Observations on the Adsorption of Caulobacter Bacteriophages containing Ribonucleic Acid

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SUMMARY

Caulobacter bacteriophages containing ribonucleic acid have been found to attach to pili of their hosts. Only when the phage is actually lytic for a given host can the phage+pilus complexes be observed; non-specific complexes are not formed. Removal of the pili from the host by shear treatment before phage adsorption resulted in effective inhibition of phage adsorption. While swarmer caulobacters frequently possessed pili which were located at the flagellated pole of the organism, stalked caulobacters were almost always devoid of these structures. Adsorption experiments with segregated populations of caulobacters indicated that stalked caulobacters adsorbed RNA phages very poorly, while in swarmer populations and unsegregated cultures the RNA phages were efficiently adsorbed.

INTRODUCTION

The demonstration by Crawford & Gesteland (1964) that R17, a male specific bacteriophage containing ribonucleic acid (RNA), attached to pili of F⁺ and Hfr strains of *Escherichia coli* drew attention to the processes of RNA-phage adsorption and infection (Brinton, Gemski & Carnahan, 1964; Bradley, 1965; Brinton, 1965; Valentine, Wedel & Ippen, 1965; Edgell & Ginoza, 1965). Among the bacteriophages lytic for Caulobacter species isolated by Schmidt & Stanier (1965) are several small spherical viruses which contain RNA. It would be of interest to know whether pili to which RNA 'caulophages' can attach occur in caulobacters. The present paper reports an investigation to determine whether the process of RNA-phage adsorption in these stalked bacteria is similar to that observed with *E. coli* + RNA-phage systems, and how stalk formation in caulobacters affects RNA-phage adsorption.

METHODS

Bacteriophages and hosts. Bacteriophages Φ Cb8r, Φ Cb12r, and Φ Cb23r, and bacterial strains *Caulobacter bacteroides* CB8 and CB11a, *C. crescentus* CB2 and CB15, and *C. fusiformis* CB27 and CB29 were used in this work. These RNA-phages were isolated and described by Schmidt & Stanier (1965); the Caulobacter strains originated from the collection of Dr Jeanne Stove Poindexter.

Media. For the propagation of host strains, preparation of phage lysates, and adsorption experiments, the standard complex caulobacter medium (PYE) was used (Poindexter, 1964).

Lysate preparation and partial purification of bacteriophages. Lysates were pre-

pared by eluting phages from confluent lysed PYE agar overlayers. The lysates were concentrated and partially purified by two cycles of differential centrifugation (Schmidt & Stanier, 1965).

Preparation for electron microscopy. PYE broth cultures of *Caulobacter* strains were grown at 30° without shaking to the late exponential phase of growth. Two ml. of the culture were sedimented by centrifugation at 7000 g, and to the bacterial pellet were added 2 ml. of a phage preparation containing 10^{10} to 10^{11} plaque-forming units (p.f.u.). The bacterial pellet was gently resuspended and phage adsorption allowed to proceed at room temperature for 10–20 min. The adsorption mixture was centrifuged at 7000 g and the supernatant fluid discarded. The pellet was gently resuspended in distilled water (1 ml.) and prepared for electron microscope observation by the negative staining method (Brenner & Horne, 1959; Bradley, 1962). The stains used were 2% sodium phosphotungstate (pH 7.0), 2% sodium tungstate (pH 7.2) and 1% uranyl acetate (pH 4.0). Specimens were placed on Formvar-coated carbon-stabilized grids and examined with a Siemens Elmiskop I electron microscope operated at 60 kV.

Shear treatment. The blending procedure for removal of bacterial pili (Brinton, 1965; Valentine *et al.* 1965) was used with *Caulobacter bacteroides* CB11a and *C. fusiformis* CB27. The bacterial cultures were subjected repeatedly to 2 min. periods of blending at 4° by using a pre-cooled MSE Atomix blender at maximum speed. Each shear treatment was followed by centrifugation at 7000 g; the bacterial pellet was then resuspended in fresh medium. After the final centrifugation, the sheared bacteria were resuspended in PYE broth to the volume of the original culture and used in phage adsorption experiments.

Segregation of Caulobacter cultures. The two morphologically distinct types of caulobacter cells, swimmers and those with stalks, can be segregated to obtain relatively homogeneous populations. The centrifugal method of Stove & Stanier (1962) was used to obtain segregated populations of *C. bacteroides* CB11a and *C. fusiformis* CB27. Four successive centrifugations, employing a Servall Superspeed with SS-34 rotor at gravitational fields of 1000 to 1500 g, gave adequate segregation of these strains.

