JOURNAL OF BACTERIOLOGY, Mar., 1966
Vol. 91, No. 3

Printed in U.S.A.

A Bacteriophage for *Myxococcus xanthus*: Isolation, Characterization and Relation of Infectivity to Host Morphogenesis[1]

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Received for publication 2 September 1965

ABSTRACT

BURCHARD, ROBERT P. (University of Minnesota, Minneapolis), AND M. DWORKIN. A bacteriophage for *Myxococcus xanthus*: isolation, characterization and relation of infectivity to host morphogenesis. J. Bacteriol. **91**:1305–1313. 1966.—A bacteriophage (MX-1) infecting *Myxococcus xanthus* FB_t has been isolated from cow dung. The bacteriophage particle is approximately 175 mμ long. A tail about 100 mμ in length is encased in a contractile sheath and terminates in a tail plate. The head is polyhedral with a width of about 75 mμ. The nucleic acid of the bacteriophage is deoxyribonucleic acid and has a guanine plus cytosine content of 55.5%. The bacteriophage requires 10^{-3} M Ca^{++} and 10^{-2} M monovalent cation for optimal adsorption. Grown on vegetative cells of *M. xanthus* FB_t at 30 C in 2% Casitone medium, the bacteriophage has a latent period of 120 min and a burst size of approximately 100. Host range studies indicate that three strains of *M. xanthus* including a morphogenetic mutant are sensitive to the bacteriophage, whereas *M. fulvus*, *Cytophaga*, *Sporocytophaga myxococcoides*, and a fourth strain of *M. xanthus* are not. Of the two cellular forms characteristic of the *Myxococcus* life cycle, the bacteriophage infect only the vegetative cells; they do not adsorb to microcysts. Ability to adsorb bacteriophage is lost between 65 and 75 min after initiation of the relatively synchronous conversion of vegetative cells to microcysts. The bacteriophage does not adsorb to spheroplasts. After the appearance of visible morphogenesis and before the loss of bacteriophage receptor sites, addition of bacteriophage results in the formation of microcysts which give rise to infective centers only upon germination. The possibility that the infected microcysts are harboring intact bacteriophages has been eliminated.

In 1955 Anacker and Ordal (2) described a bacteriophage for *Chondrococcus columnaris*, the first report of a bacterial virus for the *Myxobacterales*. Since then, five additional bacteriophages have been isolated for this same species (Kingsbury and Pacha, Bacteriol. Proc., p. 118, 1964), as well as one for *Myxococcus xanthus* (Burchard and Dworkin, Bacteriol. Proc., p. 118, 1964).

We have been investigating the developmental physiology of *M. xanthus*. Apart from our intrinsic interest in a *Myxococcus* bacteriophage, there are a number of aspects of the developmental process of the host for which a bacteriophage might prove to be a valuable tool.

To our knowledge, bacteriophage have not been utilized as a tool in the study of changes occurring in bacteria as the cells go through a morphogenetic cycle. A technique has recently been described (6) which induces *M. xanthus* vegetative cells to convert to microcysts rapidly, quantitatively, and relatively synchronously, a conversion known to occur previously only within fruiting bodies. This provides an excellent opportunity to investigate the interaction of bacteriophage with host cells during morphogenesis.

This paper describes the isolation and properties of a bacteriophage for *M. xanthus* as well as changes, during morphogenesis, in the ability of the host cell to support a complete cycle of virus replication.

MATERIALS AND METHODS

Cultivation of the host organism. M. xanthus FB (5) undergoes a yellow-tan dissociation. A tan variant (FB_t) was isolated, stabilized, and utilized in the experiments reported below. The organism was cul-

[1] A preliminary report of this work was presented at the 64th Annual Meeting of the American Society for Microbiology, Washington, D.C., 4 May 1964.

[2] Public Health Service Career Development Awardee, 1-K3-GM-5869-10.

tured as described by Dworkin (5). CT-1 (1.8×10^{-3} M $MgSO_4$, 10^{-2} M K_2HPO_4—KH_2PO_4, *p*H 7.6, and 2% Difco Casitone) was utilized in place of CT.

Isolation of the bacteriophage. Classical isolation techniques (1) with the use of samples of soil, sewage, and water failed to yield a bacteriophage for *M. xanthus* FB_t. Eventually one was isolated from a sample of cow dung from St. Paul, Minn.

Prepration of lysates and purification of the bacteriophage. Preparation of liquid lysates and plating assays were carried out according to methods described by Adams (1). Incubation temperature was 30 C. Titers of 5×10^{10} infective units per ml were attained in lysates in a broth consisting of 2% Difco Casitone, 10^{-2} M K_2HPO_4—KH_2PO_4 (*p*H 7.6), and 10^{-3} M $CaCl_2$.

