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Bacteriophage Release in a Lysogenic Strain of *Agrobacterium tumefaciens*[1]

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Bacteriophage release in a lysogenic strain of *Agrobacterium tumefaciens* V-1 is temperature-sensitive. At 25 C and 30 C, phage was released in a ratio of 1 plaque-forming unit per 100 bacteria; at 35 C, although bacterial growth was not inhibited, phage release was suppressed. Phage synthesis was induced by heat shock, 42 C for 30 min, ultraviolet irradiation, and mitomycin C. Induction by ultraviolet light was unusual—an immediate rise in phage titer followed irradiation. A large increase occurred after a 90-min latent period. The lysogenic strain was cured of the phage by incubation at 37 C, and the cured strain produced plant tumors.

Agrobacterium tumefaciens is the causal agent of crown-gall tumors in plants. The means by which this organism induces tumor formation is not known (3). We wish to determine if the presence of prophage or vegetative phage development within the bacterial cell plays a role in the transformation of the plant cells to tumor cells by this organism. Our initial approach is to study the nature of lysogeny in this species of bacteria.

We have shown that lysogeny is common among *A. tumefaciens* strains. Fourteen out of 130 *A. tumefaciens* strains examined were lysogenic (13). One of these tumorgenic lysogenic strains, *A. tumefaciens* V-1, spontaneously released phage particles into the medium in a stable, predictable fashion and, therefore, was chosen for the present study.

Lippincott has reported that the ability of *A. tumefaciens* to produce tumors can be altered by thermal treatment (10). Incubation of the cultures at 39, 42, and 45 C for various time periods up to 30 min impaired the ability of the culture to initiate tumor production but did not reduce the viability of the bacteria. Ultraviolet (UV) irradiation of the bacterial cultures also influences the infectivity of *A. tumefaciens*. Heberlein and Lippincott (4) found that a dosage of ultraviolet irradiation which killed 50 to 90% of the cells increased the number of tumors produced per surviving cell, as measured by tumor formation in primary pinto bean leaves.

Both temperature (8) and UV irradiation (11) are known to influence the prophage state of bacterial viruses. In the present study, the effect of temperature and UV irradiation upon the balance of the prophage state and vegetative phage synthesis in the lysogenic strain of *A. tumefaciens* V-1 was studied. This strain was cured of the carried phage LV-1 and compared to the lysogenic strain.

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MATERIALS AND METHODS

Bacterial strains. *A. tumefaciens* V-1, lysogenic for phage LV-1 originally isolated from *Zinnia elegans*, was obtained from R. H. Hamilton, The Pennsylvania State University. The sensitive host strain, *A. tumefaciens* B-6, was obtained from R. M. Klein, New York Botanical Garden. The sensitive host strain, *A. tumefaciens* B-6, was lysogenized with phage LV-1 by isolating colonies which formed when the bacteria were plated with an excess of phage. The colonies were restreaked to purify. The strain was designated *A. tumefaciens* B-6 (LV-1). This strain released phage particles spontaneously and was immune to lysis by phage LV-1.

Proof of lysogeny. Single colonies of *A. tumefaciens* were picked and suspended in peptone-water containing specific phage antiserum prepared against phage LV-1 to prevent carryover of free phage particles when the cultures were restreaked. The cells still spontaneously released free phage particles after four cycles of isolation. A drop of a phage lysate of LV-1 did not produce lysis upon a lawn of *A. tumefaciens* V-1; the cells were immune to infection.

Media. Peptone-broth containing 0.4% peptone (Difco) and 0.001 M $MgSO_4$ was used for liquid cultures. Peptone-agar was made with addition of 1.5% agar for plating and 0.65% agar for soft agar overlay.

Growth conditions. Liquid cultures were grown in 50 ml of peptone-broth in 125-ml Erlenmeyer flasks at 30 C or indicated temperature in a New Brunswick gyratory shaker.

