Antigenic Properties of Bacteriophage $\phi 29$ Structural Proteins

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Serological methods and electron microscopy were used to study the structural proteins of the small *Bacillus subtilis* bacteriophage $\phi 29$. This virus has a large number of fibers attached at both ends of its prolate head. A complex neck assembly is comprised of 12 symmetrically arranged appendages as the outer component. Head fibers, neck appendages, and the head surface bind anti- $\phi 29$ antibodies. Immune sera absorbed with defective lysates of suppressor-sensitive (*sus*) mutants have been used to determine the genetic control of neck appendages production. Studies on the serum-blocking power of lysates defective in different tail components showed that appendages contain the main serum-blocking protein. This finding suggests an essential role of the neck appendages in phage adsorption or DNA injection.

The small *Bacillus subtilis* bacteriophage $\phi 29$ has a prolate head with head fibers and a complex tail (1). The major structural components have been identified with specific proteins by use of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (9). The head contains three proteins, and the tail at least four proteins. The head fibers have no essential function in viral infectivity (12) and therefore might be involved only in viral assembly. In the $\phi 29$ tail, 12 spindle-shaped appendages are attached to a lower collar that constitutes part of a neck assembly; an upper collar fixes the neck assembly to the head. A tail axis runs through the neck assembly and enlarges to form a distal knob (1). The function of this unique tail as an adsorption organelle holds particular fascination.

The present investigation was undertaken in hopes of using antisera absorbed with defective lysates of $\phi 29$ suppressor-sensitive (*sus*) mutants and electron microscopy to identify structural proteins as products of specific genes. This technique has been used successfully with the much larger T4 bacteriophage (15). We describe in this investigation the interaction of antibodies with $\phi 29$ structures, and present evidence that neck appendages have a role in adsorption of $\phi 29$ to the host or injection of DNA, or both, as demonstrated by their serum-blocking activity and apparent apposition to the cell wall in thin sections.

MATERIALS AND METHODS

Phage and bacteria. Phage $\phi 29$ was the wild-type described previously (1, 10). The suppressor-sensitive (*sus*) mutants have been kindly supplied by B. E. Reilly; the isolation, burst size, and mapping of the *sus* mutants are the subject of a recent investigation (11). As nonpermissive host strains, *B. subtilis* 12A (6) or *Bacillus amyloliquefaciens* H (14) have been used. The permissive host was *B. subtilis* L15 (11).

Growth conditions. Cells were grown in Difco Antibiotic Medium 3 (PB) at 37°C in a gyratory shaker. At a cell density of 2×10^8 /ml, MgSO_4 was added to a final concentration of 5×10^{-3} M and the culture was infected at multiplicities of infection (MOI) of 5 or 10. The cultures were treated with lysozyme (10 $\mu\text{g}/\text{ml}$) 2 to 3 h after infection or as soon as the optical density dropped, and were incubated with deoxyribonuclease (2 $\mu\text{g}/\text{ml}$, DNase I, Calbiochem, La Jolla, Calif.) for 15 min at 37°C. Debris was removed by centrifugation at $5,000 \times g$ for 10 min. and sodium azide was added to 10^{-3} M before concentration or storage at 4°C. When *B. amyloliquefaciens* H was used as the host, an inhibition of lysis was obtained by addition of glucose to 1%, MgSO_4 to 5×10^{-3} M and MnCl_2 to 10^{-3} M just before infection.

Lysates for observation by electron microscopy were prepared in PB clarified by dialysis. The wild-type phage was grown on *B. amyloliquefaciens* H and purified as described previously (1), except that the polyethylene glycol concentration was omitted.

Concentration of lysates. Concentrated, debris-free lysates were required for electron microscopy or for absorption of immune sera. A 15- to 20-fold concentration was obtained by use of negative pres-

sure dialysis in collodion bags (no. 100, Schleicher & Schuell). No effect on phage titers due to interaction with the collodion bag was observed.

Antisera. Rabbits were injected intramuscularly with 3×10^{12} CsCl-purified ϕ 29 wild-type phages carefully mixed with complete Freund adjuvant (1:1). After 6 weeks a second injection was made with 1.8×10^{13} phage particles mixed with complete Freund adjuvant (1 volume of adjuvant plus 2 volumes of antigen). Bleedings were made from the ear at 10, 16, and 23 days after the second injection. The K-values for these sera were approximately 27,000/min, 11,000/min and 11,000/min, respectively, as determined in PB at 37 C. Complement was inactivated by heating at 56 C for 30 min.