Measurement of adsorption. *Caulobacter* cultures containing about 5×10^9 colony-forming units/ml. in PYE broth supplemented with 0.004 M-MgSO₄ were pre-incubated for 5 min. at 30° on a slow rotary shaker. Phage was then added at a multiplicity of infection of 0.01. At succeeding time intervals, samples were diluted 1/100 in chloroform-saturated PYE broth to stop further adsorption and to eliminate phages which had adsorbed to host caulobacters. Unadsorbed phages were then determined as plaque-forming units by appropriate dilution and plating by the agar overlayer method (Adams, 1959). For calculation of % unadsorbed phage, the initial phage titre of the adsorption mixture was determined at zero time with omission of the chloroform treatment.

RESULTS

Lytic activity

The host ranges of the caulobacter RNA-phages with the hosts used in this study are shown in Table 1. There was no overlap of host specificity among these caulophages; they are also serologically distinct (Schmidt & Stanier, 1965).

Observation of phage + pilus complexes

An electron microscope survey for formation of phage + pilus complexes was made on the RNA-phages in the presence of the various *Caulobacter* strains. This survey included specific phage + host systems, and also phage + *caulobacter* mixtures in which the phages did not cause lysis of the *Caulobacter* strains. In all cases tested, the *caulobacter* RNA-phages able to cause lysis of a given strain were found to form complexes with pili emanating from the host *caulobacters* (Table 2). In the non-specific RNA-phage + *caulobacter* mixtures, no phage + pilus complexes were observed. No exception to the correlation between ability to cause lysis and formation of phage + pilus complexes has so far been found.

Table 1. *Lytic activity of caulobacter RNA-phages*

Host strain	Caulophage		
	ΦCb8r	ΦCb12r	ΦCb23r
	Lytic activity		
<i>C. crescentus</i> CB 2	—	+	—
<i>C. crescentus</i> CB 15*	—	+	—
<i>C. bacteroides</i> CB 8	+	—	—
<i>C. bacteroides</i> CB 11a*	+	—	—
<i>C. fusiformis</i> CB 27*	—	—	+
<i>C. fusiformis</i> CB 29	—	—	+

* Host strain used in preparation of specific phage lysate.

Table 2. *Formation of RNA-phage + pilus complexes in caulobacters*

Caulobacter	Caulophage		
	ΦCb8r	ΦCb12r	ΦCb23r
	Presence (+) or Absence (—) of phage + pilus complex		
<i>C. crescentus</i> CB 2	—	+	—
<i>C. crescentus</i> CB 15	—	+	—
<i>C. bacteroides</i> CB 8	+	—	—
<i>C. bacteroides</i> CB 11a	+	—	—
<i>C. fusiformis</i> CB 27	—	—	+
<i>C. fusiformis</i> CB 29	—	—	+

Electron micrographs of phage + pilus complexes in which the pili originate from swarmer *caulobacters* are shown in Pl. 1, figs. 1–3. In all three *Caulobacter* species found to possess pili, these filamentous structures emanated from the flagellated pole of the swarmer; it is this pole which eventually gives rise to stalk development (Poindexter, 1964; Schmidt & Stanier, 1966). The pili were not found at the opposite non-flagellated pole, nor did they occur at peritrichous sites of origin. The pili occurred on most swarmers of *Caulobacter fusiformis*, but were observed rather infrequently in *C. crescentus* and *C. bacteroides*. Stalked *caulobacters* are almost always devoid of pili. In the very few instances where pili were seen on stalked *caulobacters*, these structures emanated from the terminal end of the stalk (Pl. 2, fig. 4). The

occurrence of pili on a stalked caulobacter was noted only twice, although many hundreds of stalked caulobacters have been observed in the presence of their specific RNA-phage, and with numerous phage + pili complexes occurring on swarmers present in the same preparation.

In *Caulobacter fusiformis*, the swarmers usually had several pili; up to 8 pili on one caulobacter were observed (Pl. 1, fig. 1). *Caulobacter crescentus* and *C. bacteroides* seldom possessed more than 4 pili/swarmer; more frequently only one pilus or none at all were observed (Pl. 1, figs. 2, 3).

The pili are several microns in length. The usual observable diameter is approximately 40 Å in *Caulobacter crescentus* CB 2 (Pl. 2, fig. 5) and in *C. fusiformis* CB 27. No substructure was evident. Occasionally, when several pili were present on a *C. fusiformis* organism, wider pili with apparent diameters of 75–80 Å were observed. These wider forms seemed to have a core penetrated by the negative stain (Pl. 2, fig. 6). The diameter of the pili of *C. bacteroides* was not reliably determined.