Determination of optimal ionic conditions for adsorption was carried out by assaying the supernatant fluid of infected cultures for unadsorbed bacteriophage after 10 min of gentle agitation at room temperature. Approximately 90% adsorption was considered optimal.

The bacteriophage were purified for electron microscopy and analysis of nucleic acid by the following procedure. Quantities of 10 liters of crude lysate were freed from remaining intact cells and debris by centrifugation at $10,400 \times g$ for 15 min at 4 C. The bacteriophage were sedimented by centrifugation at $24,400 \times g$ for 90 min at 4 C with a Batch head in a Spinco L-2 centrifuge. The concentrated bacteriophage preparation in 10^{-1} M K_2HPO_4—KH_2PO_4 (*p*H 7.6) and 10^{-1} M NaCl was treated with ribonuclease (bovine pancreas, five times crystallized, at 2 μg/ml) and deoxyribonuclease (crude, at 2 μg/ml) for 60 min at 30 C. This was followed by addition of trypsin (bovine pancreas, once crystallized, at 10 μg/ml) and incubation at 30 C for 60 min. Subsequently, a series of differential centrifugations at 4 C and overnight dialysis against 10^{-2} M K_2HPO_4—KH_2PO_4 (*p*H 7.6) at 4 C concluded the procedure.

Electron microscopy. Bacteriophage particles were stained with uranyl acetate. Microcysts were fixed in buffered 1% (w/v) OsO_4 overnight, followed by 2-hr fixation in buffered 0.5% (w/v) uranyl acetate (8). They were embedded in Epon 812 (9). Electron micrographs of bacteriophage and thin sections of microcysts were prepared with a Siemens Elmiskop I electron microscope.

Purification and analysis of the bacteriophage nucleic acid. Nucleic acid purification was carried out by shaking once with phenol (11), followed by a series of deproteinizations of the disrupted bacteriophage (in 1 M sodium perchlorate) with chloroform-isoamyl alcohol (24:1) (14). This was followed by two precipitations with 95% ethyl alcohol, and the deoxyribonucleic acid (DNA) was dissolved in 0.15 M NaCl—0.015 M sodium citrate.

In preparation for paper chromatography, a 2.5-mg sample of DNA was dialyzed against 4 liters of deionized water at 4 C overnight and brought to 5×10^{-3} M tris(hydroxymethyl)aminomethane buffer (*p*H 7.4) and 10^{-3} M $MgCl_2$. Amounts of 1 mg of deoxyribonuclease I (once crystallized) and snake venom phosphodiesterase (acetone fraction of lyophilized snake venom) were added to a final volume of 2.7 ml, and the mixture was incubated at 37 C over chloroform for 12 hr. The enzymatic hydrolysate was spotted on Whatman no. 1 chromatography paper. The solvent for the descending chromatogram was isobutyric acid–0.5 N ammonia (10:6) (10). The sodium salt of thymus DNA (type I; Sigma Chemical Co., St. Louis, Mo.) was used as a control. Spots were examined under ultraviolet light and eluted with 10^{-2} N HCl. The eluate was examined with a Zeiss PMQ II spectrophotometer at the absorption maxima of the mononucleotides.

The buoyant density of the bacteriophage DNA was determined by centrifugation for 22 hr at 44,770 rev/min at 25 C in a Spinco model E analytical ultracentrifuge (D head) in CsCl at a final concentration of 7.75 M (12). Two marker DNA preparations were used: *Bacillus subtilis* DNA supplied by John Spizizen and *Streptomyces griseus* DNA supplied by L. A. Jones. The former has a guanine + cytosine (G + C) content of 43.5%; the latter, 72%, as determined in the respective laboratories by melting-point and buoyant-density analyses.

Melting-point analysis on the *Myxococcus* bacteriophage DNA was carried out in sodium perchlorate solution (7). The DNA in saline-citrate was diluted 1 part into 9 parts of aqueous 8.0 M sodium perchlorate buffered with ethylenediaminetetraacetate (EDTA) at *p*H 7.0. Two preparations of viral DNA were analyzed. *B. subtilis* $phage_{11}$ DNA, supplied by Bernard Reilly, was used as a standard. Change in optical density of the solution upon heating at 260 mμ was recorded on a Gilford multiple-sample absorbance recorder adapted to a Beckman spectrophotometer.

RESULTS

The bacteriophage about to be discussed will be referred to as MX-1.