Absorption of sera with defective lysates. The method has been published elsewhere (15). Either entire sera or immunoglobulin G (IgG) enriched fractions obtained by precipitation with 17% Na_2SO_4 were used. The serum was diluted in phosphate-buffered saline, containing per liter: 7 g of Na_2HPO_4 , 3 g of KH_2PO_4 , 4 g of NaCl, and 10^{-3} M sodium azide. To 1 ml of diluted serum (K-value 2 to 10/min) was added 0.2 ml of phage lysate concentrated 15-fold. After overnight incubation at 37 C, the mixture was centrifuged at $60,000 \times g$ for 30 min and the supernatant solution (depleted serum) was used for electron microscope observations.

Electron microscopy. A thin carbon layer was evaporated onto parlodion or Formvar films on 400-mesh grids. Sodium phosphotungstate (1.5 or 2%, pH 6.8 to 7.2) was used for negative staining. The technique for the binding of antibodies to phage particles on the electron microscope grid has been described previously (15). To observe ϕ 29 adsorption in thin sections, *B. subtilis* 12A was grown in PB to about 6×10^8 cells/ml and diluted in PB to 2×10^8 cells/ml. After addition of MgSO_4 to 5×10^{-3} M, cells were infected at room temperature at an MOI of 1,500. At 5 min after infection sodium azide was added to 10^{-3} M. At 15 min after infection cells were concentrated by low-speed centrifugation ($5,000 \times g$ for 10 min), fixed in glutaraldehyde (Hagen, manuscript in preparation), and embedded in Epon 812 (8). Thin sections were poststained with uranylacetate and lead citrate. Micrographs were taken in a Philips 300 or in a Siemens Elmiskop IA electron microscope at 80 keV and instrumental magnifications between $\times 30,000$ and $\times 80,000$.

Preparation of ϕ 29-particles without head fibers. CsCl-purified wild-type ϕ 29 (about 10^{12} /ml) in 0.05 M tris (hydroxymethyl) aminomethane (Tris)-hydrochloride (pH 7.4), 0.01 M MgCl_2 , and 0.15 M NaCl were heated at 72 C for 10 to 15 min. After DNase treatment (15 $\mu\text{g}/\text{ml}$), the particles were sedimented at 40,000 rpm in the SW 65 rotor of a Spinco L2-65 ultracentrifuge for 25 min. The pellet was resuspended in the same Tris-hydrochloride buffer, layered on top of a CsCl step-gradient consisting of four steps of densities 1.55, 1.45, 1.35, and 1.25 g/cm^3 , and centrifuged for 90 min at 35,000 rpm in the SW 65 rotor. A band sedimenting slightly lower than the position of intact phage was collected. Observation of these particles in the electron microscope showed DNA-containing phages without head fibers.

SBP assay. We essentially followed the methods developed by De Mars (3) and by Edgar and Lielausis (5). Debris-free defective lysates were concentrated 20-fold as described above, frozen and stored at -60 C. For the serum-blocking protein (SBP) assay, a dilution of anti- ϕ 29 serum was prepared in phosphate-buffered saline (pH 6.8) containing 10^{-3} M NaN_3 to give a final K-value of 5 to 10/min, in order to provide conditions for observation of the absorbed sera by electron microscopy. To 0.8 ml of diluted serum, 0.2 ml of the concentrated defective lysate was added, and two- or fivefold dilutions were made in the same serum. Samples were incubated for 15 h at 45 C and then for several hours at 4 C. The residual K-value of the different samples was then determined by addition of ϕ 29 wild-type tester phage (final concentration 10^5 to 10^6 plaque-forming units/ml) to a 1:50 dilution of the absorbed sera in PB at 40 C. Samples were removed at times between 0 and 60 min, diluted in PB, and plated on *B. subtilis* 12A (nonpermissive for the *sus* mutants). Plates were incubated at 37 C for 20 to 24 h, a period sufficient for development of plaques from phage survivors that had been partially inactivated by the serum (see Results).

