Host-Bacteriophage Interaction in *Agrobacterium tumefaciens*

I. Characterization of Bacteriophage R4 and Physiological Changes in the Infected Host Cell

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Infection of *Agrobacterium tumefaciens* B6, a tumor-producing plant pathogen, by bacteriophage R4, does not immediately shut off host deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein synthesis. Viral DNA synthesis begins soon after infection, but the host DNA is not shut off until after 35 min; net RNA and protein synthesis are not inhibited until 30 min after infection. The pattern of synthesis of phage particles was confirmed by electron microscopy of thin sections during the infection cycle. The phage particle consists of a polyhedral head, 65 nm in diameter, and a long flexible tail 210 nm long and 10 nm wide with helically arranged subunits. By gel electrophoresis, four major protein components with the following molecular weights were found in the capsid: 72,000, 45,000, 28,000, and 14,500. The phage DNA has a molecular weight of 30 million and a guanine-cytosine content of 59%.

Agrobacterium tumefaciens, a bacterial plant pathogen, causes the transformation of plant cells from well differentiated cells under plant control to cells which have lost differentiation and control, i.e., tumor cells. The question is, how can a bacterial cell transform or initiate this transformation in a plant cell?

The direct role of phage in tumor formation is questionable, but reports of phage in tumorous plant tissue continue to appear. Tourneur and Morel recently summarized this data (17). All the temperate phage reported have a similar morphology to the lysogenic phage LV-1 isolated and characterized in our laboratory (22). The phage in this particular study was isolated from a lysogenic strain of *A. tumefaciens* R4, and was chosen for extensive study because Heberlein and Milani were able to transfect *A. tumefaciens* with this strain (Bacteriol. Proc., p. 35, 1971). This phage is shown in this study to be a member of a closely related group containing the four phages, omega, PS8, PB₂A, and LV-1, which are indistinguishable on the basis of deoxyribonucleic acid (DNA) homology and protein coat analysis (*manuscript in press*).

Evidence for the role of bacterial DNA as a critical factor in the induction of tumor formation is accumulating. The most recent report by Yajko and Hegeman reviews this aspect and presents additional evidence of the transfer of bacterial-

labeled DNA to plant tissue (21). This release of DNA could be indirectly associated with the production of phage by the cells. An understanding of the effect of phage infection upon the bacterial host cell may be essential to unraveling the problem of how bacteria release DNA and initiate tumor production. The interaction between phage and bacteria after infection has not been studied.

The events which follow a virulent infection from without of *A. tumefaciens* strain B-6 by phage R4 are reported here. Morphological changes were observed by examining thin sections of infected cells with the electron microscope. In this paper we report the effect of infection upon the bacterial cell synthesis and find no drastic shut-off of cell functions following infection. The results are similar to the temperate phage infection of *Escherichia coli* by lambda (3, 18).

MATERIALS AND METHODS

Bacterial strain. *A. tumefaciens* B-6, the sensitive host strain, was obtained from R. M. Klein, New York Botanical Garden.

Phage strain. Lysogenic phage R4 (referred to as PLR-4 by Heberlein) was isolated from *A. tumefaciens* R4 obtained from R. S. Dickey, Cornell University, Ithaca, N.Y.

Media. Peptone broth containing 0.4% peptone (Difco) was used for liquid cultures. Peptone agar was

made with the addition of 1.5% agar for plating and 0.65% agar for soft agar overlay.

Growth condition. Liquid cultures were grown in 50 ml of peptone broth in 125-ml Erlenmeyer flasks at 30 C in a New Brunswick rotary shaker.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Purified phage R4 was prepared by differential centrifugation followed by banding twice in CsCl, refractive index 1.380, and then dialysis against .01 M phosphate buffer, pH 7.2. Phage proteins were dissociated in the presence of 1% SDS and 1% beta-mercaptoethanol by heating to 100 C for 1 min. Procedures employed in the preparation of acrylamide gel electrophoresis, staining by Coomassie brilliant blue, and decolorization were described by Weber and Osborne (20).