UV Irradiation. A 10-ml amount of a 6-hr log-phase

peptone-broth culture was irradiated in an open petri dish placed on a vibrator at a distance of 40 cm from the light source. The samples were irradiated for 30 sec by using a General Electric 15-w germicidal lamp; the total ultraviolet dose was 6.0×10^4 erg/cm^2, which killed 99% of the bacteria.

Mitomycin C induction. A 10-ml amount of a 6-hr log-phase peptone-broth culture was sedimented by centrifugation, the supernatant fluid was decanted, and the cells were suspended in peptone-broth containing 5 μg of mitomycin C (Sigma Chemical Co., St. Louis, Mo.), per ml. After 5 min, a 1:100 dilution of the culture was made in peptone-water. The free phage in the supernatant fluid was assayed at zero-time just before addition of mitomycin C and after 5 hr. The control contained no mitomycin C (7).

Phage determination. The number of free phage particles released into the medium was measured by centrifuging the bacterial culture for 10 min in a Sorvall table-top centrifuge at 4,000 rev/min and assaying a 0.1-ml sample of supernatant liquid by the overlay method (1). Filtration of the culture through a type HA membrane filter (pore size 0.45 μm; Millipore Filter Corp., Bedford, Mass.) and assay of the filtrate gave the same number of plaque-forming units (PFU).

Samples taken immediately after UV irradiation were frozen in a dry-ice acetone bath, thawed, and titrated the following day.

Curing. *A. tumefaciens* V-1 on a peptone-agar slant was incubated at 37 C for 48 hr, transferred, and reincubated for two additional 48-hr periods. A peptone-broth culture was inoculated from the final slant, and 100 colonies were isolated, then replica-plated on peptone-agar and a lawn of *A. tumefaciens* B-6. Colonies with no zone of lysis were checked for phage sensitivity.

Spheroplast formation. The method of Iglewski and Franklin (5) was followed. Spheroplasts were disrupted by 1:100 dilution in sterile distilled water. The control was diluted in 40% sucrose in peptone-water. The preparations were centrifuged, and the supernatant fluids were assayed for free phage.

Tumor formation. Tumor-inducing ability was measured by the quantitative pinto bean assay developed by Lippincott and Heberlein (9).

RESULTS

The effect of incubation temperature (25, 30, and 35 C) upon spontaneous phage release was determined (Fig. 1). Free phage particles in the supernatant fluid at 25 C occurred at a ratio of 1 free phage particle per 100 viable bacteria. At 30 C, the phage release was similar. At 35 C, however, phage release was suppressed, even though the bacterial growth was not inhibited. The ratio of free phage particles was now less than 1 per 1,000 viable bacteria (Fig. 2). The decrease in free phage particles was not the result of heat inactivation of the virus, as no reduction in PFU was observed when the particles were held at 35 C for 8 hr.

This observed depression of phage release was not due to a critical temperature-sensitive step in vegetative phage synthesis, since phage particles were synthesized at 35 C when the lysogenic cul-

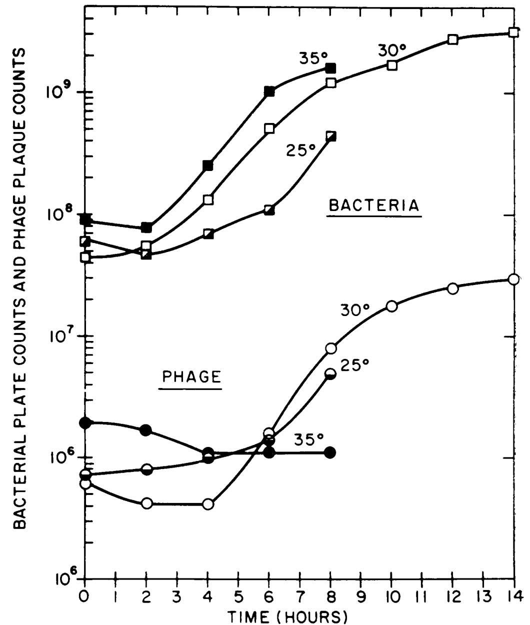

FIG. 1. *Growth curve of A. tumefaciens V-1 and number of spontaneously released phage particles (PFU) at 25, 30, and 35 C in peptone-water.*