Bacteriophage morphology. MX-1 has an overall length of about 175 mμ with a polyhedral head about 75 mμ in diameter and a tail 100 mμ long (Fig. 1). The tail terminates in a base plate and spike. A sheath in either extended or contracted configuration is also present. The size and morphology are grossly similar to that of coliphage T2 (4). In the same preparation, one field contained only contracted sheaths (Fig. 2). A core channel can be observed in both side and end-on views; it has a diameter of approximately 50 A.

Adsorption conditions. Adsorption of bacteriophage to host cells was studied in a 2% Difco Casitone medium. Addition of 10^{-3} M Ca^{++} and 10^{-2} M K_2HPO_4—KH_2PO_4 (*p*H 7.6) to the salts normally present in Casitone is requisite for optimal, irreversible adsorption. Mg^{++} does not substitute for Ca^{++}.

Plaque morphology. After approximately 20 hr of incubation at 30 C, plaques of MX-1 generally have a diameter of 1 to 2 mm and are clear.

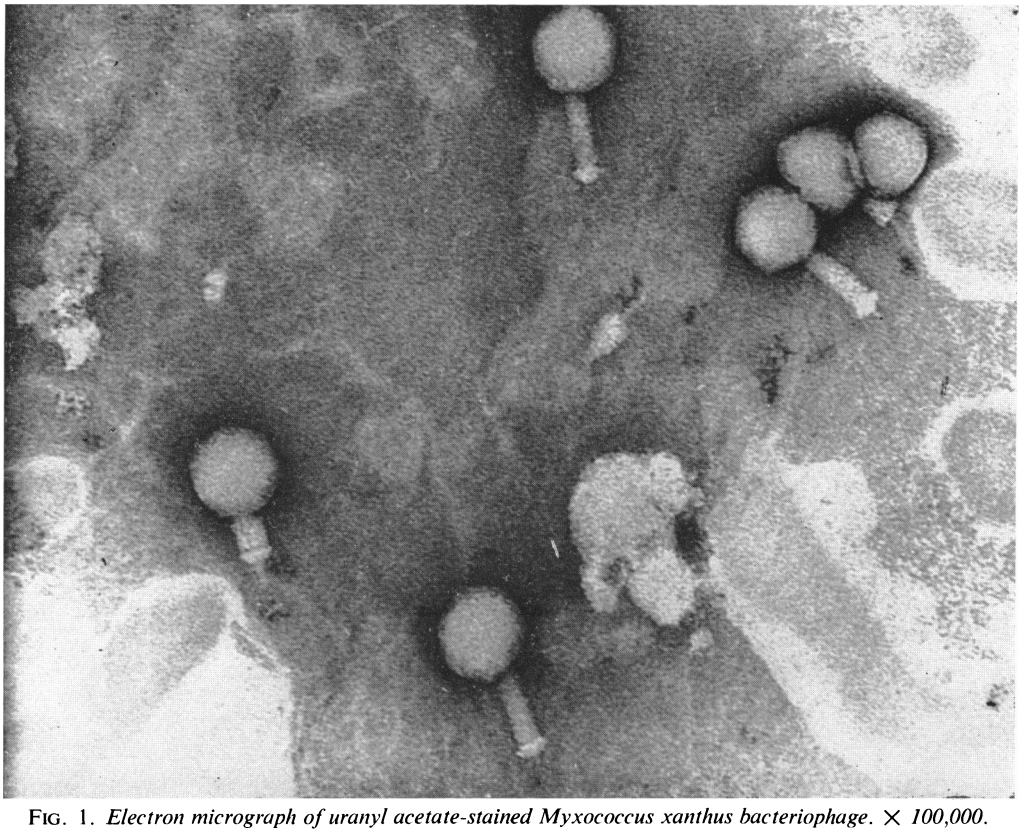

FIG. 1. *Electron micrograph of uranyl acetate-stained Myxococcus xanthus bacteriophage.* × *100,000.*

Halos have been observed around the plaques after longer incubation periods.

Bacteriophage host range. The bacteriophage includes in its host range three laboratory strains of *M. xanthus*, including a morphogenetic variant (strain MC; 5) of the original strain, *M. xanthus* FB. *M. fulvus* (strain MF, kindly supplied by Walter Fluegel), a species of *Cytophaga*, and *Sporocytophaga myxococcoides* (ATCC 10010) are not infected.

One-step growth curve and burst size. MX-1 exhibits a typical one-step growth curve (Fig. 3). A latent period lasting 2 to 2.5 hr at 30 C is followed by a 2-hr rise, culminating in a burst of about 100 particles per cell.