Mitomycin C inhibition of host DNA. An overnight culture was suspended in peptone broth to give 1×10^8 cells/ml and incubated for 1 hr at 30 C. Fifteen minutes before infection, 10 μ g of mitomycin C per ml (Schwarz/Mann) was added. After 10 min the cells were centrifuged and resuspended in peptone broth and infected with a multiplicity of infection (MOI) of 10 plaque-forming units (PFU)/cell. ^3H -thymidine, 2 $\mu\text{Ci}/\text{ml}$, specific activity 15 Ci/mm (Schwarz/Mann) was added; samples were removed at 5-min intervals.

Radioactive assay of DNA, RNA, and protein. Thirty minutes prior to infection, 2 μCi of ^3H -thymidine per ml was added to log-phase cells in peptone broth. At 15-min intervals, duplicate 1.0-ml and 10-ml samples were removed and precipitated with cold 5% trichloroacetic acid in the presence of carrier DNA. At zero time the cells were infected at an MOI of 10 PFU/cell, and 1.0-ml samples were removed at 5-min intervals. Samples were collected onto a membrane filter (Millipore Corp.), washed with 5% trichloroacetic acid followed by an ethanol wash, combusted in a Packard tritium oxidizer, and counted in a Packard scintillation counter.

Radioactive uridine, 3 $\mu\text{Ci}/\text{ml}$, specific activity 27 Ci/mm (New England Nuclear), was added 10 min prior to infection to assay ribonucleic acid (RNA). Cells were infected at zero time with an MOI of 10, and 1-ml samples were removed at 10-min intervals and processed as described in the previous paragraph.

Protein hydrolysate, 4 $\mu\text{Ci}/\text{ml}$ (Amersham-Searle), was added 10 min prior to assay protein synthesis. Cells were infected at zero time with an MOI of 10. One-milliliter samples were removed at 10-min intervals and processed as for DNA analysis.

Preparation of DNA and sedimentation in CsCl. An overnight culture of cells was resuspended in fresh peptone broth at a concentration of 1×10^8 cells/ml in the presence of 200 μg of bromodeoxyuridine (BUdr) per ml (Schwarz/Mann) and incubated for 60 min prior to infection (7). The cells were then washed, resuspended in fresh peptone broth, and infected at a multiplicity of 10 PFU/cell. At the appropriate times, 5-ml samples of culture were removed and labeled for 5 min with 2 μCi of ^3H -adenine per ml, specific activity 16 Ci/mole (Schwarz/Mann) and 2 μCi of ^3H -thymidine per ml. The reaction was stopped by adding ice-cold 0.1 M ethylenediaminetetraacetic acid

(EDTA), the cells were lysed upon addition of 1.0 ml of 20% SDS. The DNA was treated with ribonuclease (40 $\mu\text{g}/\text{ml}$) and Pronase (40 $\mu\text{g}/\text{ml}$), layered on CsCl, average density 1.72 g/cm³, and centrifuged 40 hr at 40,000 rev/min in the SW65 rotor. Twelve-drop fractions were collected and treated as described earlier.

Colorimetric determination of DNA. One milliliter of cold 50% trichloroacetic acid was added to each 10-ml sample and placed at 4 C overnight. The precipitates were washed twice with cold 5% trichloroacetic acid and resuspended in 2 ml of 5% trichloroacetic acid, heated to 90 C for 30 min, and centrifuged. The supernatant fluid was assayed for DNA using diphenylamine (2).

Sucrose gradient centrifugation. The method of Thomas and MacHattie was followed for determination of the molecular weight of R4 DNA (15). A 5 to 20% (w/v) sucrose solution in a .01 M tris(hydroxymethyl)aminomethane (Tris) buffer solution containing 1 M NaCl, pH 7.8, was overlaid with 50 μl of an R4 DNA sample and 25 μl of purified ϕX174 DNA (obtained from W. D. Taylor), and centrifuged in a SW65 rotor for 90 min at 35,000 rev/min. The DNA bands were observed with an ISCO model D density gradient fractionator measuring adsorption at 254 nm.

Electron microscopy. Bacterial cells were fixed using Kellenberger's technique (5), stained for 2 hr in 0.5% uranyl acetate, and embedded in Spurr's epoxy resin embedding medium (14). Cells were sectioned on an LKB 8800 A ultramicrotome. The sections were stained with 0.5% uranyl acetate for 10 min and then 0.5% lead citrate for 4 min and examined with a Phillips model 300 electron microscope. The virus was negatively stained with a 2% phosphotungstic acid solution, adjusted to pH 7.2 with NaOH, containing 500 μg of bovine serum albumin per ml.