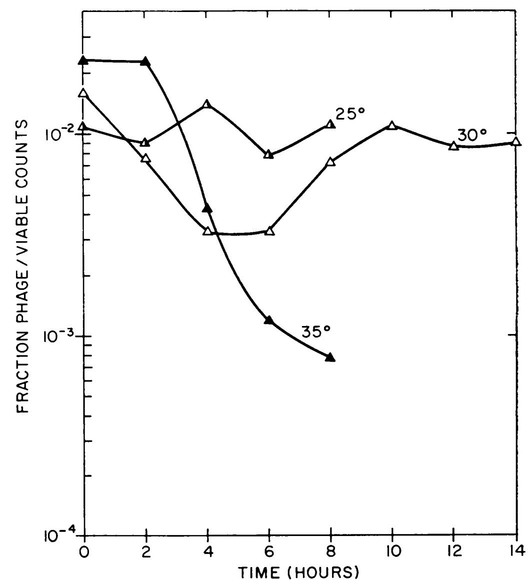

FIG. 2. *Ratio of free phage (PFU) released by A. tumefaciens V-1 to the number of viable bacterial cells in peptone-water at 25, 30, and 35 C incubation temperatures.*

ture was irradiated with UV light (Fig. 3). Irradiation reduced the bacterial viable count by 99%. Plaque counts made at 10-min intervals after UV irradiation demonstrated that, within 10 min, the free-phage titer increased, reaching a 10-fold increase in 30 min; it decreased slightly, and then, 90 min from time of irradiation, a rise in phage titer of over 100-fold was observed. This second rise corresponds to the normal 90-min latent period for the synthesis of LV-1 phage when the sensitive host, *A. tumefaciens* B-6, is infected from without. UV light induction of phage synthesis at 30 C showed a similar pattern of phage release (Fig. 4).

The depression of phage release by temperature of incubation (35 C) was reversed when the cultures were shifted to a 30 C water bath. After a delay of 90 min, the normal latent period, an increase in the spontaneous release of phage particles occurred.

Thermal shock (incubation at 42 C for 30 min)

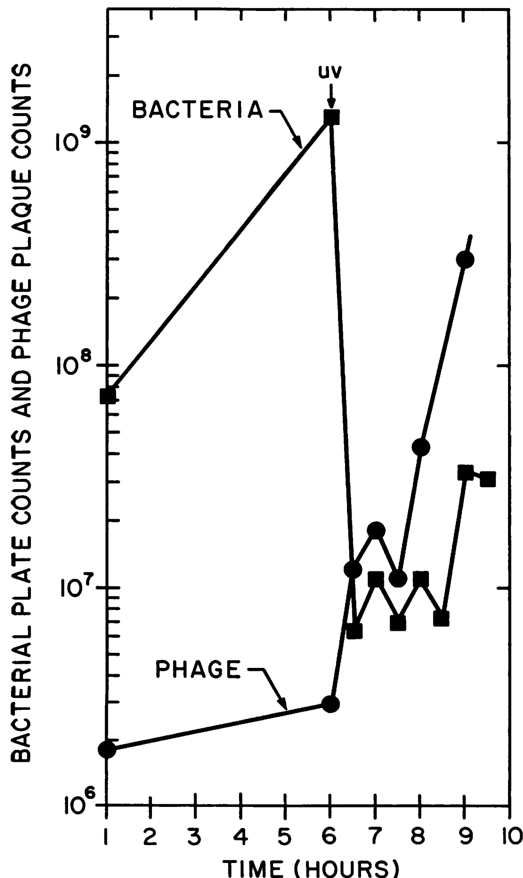

FIG. 3. *Ultraviolet light induction of phage synthesis in A. tumefaciens V-1 at 35 C in peptone-water.*