Table 3. *RNA-phage adsorption with sheared and normal caulobacter cultures*

Expt. no.	Caulophage	Caulobacter host	Shear treatment (2 min.)	Phage remaining unadsorbed at	
				7 min. (% unadsorbed)	15 min.
1	ΦCb8r	CB 11a	—	58	20
			× 3	100	99
2	ΦCb32r	CB 27	—	48	17
			× 3	85	75
3	ΦCb23r	CB 27	—	54	28
			× 4	100	97

Adsorption of caulophages to sheared hosts

To determine whether RNA-phage adsorption in caulobacters is dependent on the presence of shear-sensitive structures as has been demonstrated for the adsorption of male-specific RNA-phages of *Escherichia coli* (Brinton, 1965; Valentine *et al.* 1965), adsorption experiments with sheared and untreated caulobacter cultures were made. Two systems, *Caulobacter bacteroides* CB 11a and ΦCb8r, and *C. fusiformis* CB 27 and ΦCb23r, were examined. The results are shown in Table 3. In both systems, host cultures which had undergone shear treatment just before the adsorption experiment were greatly decreased in their capacity to absorb the RNA-phages. With adequate shear treatment, phage adsorption was negligible.

Phage adsorption after chloramphenicol treatment of caulobacters

Edgell & Ginoza (1965) showed that R17, a male-specific RNA-phage of *Escherichia coli*, has an adsorption requirement for metabolically active host organisms. Pre-treatment of the host with chloramphenicol or other metabolic inhibitors prevented irreversible adsorption of phage R17. An analogous situation appears to exist with caulobacter RNA-phage adsorption. Growing cultures of *Caulobacter fusiformis* CB 27 and *C. bacteroides* CB 11a were exposed to chloramphenicol (150 µg./ml.)

for 2 hr, during which time increase in turbidity of the cultures ceased. Phage-adsorption experiments were made with chloramphenicol-inhibited cultures and with control cultures. In both phage systems pre-treatment of the host caulobacters with chloramphenicol prevented phage adsorption.

Adsorption of caulophages in segregated host populations

Because stalked caulobacters had so rarely been observed to possess pili it was of interest to determine how RNA-phage adsorption in populations of stalked caulobacters would be affected by the natural absence of pili. Segregated populations of *Caulobacter fusiformis* CB27 were more than 90 % homogeneous at the beginning of the adsorption experiment; however, division of stalked caulobacters, giving daughter swarmers did occur during the experiment. Division was not prevented by the addition of a metabolic inhibitor because of the adsorption requirement for metabolically active host caulobacters. Despite the increasing numbers of swarmers, the adsorption of caulophage Φ Cb23r was sharply decreased in the stalked caulobacter populations, as compared to that in swarmer populations and in control unsegregated cultures of *C. fusiformis* CB27 (Table 4). Similar results were obtained with caulophage Φ Cb8r and segregated *C. bacterioides* CB11a populations.

Table 4. *RNA-phage adsorption with segregated Caulobacter fusiformis* host populations

Host caulobacters	Unadsorbed caulophage (Φ Cb23r) at time	
	7 min	15 min
	% unadsorbed phage	
Unsegregated control	61	33
Stalked caulobacters	95	82
Swarmers	45	25

DISCUSSION

The attachment of caulobacter RNA-phages to pili of their hosts and the dependence of their adsorption upon the presence of these pili are features consistent with the situation described for male-specific RNA-phages of *Escherichia coli* (Crawford & Gesteland, 1964; Brinton, 1965; Valentine *et al.* 1965). Bradley (1965) reported a similar observation for attachment sites of an RNA-phage of *Pseudomonas aeruginosa*. Thus for all known RNA-phage systems the phage + pilus association is a general characteristic. Brinton (1965) suggested that bacterial pili participate in the conjugation process. It becomes evident that bacteria of the *Caulobacter* group should be investigated for ability to conjugate, based on the presence in these organisms of RNA-phage + pilus complexes such as occur in *E. coli* and *P. aeruginosa*, which are both known to possess sexuality.

The data presented here do not eliminate the possibility that receptor sites for the caulobacter RNA-phages apart from the pili might exist on the surface of the organisms. However, the sensitivity of RNA-caulophage adsorption to shearing treatment of the host, and the poor phage adsorption with stalked caulobacter

populations which largely lack pili, implicate the pili as significant phage receptors. The relationship of the phage + pilus association in the actual infection of the caulobacters with viral RNA remains obscure.