RESULTS

Properties of R4 phage. Centrifugation of phage particles in a CsCl density gradient produced two phage bands. Electron microscopy revealed the heavier band, density 1.51 g/cm³ as determined by refractive index, to consist of complete phage particles, whereas the band of lesser density, 1.49 g/cm³, was found to consist primarily of ghosted phage particles. Infectivity was confined to the band of greater density. The phage consists of a polyhedral head, 65 nm in diameter, and a long flexible tail 210 nm long and 10 nm wide (Fig. 1).

Properties of phage R4 DNA. The properties of phage R4 DNA were found to be similar to those of *Agrobacterium* phage LV-1 (6). Phage DNA was extracted and sedimented in sucrose at neutral pH, which revealed an $S_{20, w}$ of $34S \pm 3\%$ (data not shown). This corresponds to a molecular weight of 30 million for the native molecules according to Studier (13).

Density gradient ultracentrifugation was per-

FIG. 1. *Electron micrograph of bacteriophage R4.*

formed on the purified phage DNA. The DNA was found to possess a density of 1.718 g/cm^3 . The guanine-cytosine content calculated using the relationship of Mandel gave a value of 59% (10). This is in agreement with the value of 1.7186 g/cm^3 and a guanine-cytosine (GC) content of 61% for the host bacterium, B-6, which this phage is capable of lysogenizing (4).

The mid-point of the melting transition (T_m) for the phage DNA is 79 C. This corresponds, in standard saline citrate (SSC)/10, to a GC content of 59% for the phage DNA (10).

Structural proteins of R4. The analysis of the structural proteins of R4 was carried out in 10% acrylamide gels, in which the distance migrated is inversely related to the log of the molecular

weight of the protein (20). The molecular weights were determined by comparison with migration of standard proteins run concurrently on separate gels.

Four major proteins, designated A, B, C, and D can be identified on acrylamide gels (Fig. 2). These correspond to molecular weights of about 72,000 for A band, 45,000 for the B band, 28,000 for the C band, and 14,500 for the D band.

Morphology of *A. tumefaciens* during infection with R4 virus. The one-step growth curve of phage R4 was found to be similar to that of the *A. tumefaciens* phage LV-1 (6). The latent period was 100 min, with a subsequent burst size of 75 PFU/infectious center (Fig. 3).

The infected cells were prematurely lysed with chloroform during R4 infection. Mature intracellular phage appear about 50 min after infection, a result confirmed by electron microscopy of cell sections.

To view the morphological changes which occur after infection with R4, samples were removed at approximately 15 min intervals for electron microscopy.

For the first 30 min after infection, no significant internal changes were noted in cells. The cytoplasm seems to become less dense in some cells, but this may have been due to the plane of the section. The first significant visible change in the cell takes place 40 to 50 min after infection; developing phage heads become visible as hexagonal bodies in the cytoplasm of some of the infected cells in the periphery near the cell wall.

After 80 min the cells observed possess a number of complete or developing phage heads, predominately in the peripheral areas of the cell.

At 100 min after infection, complete heads, which have acquired their DNA, can be dis-

FIG. 3. One-step growth and intracellular growth curves of R4 at MOI = 0.1.

tinguished in the cytoplasm by their electron opacity as well as empty heads. All heads can be seen associated with the nuclear material of the cell. Cells at this time are beginning to lyse; disrupted cell membrane and cell wall are present. The DNA fibers have now become condensed and numerous; heads can be seen in close association with ribosomal material, which is probably involved in head and tail synthesis.

By 150 min after infection, the cells which have not lysed are packed with phage heads. The nuclear area has become very condensed and numerous complete or developing heads are visible. By 180 min after infection, most cells have undergone lysis.