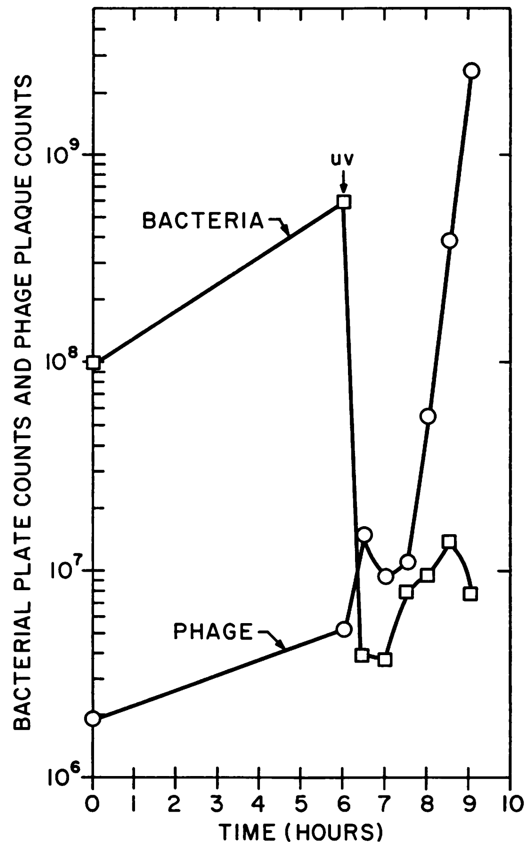

FIG. 4. *Ultraviolet light induction of phage synthesis in A. tumefaciens V-1 at 30 C in peptone-water.*

induced phage release in peptone-broth cultures grown at 30 or 35 C. The pattern of phage release was similar to UV induction: an immediate increase in free phage particles, followed by a larger increase in free phage particles 1.5 to 2 hr later (Fig. 5 and 6). Higher temperatures, 43, 44, and 45 C, did not result in induction of phage synthesis (Table 1). At 45 C, the growth of the bacterial culture was inhibited.

Mitomycin C, an inhibitor of deoxyribonucleic acid synthesis also induced phage synthesis in this system. The number of PFU/ml in the supernatant fluid of a lysogenic culture exposed 5 min to mitomycin C rose from 2.5×10^6 at time of addition of the drug to 1.6×10^{10} 5 hr later. The control culture, after 5 hr, contained 8.9×10^7 PFU/ml.

The effect of UV irradiation upon the turbidity of *A. tumefaciens* V-1 was an initial slight decrease in optical density (OD) at 10 min, followed by an increase until 45 min, when lysis ensued and the OD decreased.

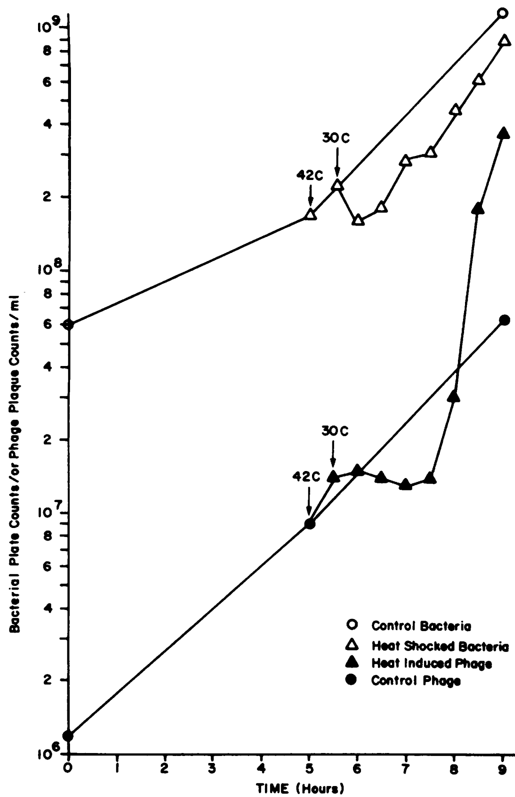

FIG. 5. *Effect of thermal shock (42 C for 30 min) of A. tumefaciens V-1 on phage release using 30 C as a base temperature.*

The initial 15-fold rise in PFU in the supernatant fluid was found immediately after UV irradiation, at 30 and 35 C (Table 2).