Bacteriophage nucleic acid. The nucleic acid of the virus is DNA, as demonstrated by positive diphenylamine and negative orcinol tests (14) and by paper chromatographic separation of the mononucleotides. Four mononucleotides, corresponding to adenylic, cytidylic, guanylic, and thymidylic acids, were clearly separated on the chromatogram. A G + C content of 55.5% was calculated for the eluates of the mononucleotides. Table 1 contains the base composition of MX-1 DNA.

Initial DNA base analyses performed on formic acid hydrolysates (17) revealed a fifth spot upon chromatography with isopropanol—HCl—water (15). The spot was still present after hydrolysis with 6 N HCl, but did not appear after perchloric acid hydrolysis of the DNA (3). Only four mononucleotides were identifiable after enzymatic hydrolysis of the MX-1 DNA. Chromatography of a formic acid hydrolysate of host cell nucleic acids revealed no spot corresponding to that which appeared in bacteriophage DNA chromatograms, ruling out contamination from this source. The identity of the material in the spot of the acid hydrolysate remains undetermined.

Buoyant density of the bacteriophage DNA was 1.716, assuming a linear density gradient between the *B. subtilis* marker DNA and *S. griseus* marker DNA. This value corresponds to 56% G + C (12).

Two viral DNA preparations demonstrated a melting temperature of 50.8 C in 7.2 M sodium perchlorate. This corresponds to a double-stranded molecule having a G + C content of 55% (7).

Relation between host morphogenesis and bacteriophage adsorption. We were interested in investigating the interaction of the bacteriophage

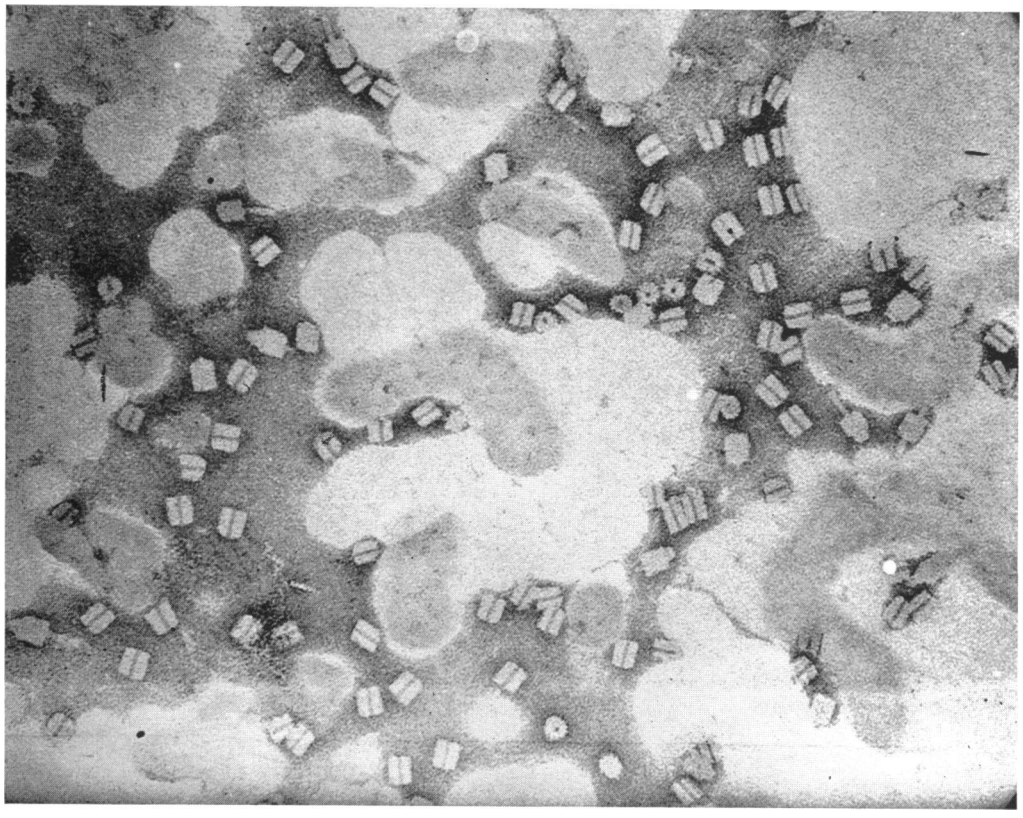

FIG. 2. *Electron micrograph of Myxococcus xanthus bacteriophage tail sheaths stained with uranyl acetate.* × *100,000.*