The residual K-value for each sample was calculated from the slope of its inactivation kinetics as compared to a control (the same serum without lysate). Generally, three points (times 0, 20, and 40 min) were used for the determination of each slope.

RESULTS

Characterization of anti- ϕ 29 serum by electron microscopy. Association of anti- ϕ 29 antibodies to intact ϕ 29 particles is shown in Fig. 1. For reference of structure, ϕ 29 particles and detached ϕ 29 tails with neck are shown in Fig. 2. Details of ϕ 29 morphology were presented previously (1). Figure 1 shows that a dense antibody layer is formed around the head and completely covers the neck of the phage. The ϕ 29 antigen-antibody complex appears to be heavier at both ends of the head and in the neck region. The distal tail enlargement (tail knob) did not seem to bind an appreciable amount of antibodies in studies with several sera.

The specificity of this complex has been demonstrated in the case of an anti-T4D+ serum reacted with T4D+ particles by comparison with nonspecific and with totally absorbed sera (15). We have performed similar controls: Figure 3 shows that no complex is formed on ϕ 29 by anti-T4 serum. Moreover, after absorption of the diluted anti- ϕ 29 serum with 10^{11} to 2×10^{11} wild-type ϕ 29 phages/ml under the conditions described in Materials and Methods, no antigen-antibody complex was visible on the electron microscope grids.

If ϕ 29 particles without head fibers are adsorbed to the carbon film instead of intact

FIG. 1. Electron micrograph of $\phi 29$ wild-type phage incubated with anti- $\phi 29$ serum on the electron microscope grid. Head fibers and neck appendages are covered by a dense layer of antibodies. Magnification $\times 210,000$.

FIG. 2. $\phi 29$ phages and $\phi 29$ tails with neck. Magnification $\times 200,000$.

FIG. 3. T4D+ particles and $\phi 29$ particles incubated with anti-T4D+ serum. Magnification $\times 130,000$.

phage, only a thinner layer of antibodies is present around the head surface, and the complex is reduced also in the neck region (Fig. 4). Therefore, head fibers are mainly responsible for the heavy clusters of antibody-antigen com-

plex on intact phages reacted with anti- $\phi 29$ serum. Moreover, the presence of a thinner antibody layer in the elongated portion of the head (Fig. 1) indicates that head fibers are assembled only on the closing portions of the

FIG. 4. Fiberless ϕ 29 particles incubated with anti- ϕ 29 serum on the electron microscope grid. Antibodies are associated with the head surface and the neck appendages. Magnification $\times 130,000$.

head, in agreement with previous electron microscopic observations (1).

Figure 4 also shows that there is a class of

antibodies that interacts with the neck region. These are likely to be anti-neck appendages antibodies, because all the other structures in

the neck are much smaller and are almost completely covered by the appendages in the intact phage. We therefore have an experimental system in which we can distinguish at least three presumably different species of antibodies: anti-head fibers, anti-head surface, and anti-neck appendages. More evidence in favor of the assignment of the last species to the neck appendages will be presented below.

Determination of gene product positions. Anti- ϕ 29 serum was depleted with defective lysates of *sus* mutants in hopes of obtaining antibodies directed against specific structural components; localization of such antibodies on wild-type phages by electron microscopy would thus identify the antigens as products of specific genes. Thirteen *sus* mutants were investigated, representing the six cistrons D, F, H, I, J, and K (11). Positive results have been obtained in the case of the mutants J305 (Fig. 5 and 6) and J602 (Fig. 7).

The residual antibodies after depletion of anti- ϕ 29 serum with a J305-defective lysate (LY305) are specific for the neck region. Occasionally, headless particles with bound antibodies can be seen (Fig. 5). The specificity of these antibodies for the neck appendages is suggested by the thickness and the dimensions of the complex, and by the observation of "bridges" between adjacent particles at the level of the appendages over distances that exclude binding of antibodies to inner structures of the neck as a possible explanation (Fig. 6).

Mutant 602 has also been mapped in cistron J, of which mutant 305 was chosen as representative (11). As with mutant J305, antibodies remaining after depletion of anti- ϕ 29 serum with a J602-defective lysate (LY602) are specific for the tail appendages (Fig. 7). The assignment of the appendages protein to cistron J has been confirmed by our SDS polyacrylamide gel electrophoresis studies of mutants J305 and J602 (2).