Effect of R4 infection on kinetics of DNA synthesis. Net synthesis of DNA was not inhibited following infection of *A. tumefaciens* except for an apparent plateau occurring 40 min after infection (Fig. 4). However, when the amount of DNA estimated to be present in the phage progeny is compared with the net amount of DNA synthesized in the culture after infection, it is evident that the synthesis of host DNA is considerably decreased during the vegetative synthesis of the phage. At least 50% of the increase in DNA during the cycle is accounted for by the progeny phage; thus most DNA synthesized after infection is phage specific, and host DNA replication is reduced.

An apparent feature of the DNA replication is

FIG. 2. Electrophoretic separation of R4 proteins on SDS-acrylamide gels.

FIG. 4. DNA synthesis in R4-infected *A. tumefaciens*. Zero time represents the time of infection.

the presence of a plateau occurring about 40 min after infection, which would indicate that no net increase in DNA is occurring. This may be due to either an equilibrium between host DNA breakdown and phage DNA synthesis, or a period in which host DNA synthesis is shut off prior to a period of increased rate of phage DNA synthesis. To test for the possibility of degradation of cell DNA, a culture of *A. tumefaciens* was grown in the presence of ³H-thymidine for 16 hr prior to infection, followed by a 2-hr chase; no decrease in the quantity of acid-precipitable counts occurred, and less than 1% of the counts appeared in phage DNA (data not shown). It was concluded host cell DNA is probably stable during infection by R4.

To observe the time for onset of phage DNA synthesis, net synthesis was observed in phage-infected, mitomycin C-treated cells (Fig. 5). Mitomycin C has been found to covalently link to the double-stranded DNA structure, thereby blocking replication. An interesting feature is the capacity of the drug to block synthesis of host cell DNA, but permit viral DNA synthesis (19). As seen in Fig. 5, host DNA synthesis is completely shut off by the drug, whereas phage DNA synthesis commences minutes after infection.

Incorporation of ³H-nucleotides into R4 DNA. The distribution of ³H-thymidine and ³H-adenine at various times after infection was determined by preparative ultracentrifugation in CsCl gradients.

Since host cell DNA and phage DNA possess similar buoyant densities, host cells were grown in the presence of BUdr prior to infection (7). The labeled nucleotides were introduced for 5-min pulses at various periods after infection.

Label introduced between 5 and 10 min after infection is incorporated predominately into host DNA (Fig. 6a), which bands at a density of 1.728 g/cm³ based on refractive index; a second band at 1.718 g/cm³ equivalent to the density of phage DNA is also present. Since short regions of *Agrobacterium* DNA may not have incorporated BUdr and thus may band at a lighter density, host cells were grown in the presence of BUdr, but not infected. Although a small percentage of trichloroacetic acid-precipitable material was found in a band of lighter density, the DNA banded predominately at the higher density. Although some host cell DNA may band at a lighter density, the smaller peak represents predominately phage-specific DNA.

At 25 min after infection (Fig. 6b), replication of host cell and R4 DNA is taking place; a shoulder appears on the band of greater density due to a shifting of the host DNA to a hybrid density.

By 40 min after infection, host cell DNA synthesis seems to be shut off, with label in the single peak corresponding to phage DNA, whereas the peak at higher density has disappeared (Fig. 6c). Sixty minutes after infection, all label appears in the phage DNA (Fig. 6d).

FIG. 5. DNA synthesis of R4-infected and uninfected *A. tumefaciens* treated with mitomycin C.

FIG. 6. Equilibrium centrifugation of ³H-labeled DNA from R4-infected *A. tumefaciens*. At various times after infection, 5-ml amounts were removed and added to radioactivity as described. Incorporation was terminated by addition of cold buffer and immersion in dry ice. Thirty fractions were collected from each tube, although only the middle fractions are shown here. a, Label introduced 5 to 10 min after infection; b, 25 to 30 min after infection; c, 40 to 45 min after infection; d, 60 to 65 min after infection. Seven micrograms of DNA was applied to the gradient in samples a and b, and 6 μ g of DNA was applied for samples c and d, since solutions of DNA were not of equal concentration (12).

Effect of infection on RNA and protein synthesis. Net synthesis of RNA is normal for the first 10 min after infection, at which time a gradual reduction in rate occurs until 30 to 40 min after infection, when no change in net synthesis of RNA is observed until lysis of the cells (Fig. 7). This may reflect shutdown of host ribosomal RNA synthesis.