Bradley (1965) reported that the pili of *Pseudomonas aeruginosa* to which RNA-phages attach are polar, as found for the pili of the three *Caulobacter* species studied here. Both the genera *Pseudomonas* and *Caulobacter* are characterized by polar flagellation. The diameter of the *P. aeruginosa* pilus is about 40 Å (Bradley, 1966), similar to the diameter observed for most pili of *Caulobacter fusiformis* and *C. crescentus*. The F pili of *E. coli* are wider, 85 Å, and have peritrichous sites of origin (Brinton, 1965). There is a possibility that the few wider pili of *C. fusiformis* which have been observed were actually two pili intertwined, since they have been found only where several pili emanated from the pole of a swarmer and where entanglement of pili was notable. The uneven nature of the negative stain frequently results in apparent variation in the diameter of a pilus. Sodium tungstate was used for negatively-stained preparations of caulophages ΦCb8r and ΦCb12r since these phages disintegrate in sodium phosphotungstate. The phage + pilus complexes were not observable in preparations stained with uranyl acetate, although the phages remained intact.

It had been previously noted in one-step growth experiments that RNA-phages did not multiply in segregated populations of stalked host caulobacters (unpublished data). The very infrequent occurrence of pili on stalked caulobacters, and the poor adsorption of RNA-phages to the stalked caulobacters provide a possible basis for explanation of this observation. The stalked caulobacters may represent a form relatively resistant to RNA-phages which are, however, able to infect the piliated swarmer before it has undergone stalk development.

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REFERENCES

- ADAMS, M. (1959). *Bacteriophages*. New York: Interscience Publishers, Inc.
- BRADLEY, D. E. (1962). A study of the negative staining process. *J. gen. Microbiol.* **29**, 503.
- BRADLEY, D. E. (1965). The morphology and physiology of bacteriophages as revealed by the electron microscope. *Jl R. Microsc. Soc.* **84**, 257.
- BRADLEY, D. E. (1966). The structure and infective process of a *Pseudomonas aeruginosa* bacteriophage containing ribonucleic acid. *J. gen. Microbiol.*
- BRENNER, S. & HORNE, R. W. (1959). A negative staining method for high resolution electron microscopy of viruses. *Biochem. biophys. Acta* **34**, 103.
- BRINTON, C. C. (1965). The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in Gram negative bacteria. *Trans. N.Y. Acad. Sci.* **27**, 1003.
- BRINTON, C. C., GEMSKI, P. & CARNAHAN, J. (1964). A new type of bacterial pilus genetically controlled by the fertility factor of *E. coli* K12 and its role in chromosome transfer. *Proc. natn. Acad. Sci. U.S.A.* **52**, 776.
- CRAWFORD, E. M. & GESTELAND, R. F. (1964). The adsorption of bacteriophage R17. *Virology* **22**, 165.
- EDGEELL, M. H. & GINOZA, W. (1965). The fate during infection of the coat protein of the spherical bacteriophage R17. *Virology* **27**, 23.

J. M. SCHMIDT

- POINDEXTER, J. S. (1964). Biological properties and classification of the *Caulobacter* group. *Bact. Rev.* **28**, 231.
- SCHMIDT, J. M. & STANIER, R. Y. (1965). Isolation and characterization of bacteriophages active against stalked bacteria. *J. gen. Microbiol.* **39**, 95.
- SCHMIDT, J. M. & STANIER, R. Y. (1966). The development of cellular stalks in bacteria. *J. cell. Biol.* **28**, 423.
- STOVE, J. L. & STANIER, R. Y. (1962). Cellular differentiation in stalked bacteria. *Nature, Lond.* **196**, 1189.
- VALENTINE, R. C., WEDEL, H. & IPPEN, K. A. (1965). F-pili requirement for RNA bacteriophage adsorption. *Biochem. biophys. Res. Comm.* **21**, 277.

EXPLANATION OF PLATES

PLATE 1

Figs. 1–3. *Caulobacter* swimmers with RNA-phage attachment to pili. Sodium tungstate stain. $\times 68,000$.

Fig. 1. *C. fusiformis* CB27 and caulophage Φ Cf23r.

Fig. 2. *C. crescentus* CB2 and caulophage Φ Cb12r.

Fig. 3. *C. bacteroides* CB11a and caulophage Φ Cb8r.

PLATE 2

Fig. 4. *C. fusiformis* CB27 stalked organisms and caulophage Φ Cb23r. Sodium tungstate stain. $\times 68,000$.

Fig. 5. Pili of *C. crescentus* CB2 and caulophage Φ Cb12r. Sodium tungstate stain. $\times 144,000$.

Fig. 6. Pili and flagellum of *C. fusiformis* CB27 and caulophage Φ Cb23r. Sodium tungstate stain. $\times 144,000$.