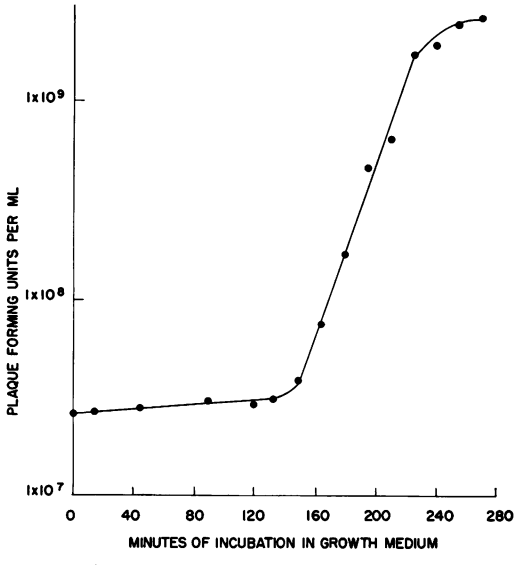

FIG. 3. *One-step growth curve of Myxococcus xanthus bacteriophage at 30 C; 3.8* × 10^8 *cells per milliliter were infected at a multiplicity of three.*

TABLE 1. *Base composition of MX-1 DNA*

Nucleotide base	Per cent of total nucleotides
Guanine	25.0
Cytosine	30.8
Thymine	20.0
Adenine	24.1

with *M. xanthus* at various stages of the life cycle of the host. Long vegetative cells are transformed to spherical, encapsulated microcysts, relatively resistant to heat, desiccation, and physical disruption. Normally, the microcysts develop within aggregates (fruiting bodies) on agar containing a low concentration of nutrient [about 0.05% Difco tryptone; (5)]. It has been recently demonstrated that conversion of vegetative rods to microcysts also occurs in liquid CT growth medium following the addition of 0.5 M glycerol (6). Furthermore, spheroplasts, not part of the life cycle, will form from logarithmic-phase cells in CT upon the addition of 3.0 M glycerol.

The bacteriophage were not adsorbed by spheroplasts or by either plate-grown or glycerol-CT-1 microcysts.

By use of the glycerol induction technique, it was possible to determine at what specific stage of microcyst formation the alteration in capacity to adsorb bacteriophage occurred since, under these conditions, morphogenesis was relatively synchronous (Fig. 4). Glycerol added to the CT-1 culture at zero-time stopped further cell multiplication. Then, during the first 75 min, the vegetative cells shortened to nonrefractile spheres. After this, the spheres became increasingly refractile, and the morphological conversion was essentially complete after about 120 min.

Bacteriophage was added to vegetative cells at various times during their conversion to microcysts. After a 10-min adsorption period, the supernatant culture fluid was assayed for free bacteriophage. No significant change in the ability to adsorb bacteriophage occurred until 65 min had elapsed. It was of considerable interest that, in the 10-min period during which the ovoids became spheres, bacteriophage adsorption declined by 50% (Fig. 4). The remaining 15% adsorption is probably accounted for by the presence of a low percentage of vegetative cells and some reversible (nonspecific) adsorption.

Relationship between host morphogenesis and bacteriophage infectivity. It was of interest to investigate the fate of the cells which adsorb bacteriophage during the first 65 min of glycerol-induced morphogenesis. Bacteriophage, at a multiplicity of infection of 3, were added to samples of a culture at intervals after the addition of glycerol, and the infected cultures were incubated further at 30 C until all cells undergoing morphogenesis had completed their microcyst