Results shown in Fig. 8 indicate protein synthesis is also not affected by infection until 20 to 30 min after infection.

DISCUSSION

The lysogenic *A. tumefaciens* phage R4 is morphologically similar to the other lysogenic phage LV-1 and resembles the electron micro-

graph of the phage obtained by Tourneur (16). Kurkdjian also observed a morphologically similar phage in pea plants inoculated with virulent or avirulent cells of *A. tumefaciens*, strain 806 (8). The common morphology shared by these phages consists of a polyhedral head with a six-sided outline and a long noncontractile tail. In the electron micrographs where the tails are detached

FIG. 7. RNA synthesis during R4 infection of *A. tumefaciens*. Zero time represents the time of infection (arrow).

FIG. 8. Protein synthesis during R4 infection of *A. tumefaciens*. Zero time represents the time of infection (arrow). Protein was also quantitated by the method of Lowry (9).

from the phage head, a knob is present at the end of the tail which projects into the head. This knobbed structure could function in the attachment of the head and tail structures.

The DNA of R4 phage was found to have a molecular weight of 30 million, which is within experimental error of the molecular weight found for LV-1 by Korant (6). The buoyant density of the DNA was found to be 1.718 g/cm³. This corresponds to a GC content of 59% which is similar to the DNA of the host species *A. tumefaciens* (61%), in which this phage is capable of insertion in a lysogenic state. Such similarity of base composition is common among temperate phages and host cells, although it must be kept in mind that base sequence homology need not necessarily be the case.

The major structural proteins of R4 were characterized by SDS gel electrophoresis. Most of this protein structure is comprised of four major polypeptides with estimated molecular weights of 72,000 (A), 45,000 (B), 28,000 (C), and 14,500 (D).

An estimate of the relative amounts of polypeptide was made by cutting out the area under the peaks of the analytic scan and weighing them. Polypeptide B represented 40% of the total major protein, polypeptides A and C each represented about 20%, and polypeptide D, about 10%. These undoubtedly represent the major head and tail proteins of the phage.

R4 closely resembles phage LV-1 in core composition and physical characteristics of the DNA as well as general morphology. Phage LV-1 was found to be part of an indistinguishable group of phage containing phage omega, PS8, and PB₂A (*manuscript in press*). Omega is the original lysogenic strain isolated by Beardsley in 1955. Phage PS8 was isolated by Parsons and Beardsley from a homogenate of cultures of S8 crown gall tumor tissue. Phage PB₂A was isolated from a lysogenic strain of *A. tumefaciens* B₂A, a subclone from strain B2 originally isolated from apple seedling crown gall by G. C. Kent. Beardsley extensively discusses these phage in relation to plant virulence studies in a recent review (1). One can predict that all these phage will interact in the same manner with the sensitive host strain *A. tumefaciens* B6.

Phage R4 infection of *A. tumefaciens* is typical of temperate phage, such as lambda, in that it has no immediate drastic effect on host cell metabolism (3, 11, 18).

Viral DNA was found to begin synthesis soon after infectio (Fig. 5 and 6), and host DNA shut-off by 35 min after infection; thus, the major fraction of DNA made during R4 development corresponds to bacteriophage DNA.

Host protein synthesis was found not to decrease immediately following infection similar to results found with lambda (3).

Since the shut-off of host DNA synthesis by R4 is not as quick as bacterial protein synthesis cessation and possibly slower than shut-off of host RNA synthesis, the inhibition of host DNA may only be a secondary effect of the host protein shut-off, although the possibility of a phage-specific enzyme being involved cannot be discounted.

Unlike lambda infection of *E. coli* where net synthesis of RNA is affected a few minutes after infection (3), RNA synthesis during R4 infection of *A. tumefaciens* is apparently not inhibited until 30 to 40 min after infection.

In conclusion, the lytic infection of *Agrobacterium tumefaciens* by R4 has similarities with lambda infection of *E. coli*. Upon infection, host protein synthesis is reduced, and possibly ceases. Host DNA replication is reduced, and is ultimately shut off, whereas phage DNA synthesis commences soon after infection and appears to continue throughout the latent period.

In summary, virulent phage infection does not have an immediate drastic effect upon the host cell.

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