Electron microscope observation of lysates. Empty heads without tails are the major component in lysates of mutant J305 prepared on the nonpermissive bacteria and observed by negative staining (Fig. 8). No particles with appendages were observed. Lysates of mutant J602 prepared and observed under the same conditions show, in addition to empty heads, a small but significant proportion of DNA-containing particles with tail and normal collars, but no neck appendages (Fig. 9). Inability to observe substantial numbers of these particles in mutant J305 lysates may reflect an even more fragile head-tail junction (Fig. 1-9).

SBP assay. We have observed that antisera depleted with the defective lysates LY305 or LY602 (cistron J), under the conditions used for electron microscopy, still have more than 30% of the original K-value as compared with a control serum to which broth was added. We therefore investigated the contribution of anti-neck appendages antibodies to the phage neutralizing activity of the serum, using the SBP assay (3, 5). The type of information that can be expected from this approach has been discussed by Edgar and Lielausis (5). Under the conditions we used, the inactivation of tester phage by anti- ϕ 29 serum followed first order exponential kinetics, at least in the range extending from 95% to about 5% survivors that was typical of our experiments.

After exposure of tester phages to the serum, survivors were often inhibited in formation of normal size plaques. This observation suggests a mechanism of partial inactivation characterized by delayed infection (see Discussion). Plates were therefore incubated for a time sufficient for counting all survivors. Agreement of the experimental points with a first order exponential kinetics was taken as an indication that survivors had been counted correctly (this was generally the case after 24 h of incubation at 37 C).

The *sus* mutants tested for SBP were mainly tail mutants, as indicated by our SDS polyacrylamide gel electrophoresis data (2). Table 1 summarizes the essential characteristics of these *sus* mutants.

Figures 10 and 11 show the results of our SBP experiments. The significant features of the SBP curves can be summarized as follows. The control curve shows a total blocking of serum activity occurring in the range from about 10^8 to 2×10^{10} added plaque-forming units/ml, corresponding to dilutions from 700-fold to 25-fold of added lysate. The LY305 curve (neck appendages mutant) stays at the 100% residual K-value level at least until a 15-fold dilution of added lysate is reached (Fig. 10). Thus, this appendage-defective lysate is not able to block the serum in any detectable amount up to concentrations about 50 times higher than the initial activity of the control. The LY704 and the LY756 curves (tail knob mutants) are displaced toward higher lysate concentrations than the control, but both defective lysates seem to be able to block the serum completely (Fig. 10). The LY710 curve (multiple-head mutant) shows an aberrant behavior in the slope in that blocking of serum extends over a larger concentration range, from a 500-fold to a 1.5-fold dilution (Fig. 11).

FIG. 5. Phage $\phi 29$ wild-type particles were incubated with anti- $\phi 29$ serum depleted with a mutant J305 defective lysate. Antibodies are associated with the neck appendages. The arrow indicates antibodies associated with appendages on a free tail. Magnification $\times 170,000$.

The presence of a tail of serum-blocking activity at very high lysate concentrations has been discussed by Edgar and Lielausis (5) and

was explained as the contribution of input phages. Input phages were therefore expected to cause formation of tails at defective lysate

FIG. 6. Phage $\phi 29$ wild-type particles incubated with $\phi 29$ -antiserum depleted with a mutant J305 defective lysate. Antibodies sometimes form bridges between adjacent particles at the level of the neck appendages. Magnification $\times 170,000$.

concentrations about 50 times higher than those providing the initial activity of the control (as calculated by comparing the multiplicity of infection with the burst size of the control as shown in Table 1). In our experiments the tails of serum-blocking activity of the appendage defective lysates are located at concentrations

about 100 times higher than the blocking range of the control (if the different slopes are considered).

The tail knob mutants H704 and H756 have low burst sizes (Table 1), and the residual K-value curves of lysates LY704 and LY756 are accordingly displaced from the control toward

FIG. 7. Phage $\phi 29$ wild-type particles incubated with anti- $\phi 29$ serum depleted with a mutant J602 defective lysate. Antibodies are associated with the neck appendages. Magnification $\times 130,000$.

FIG. 8. Empty heads in a mutant J305 defective lysate. Magnification $\times 130,000$.