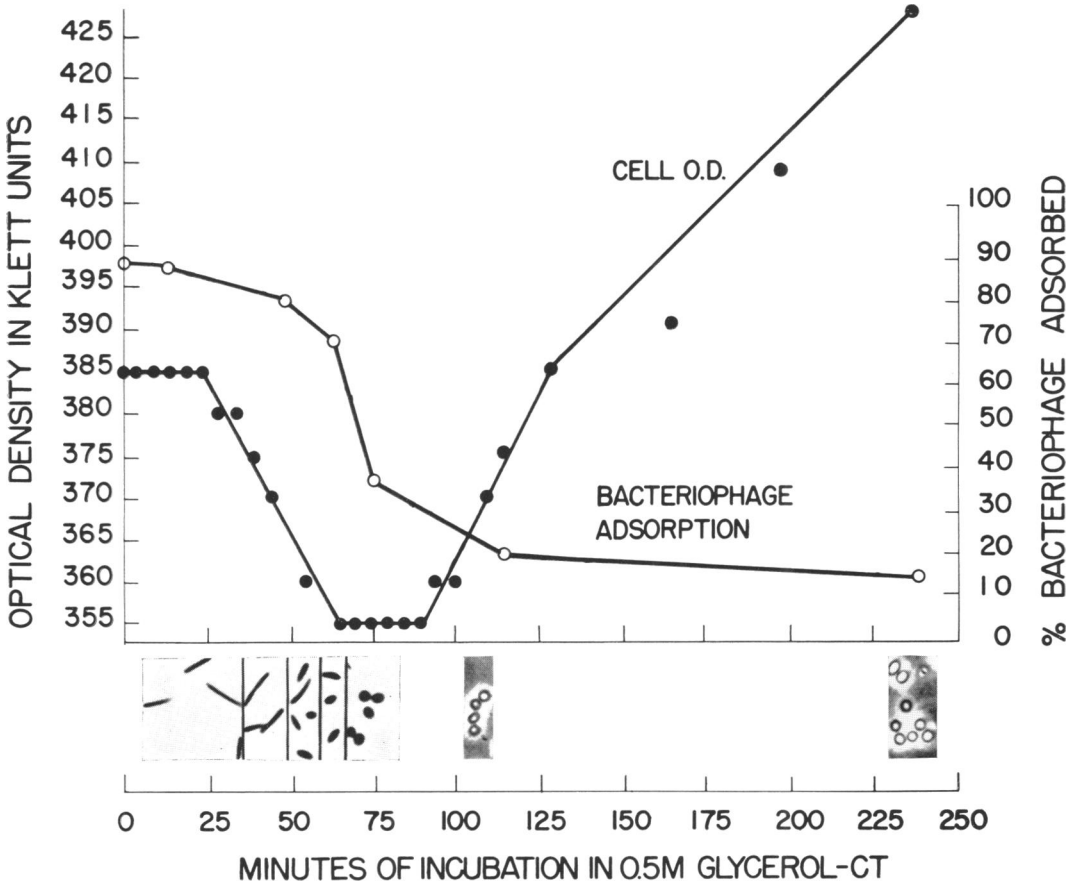

FIG. 4. *Loss of ability to adsorb bacteriophage during Myxococcus xanthus morphogenesis. Glycerol-induced conversion of vegetative cells to microcysts is followed by change in optical density of the suspension and by visible alterations of cells (Zeiss phase-contrast microscope, × 540). Bacteriophage adsorption is measured by assay of residual, free bacteriophage particles.*

formation (150 to 250 min). Each culture was then subjected to sonic treatment for 30 sec with a 20-kc MSE ultrasonic disintegrator. The morphologically mature microcysts, which were resistant to sonic treatment, were not affected by this treatment; all cells which had not completed morphogenesis were ruptured. Surviving cells (i.e., microcysts) were counted with a Petroff-Hauser counting chamber. Figure 5 depicts vegetative cells which form microcysts after bacteriophage infection at various times following addition of glycerol, as percentage of the number of microcysts formed in the uninfected culture. Each experimental point represents addition of bacteriophage to one portion of the culture. During the first 20 min of glycerol-induced morphogenesis, there is a small increase in the number of cells completing microcyst formation. Between 20 and 50 min there is an approximately linear increase to 100% in the number of cells completing morphogenesis after addition of bacteriophage. The conditions reported as being optimal for synchronous microcyst formation (6) were altered somewhat to conform to the requirements of the present experiment. This accounts for the decrease in synchrony and the gradual linear increase in microcyst formation. Thus, between 30 and 65 min after addition of glycerol, the cells adsorb bacteriophage; yet, they complete microcyst formation.

To determine whether the microcysts which have formed in the presence of bacteriophage are infected, the following experiment was carried out. A culture of vegetative cells (5×10^8 per milliliter) was induced to form microcysts by the glycerol technique. Converting cells were infected

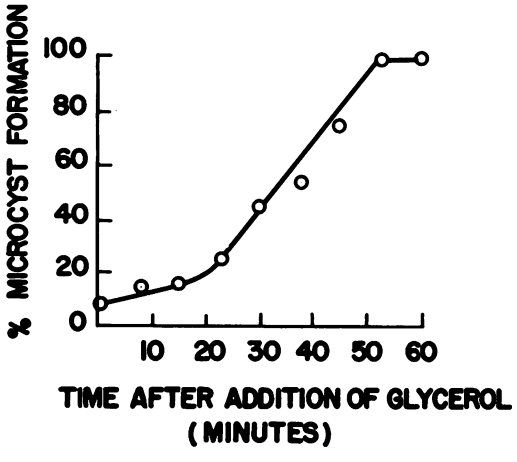

FIG. 5. *Microcyst formation after addition of bacteriophage at various times after onset of glycerol-induced morphogenesis.*

