversible assay
	Total	Free	
0.002	5.4×10^6	1.8×10^6	1.4×10^6
0.1	5.4×10^6	3.6×10^6	1.2×10^6

Table 7. *Suppression of actinophage MSP 2 replication in Streptomyces venezuelae s13*

Washed *S. venezuelae* s13 mycelium was infected in salt solution; attachment was stopped by antiserum; infected mycelium was sedimented and suspended in peptone yeast-extract broth (to give 2×10^6 p.f.u./ml.) with the indicated compound. After 4 hr, the amount of phage was assayed.

Treatment	Concentration (μ g./ml.)	No. MSP 2 p.f.u./ml.	Effect on host
Control, 30°	—	1.4×10^8	None
37°	—	2×10^6	None
Acriflavine*	15	8×10^6	None
Endomycin†	200	1.9×10^7	Inhibited
Novobiocin†	20	2.1×10^7	Inhibited
Bacitracin‡	5§	3×10^8	Inhibited
Streptomycin‡	125	1.3×10^9	None

* An equal mixture of euflavine and proflavine.

† From the Upjohn Company.

‡ From the California Corporation for Biochemical Research.

§ Units/ml.

Table 8. *Effect of acriflavine on actinophage MSP 2 replication*

Washed *S. venezuelae* s13 mycelium was infected in salt solution; attachment was stopped by adding antiserum; infected mycelium was sedimented and suspended in peptone yeast-extract broth with acriflavine as indicated. Infected hyphae were disrupted by ultrasonic treatment.

Time (min.)	No. MSP 2 p.f.u./ml.			
	20 μ g. acriflavine/ml.		Control	
	Intact	Disrupted	Intact	Disrupted
0	3×10^7	1×10^7	4.5×10^7	8×10^6
90	2.5×10^7	1×10^7	4.5×10^7	5×10^6
180	5×10^7	1×10^7	7.7×10^9	5×10^9
270	9×10^7	2×10^7	4×10^{10}	2×10^{10}
270 (drug last 180 min.)	1×10^9	Not done	4×10^{10}	Not done
270 (drug last 120 min.)	1.8×10^{10}	Not done	4×10^{10}	Not done

number of p.f.u. in newly infected cultures. The time between infection and the first appearance of mature phage intracellularly, that is, the eclipse period, was about 1 hr. The period of rise was 2–3 hr in cultures in which phage growth was interrupted by ultrasonic treatment. The maximal titre achieved was the same in artificially-lysed cultures as in cultures which had lysed normally.

Various factors were tested for ability to suppress phage replication. Only acriflavine (equal parts euflavine and proflavine) and elevated temperature sharply

Fig. 1

Fig. 2

Fig. 1. One-step growth curve (●—●) and intracellular growth curve (○—○) of actinophage MSP2 in host (*Streptomyces venezuelae* s 13) mycelium. Cultures were disrupted by ultrasonic treatment to determine intracellular phage.

Fig. 2. Diagram of agar gel diffusion plate of actinophage MSP2. Wells labelled 1 contained hyperimmune antiserum against actinophage MSP2; 2 contained purified, intact MSP2; 3 contained a fraction (35,000g sediment) from an acriflavine-treated, phage-infected *Streptomyces venezuelae* culture; 4 contained a comparable fraction (35,000g sediment) from an acriflavine-treated *S. venezuelae* culture without phage; and 5 contained purified MSP2 which had been disrupted by prolonged (2 hr) ultrasonic treatment.

inhibited phage growth without interfering with host growth (Table 7). Acriflavine did not kill intact phage, nor did it inhibit attachment or bactericidal action of phage upon the host. The intracellular development of intact phage was prevented

(Table 8). Infected host mycelium incubated for 90 min. without acriflavine and then for 3 hr with acriflavine, supported a 30-fold increase in phage. Apparently, delayed addition of acriflavine allowed release of preformed phage. Attempts to remove acriflavine by sedimentation of the host mycelium and its resuspension in fresh medium did not permit development of mature phage. The mycelium, once exposed to acriflavine, remained markedly yellow in spite of repeated washing.

Infected cultures, exposed to acriflavine, were disrupted by ultrasonic treatment and fractionated by alternate cycles of centrifugation at 8000g for 10 min. and at 35,000g for 30 min. The 8000g residue, which contained host cell walls, was discarded. The 35,000g sediment contained particles which resembled phage heads and tails when viewed in the electron microscope and which gave precipitate bands in diffusion gel experiments with antiserum directed against intact MSP 2 (Fig. 2). Uninfected cultures, treated or untreated with acriflavine, were similarly processed, but did not yield particles which resembled phage components or give bands of precipitate when tested with antiserum directed against the actinophage MSP 2.

Lysis

Incompletely lysed cultures often cleared and gave increased titres of phage during refrigeration. Accordingly, the effect of continuous and discontinuous chilling of phage-infected cultures was studied. Phage-infected cultures incubated continuously at 1° did not produce phage. The shift from 1° to 30° merely delayed the normal lytic cycle for 1 hr. The shift from 30° to 1° after 1 hr permitted a 100-fold increase in phage titre.

DISCUSSION

Two types of attachment of actinophage to host *Streptomyces venezuelae* s13 mycelium were observed. Reversible attachment was relatively independent of time, temperature and phage concentration. The principal controlling variable in the extent of adsorption was the amount of host mycelium. Irreversible attachment was time dependent and temperature dependent, clearly differentiating it from reversible adsorption. Also, reversible attachment was inhibited by high concentrations of NaCl, whereas the irreversible type of attachment was not. It is conceivable that reversible attachment is an ion-exchange reaction and irreversible attachment an enzymic process. To date, irreversible attachment has not been experimentally separated from the injection process.

Intracellular growth of actinophage MSP 2 in *Streptomyces venezuelae* s13 was differentiated into an early phase which did not proceed at 1° and a later stage which was inhibited by acriflavine. Fractions of acriflavine-treated, phage-infected cultures contained particles which when viewed in the electron microscope resembled purified actinophage that had been disrupted by ultrasonic treatment (Anderson & Bradley, 1964) and by certain chemical compounds (Painter & Bradley, 1965). These same samples gave two precipitate bands with specific antiserum in diffusion gel experiments; these bands were immunologically identical to those obtained with the actinophage disrupted by ultrasonic treatment. Because these preparations contained empty heads and tails, acriflavine probably inhibited the assemblage of phage components into mature phage particles. The action of acriflavine on actinophage,

therefore, resembles that on T-even coliphages of *Escherichia coli* (DeMars, Luria, Fisher & Levinthal, 1953).

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