FIG. 9. A DNA-containing particle without appendages in a mutant J602 defective lysate. Magnification $\times 260,000$.

higher lysate concentrations (Fig. 10). The fact that lysates of both mutants are able to block the serum indicates that they produce the normal SBP, and that (at least with the immune sera used) the tail knob protein is not essential for blocking of serum activity.

Observation of $\phi 29$ phages adsorbed to *B. subtilis* 12A. The finding of most of the serum-

blocking activity in the neck appendages protein suggests a role for this structure in adsorption or injection of DNA, or both. At high multiplicities of infection, adsorbed $\phi 29$ phages can be observed in thin sections (Fig. 12). These particles are generally oriented toward the cell wall. If appendages are still present on the phage particles at this stage of infection, they

TABLE 1. *Sus* mutants used in the SBP experiments

Cistron	Mutant	Structural mutation ^a	Burst size ^b (phages/cell)
J	305	Neck appendages	321
J	602	Neck appendages	52
H	704	Distal tail knob	38
H	756	Distal tail knob	81
F	710	Head proteins	15
K	330	No structural mutation has been detected	537 ^c

^a From polyacrylamide gel electrophoresis (2).

^b From Reilly et al. (11), except for the mutant 602 value which was determined here and normalized to the other values by comparing *sus*⁺ bursts.

^c Used as a control lysate when grown on *B. subtilis* L15 (permissive strain).

FIG. 10. Blocking of anti- ϕ 29 serum by defective *sus* lysates. The highest amount of added lysate corresponds to 0.2 ml of a 20-fold concentrated lysate added to 0.8 ml of diluted serum. The phage equivalents scale refers to the number of plaque-forming units added to 1 ml of diluted serum in the case of the control. Symbols: ●, control (mutant K330 grown on the permissive strain L15); ▲, LY305 (appendages); △, LY756 (tail knob); ▽, LY704 (tail knob); ○, uninfected *B. subtilis* 12A lysate.

must occupy the small region between the phage head and the cell wall.

In this experiment the titration of free phage in the supernatant after removal of the infected cells by centrifugation indicated that approximately 400 phages/cell were able to adsorb. The proportion of phages that had injected their DNA was only about 10 to 20% as estimated from the observation of a large number of thin sections.

DISCUSSION

Antigenic properties of the phage surface.

The combination of serological methods and electron microscopy has revealed the presence of at least two and possibly three different antigens on the ϕ 29 structure. These are located on the head fibers, the head surface, and the neck appendages, respectively. The specificity of the antigenic sites on the neck appendages is clearly demonstrated by our results. The observations presented in this investigation do not definitively demonstrate that the head fibers and the head surface have a different antigenic specificity. Nevertheless, the distinct nature of the head fiber protein as compared to the major head protein has been demonstrated previously (12). Head fibers can be removed from the head by physical or chemical methods without essentially altering the antigenic properties of the head surface or the infectivity of the phage (our results and ref. 12). The head fiber protein differs in molecular weight and chemical composition (12) from the major head protein.

The strongest antibody binding on the intact phage occurs to the head fibers in the reaction on the electron microscope grid. This is also suggested by our observation that incompletely absorbed sera always show some residual complex with the fibers, and that full depletion requires addition of purified phages or phage equivalents in amounts at least 10 times higher than those required for complete blocking of phage neutralization activity. It seems, therefore, that either the reaction on the electron microscope grid selects strongly in favor of

FIG. 11. Blocking of anti- ϕ 29 serum by defective *sus* lysates. Symbols: ●, control (mutant K330 grown on the permissive strain L15); ▼, LY602 (appendages); □, LY710 (multiple-head mutant).

FIG. 12. *B. subtilis* 12A cell after adsorption of phage $\phi 29$ wild-type at a multiplicity of infection of 1,500. Magnification $\times 110,000$.

anti-head fiber antibodies, or fibers are more immunogenic than the other structural components. The latter explanation is consistent with the large number of filamentous fibers on the virus which provide an ideal exposure of antigenic sites.

The tail knob behaves as a poor antigen with all the sera tested. Although a very small number of antibody molecules cannot be detected by the electron microscope technique used (15), both electron microscopy and the serum blocking assay suggest that the antigenic power of the tail knob is low, probably because of its compact structure and small number of subunits.