with bacteriophage (multiplicity of infection of 1 to 2) 40 min after addition of glycerol. Microcyst formation was allowed to proceed to completion (300 min after addition of glycerol), after which the cultures were centrifuged at 5,900 $\times$ *g* for 10 min. Pellets were resuspended in 5 ml of Mg-P-1 (10^{-2} M K_2HPO_4—KH_2PO_4, *p*H 7.6, and 1.8×10^{-3} M $MgSO_4$) and were sonically treated for 30 sec; centrifugation and sonic treatment were repeated twice. This was done to remove remaining vegetative cells and unadsorbed bacteriophage. The microcysts were then counted and plated on a germination medium (CT-1) to determine the number of viable (presumably uninfected) cells and on a lawn of bacteriophage-sensitive vegetative cells (on CT-1) to assay the number of infective centers. The data are presented in Table 2. Infection has clearly taken place, and there are approximately equal numbers of infected and uninfected microcysts (1.2×10^{10} and 1.4×10^{10}, respectively). The control flask contained microcysts which had been sonically treated prior to the addition of bacteriophage at a low multiplicity of infection.

Since the infected microcysts had been subjected to sonic oscillation and centrifugation to eliminate loosely bound viral particles (free bacteriophage lose infectivity after sonic treatment), there must then be a close relationship between the host cell and the infecting virus. Several models are possible. (i) The bacteriophage may be irreversibly adsorbed to the cell wall, but a change in the wall during microcyst formation may prohibit entrance of the viral nucleic acid into the cell. As microcyst development continues, capsular material may envelop the viral particle, protecting it from shearing by sonic treatment. Upon germination and re-establishment of the vegetative cell wall, the bacteriophage nucleic acid may then be injected into the cell to initiate an infectious cycle. (ii) One or more complete bacteriophage may be formed following immediate entrance of viral DNA into the host cell. A change in the wall during the vegetative cell-microcyst conversion may prevent release of the bacteriophage until germination has occurred. (iii) The adsorbed bacteriophage particle may inject its DNA into the host cell; as a result of an alteration in the biosynthetic capability of the system, the viral nucleic acid moiety may remain dormant until germination occurs. (iv) Viral DNA may be injected and one or more rounds of DNA replication initiated; protein coats of the bacteriophage may also be synthesized. No mature virus particles would be constructed until germination.

Preliminary experiments have been carried out

TABLE 2. *Interaction of bacteriophage MX-1 with cells undergoing morphogenesis**

Time (min)	Procedure	Experimental		Control	
		Total viable cells	Total infective centers	Total viable cells	Total infective centers
0	Glycerol (0.5 M) added to vegetative cells	2.5×10^{10}		2.5×10^{10}	
40	Bacteriophage added to experimental		4.0×10^{10}		—
	$CaCl_2$ (10^{-3} M) added to all				
100	Assay unadsorbed phage		3.5×10^{8}		—
300	Bacteriophage added to control		—		4.0×10^{9}
	Assay total microcysts	1.8×10^{10}		2.8×10^{10}	
	Assay viable cells (colony-forming units)	1.4×10^{10}		1.5×10^{10}	
	Assay infective centers		1.2×10^{10}		3.0×10^{9}
	Minimill for 10 min	$<2.5 \times 10^{4}$	1.5×10^{8}	—	1.3×10^{9}
	Assay infective centers in 5,900 × *g* supernatant fraction		1.2×10^{6}		

* Glycerol-induced microcyst formation was allowed to proceed for 300 min, at which time both the bacteriophage-infected experimental and the control cultures were centrifuged at 5,900 × *g* for 10 min. Pellets were resuspended in 5 ml of Mg-P-1 and sonically treated 30 sec; centrifugation and sonic treatment were repeated twice. Bacteriophage were added to the control microcysts. Both preparations were subjected to ballistic disintegration for 10 min.

to attempt to select one of the proposed models. Electron micrographs of uranyl acetate-stained thin sections of microcysts exposed to bacteriophage (multiplicity of infection, 5) at 45 min after addition of glycerol demonstrated little detectable difference from similar preparations of cells not exposed to bacteriophage.

To determine whether the bacteriophage-microcyst complexes had complete, infective viral particles attached to the microcyst surface or within the cell (models 1 and 2), ballistic disintegration was used to disrupt the infected microcysts and presumably to release any epicellular or intracellular bacteriophage. Infected microcysts were prepared as described above. Bacteriophage at low multiplicity of infection were added to the control culture so that the effect of subsequent treatment on free viral particles could be determined. Acid-washed, glass beads (7 ml, 85-μ diameter, Minnesota Mining and Manufacturing Co.) were added to 5-ml cell volumes, and the mixture was subjected to treatment in a Minimill for 10 min in the cold. This treatment disrupts microcysts with high efficiency but inactivates free bacteriophage only slowly. The preparations were then centrifuged at 5,900 × *g* for 10 min. Viable cells, total infective centers, and infective centers in the 5,900 × *g* supernatant fraction of the disrupted microcyst preparations were assayed. The data of one such experiment are presented in Table 1. Upon ballistic disruption of the bacteriophage-microcyst complexes, the number of infective centers decreased by 99%. Almost all of the remaining infective centers were associated with the 5,900 × *g* pellet fraction of the disrupted cells. Longer treatment in the Minimill (additional 10 min) did not give rise to any significant increase in the number of infective centers.

DISCUSSION

In terms of morphology, nature of the nucleic acid, and infective cycle, the bacteriophage described above has no unusual characteristics in comparison with other previously described bacterial viruses.

Our results demonstrate that only after the vegetative cells have undergone a considerable change in form does the alteration in ability to adsorb bacteriophage occur. During the interval when ovoid cells become nonrefractile spheres, a sharp drop in bacteriophage adsorption occurs. Since capsule formation does not occur prior to the acquisition of refractility, it is evident that it is not the microcyst capsule which prevents bacteriophage adsorption. We have, thus far, been unable to isolate receptor sites from vegetative cells, and, therefore, do not know whether the loss of ability to adsorb bacteriophage results from a loss, alteration, or masking of the receptor sites. If, however, a virus receptor site is lost during the ovoid-sphere conversion, the isolation and characterization of this site should give some insight into the nature of the cell wall changes associated with morphogenesis.

Prior to the loss of bacteriophage receptor sites, viral particles are irreversibly adsorbed to host cells, but only during the first 30 min of mor-

phogenesis do most of the bacteriophage initiate a virulent infection cycle. Between 30 and 65 min, cells complete their morphogenesis but are potential infective centers. The fact that the number of infective centers (1.2×10^{10}) plus the number of viable microcysts (1.4×10^{10}) add up to a larger number than the number of total microcysts (1.8×10^{10}) suggests that some of the infective centers may be capable of giving rise to colonies. One might expect that, if this were the case, some of the plaques would have been turbid. This was not observed.

If complete bacteriophage particles were tightly bound to the peripheral structure of the microcyst, or were included within it, a substantial release of bacteriophage would have been expected after ballistic disruption of the infected microcysts. The data indicated that only one infective particle per 80 infected cells was present after disruption of the cells. While it is possible that the ballistic treatment only freed a small fraction of the viral particles from their complex with the cell wall or capsule, or both, the data do tend to exclude the possibility of the presence of intracellular intact bacteriophage. It is not clear why disruption with the Minimill resulted in a reduction of the viable count from 1.4×10^{10} to $<2.5 \times 10^{4}$ total microcysts, but only in an 80-fold decrease in the number of infective centers. This suggests that perhaps 1 in 80 cells does indeed contain an intact viral particle which is released upon cell disruption.

Sadler and Dworkin (J. Bacteriol., *in press*) have found that during glycerol-induced microcyst formation in *M. xanthus* FB, under slightly different conditions from those utilized here, net synthesis of DNA, ribonucleic acid, and protein temporarily ceases. This period coincides with the interval during which nonvirulent infection may take place. This cessation of net synthesis has been shown to precede the appearance of microcyst protein, and may represent a switch-over to a microcyst genome. It may be that the transition to the synthesis of microcyst macromolecules or the earlier block in net synthesis of vegetative cell macromolecules prevents synthesis of bacteriophage structural protein, DNA, or the biosynthetic enzymes required for virus production. If the bacteriophage is added shortly before the transition, one or a few rounds of DNA replication might occur; if added still earlier, the infective process may continue to completion. When the transition is completed, the biosynthetic capability of the cell may have become committed to microcyst formation. The viral DNA moiety(s) might then be carried in a nonvirulent state in the microcyst cytoplasm until germination permits completion of a lytic cycle.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Bernard Pollara and James Isherwood in carrying out DNA buoyant-density and melting-curve analyses, respectively. We are indebted to Barbara Painter for preparation of the electron micrographs.

This investigation was supported by grant NSFGB-9 from the Developmental Biology Section of the National Science Foundation and by Public Health Service training grant 2TIA190-06.

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