Our observations were not detailed enough to determine how individual antibody molecules are bound to the head fibers and to the neck appendages. The idea of cross links between fibers and between appendages is consistent with previous data on the dimensions of IgG molecules (7, 13), the flexibility at the junction between F_{ab} segments (16), and the dimensions and structural organization of fibers and appendages (1).

Serum-blocking protein. Our results show that production of neck appendage protein is under the control of cistron J, and that appendages defective lysates are unable to block anti- $\phi 29$ serum. The persistence of serum activity at

the 100% residual K-value over a 50- to 100-fold concentration range after absorption with LY305 (appendages defective lysate) is significant enough to suggest that lack of serum-blocking protein is total. The appearance of tails of serum-blocking activity can be consistently explained by the contribution of input phages and input free appendages. Either low-speed centrifugation to remove bacterial debris was not efficient in eliminating input phages, or the appearance of these tails is due in large part to the presence of appendages added at the time of infection. Serum-blocking activity in the *sus* polypeptide fragments would be expected to follow a different type of curve (either of the altered antigen type [5] or with an intermediate plateau). This kind of serum-blocking activity may be a reason for the different behavior of LY602 (Fig. 11) as compared to LY305 (Fig. 10), as suggested by the observation of a large *sus* fragment of the appendage protein in SDS gel electrophoresis studies with mutant J602 (2). In the appendage-defective mutants J305 and J602, no other structural mutation has been detected by SDS gel electrophoresis (2). The absence of intermediate plateaus in the LY305 curve, therefore, proves that no other structural protein has a measurable serum-blocking power. The investigation of lysates defective in the tail knob (the next candidate for serum-blocking power) confirms this interpretation by showing that this component does not contribute to serum-blocking activity in a detectable amount. We, therefore, conclude that the appendages contain the main serum-blocking protein for immune sera obtained by injection of the entire ϕ 29 phage.

One of the *sus* mutants investigated (F710) shows a peculiar behavior in the SBP experiment. Its defective lysate has a serum-blocking activity characteristic of an altered antigen (5). Our electrophoretic data indicate that mutants of this cistron are defective in head proteins but not in the appendages protein (2). Our working hypothesis is that this serological behavior is typical of free appendages. Unassembled appendages might be expected to block serum less efficiently than assembled appendages, because the structure of the normal neck assembly would facilitate formation of stable antibody cross links.

Partial inactivation of ϕ 29 phage by anti- ϕ 29 serum. The observed reduction in plaque size of the survivors after long exposure to anti- ϕ 29 serum suggests partial inactivation of the phage under these conditions. By this we mean that adsorption to the host and DNA injection are delayed. A different serum compo-

nent than the anti-appendages antibodies may be responsible for this phenomenon (either a minor component, or, for example, binding of antibodies to the fibers on the lower part of the head). Alternatively, a critical number of antibodies have to be attached to the appendages for total inactivation, or antibodies can be attached to the appendages in an unstable way and released during incubation of the plates. Our present data do not permit us to select among these possibilities.

Possible role of the appendages in infection. The distance between heads of adsorbed ϕ 29 and the *B. subtilis* cell wall in thin sections (Fig. 12) is consistent with the possibility that the neck appendages may be in contact with the cell wall. It is also possible that the neck appendages may function in DNA injection. The small size of the tail knob (about 6 nm in diameter [1]) might facilitate its insertion into a porous wall surface. It should be noted that the absence of detectable serum-blocking power in the tail knob protein does not exclude an essential role of this structure during adsorption, but may only reflect the low immunogenic capacity of the tail knob.

A peculiar plaque morphology has been observed for most of the mutants grouped by complementation in the J cistron (neck appendages). Both *ts* and *sus* mutants generally produce clear plaques under permissive conditions. A variant of the ϕ 29 wild-type phage has also been isolated which has the same plaque morphology. Preliminary evidence indicates that in this case the clear phenotype locus is in the appendages region of the genome.

Plaque morphology reflects the phage-bacterium physiological relationship, as demonstrated in the case of the r-mutations of the T-even phages where a clear plaque morphology is due to the absence of lysis inhibition (4). We presently do not have a clear cut explanation that links plaque morphology to the function of the neck appendages. Mechanisms other than lysis inhibition may have a role in determining plaque morphology. A modification of the neck appendage protein may change the ability of the phage to infect host cells during plaque development.

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