A similar pattern of phage release occurred upon UV irradiation of the artificially lysogenized strain of *A. tumefaciens* B-6 (LV-1). An initial rise of PFU/ml from 5.5×10^5 to 2.8×10^7 occurred immediately after UV irradiation, followed by an additional increase after the normal latent period.

Addition of chloramphenicol (final concentration, 100 μg/ml) and ethylenediaminetetraacetic acid (EDTA; final concentration 5×10^{-5} M) to *A. tumefaciens* V-1 10 min before irradiation did not abolish the immediate release of phage after UV irradiation (Table 2). Chloramphenicol (100 μg/ml) inhibited growth of *A. tumefaciens* V-1 and phage synthesis by *A. tumefaciens* B-6 when infected from without by phage LV-1.

Spheroplasts disrupted by osmotic shock immediately released free phage particles into the medium (Table 3). As a control, lysogenic *Escherichia coli* K-12 (λ) was converted to spheroplasts and disrupted by osmotic shock. No preformed phage particles were released by this treatment.

After many trials, the lysogenic strain, *A. tumefaciens* V-1, was "cured" of the prophage by incubation at 37 C. Many colonies were picked and tested. Cells from two of these colonies did not spontaneously release phage into the medium

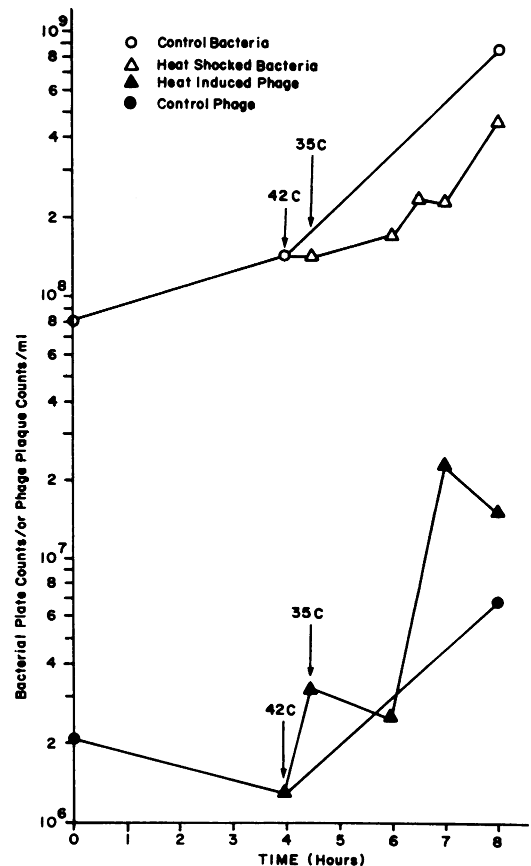

FIG. 6. *Effect of thermal shock (42 C for 30 min) of A. tumefaciens V-1 on phage release using 35 C as a base temperature.*

TABLE 1. *Ratio of final phage concentration to final bacterial concentration at 3.5 hr after exposure to thermal shock for 0.5 hr*

Temp[a] (0.5-hr treatment)	Ratio after 3.5 hr[b]
C	
30	3.7
42	38.5
43	3.9
44	4.1
45	3.9

[a] Incubation temperature was 30 C.
[b] PFU per viable bacterial counts $\times 10^{-2}$.

TABLE 2. *Inital rise in phage titer immediately following ultraviolet irradiation of A. tumefaciens V-1*

Conditions	PFU/ml of supernatant fluid		Increase
	Before UV irradiation	Immediately after UV irradiation	
30 C	2.0 (× 10^5)	28.0 (× 10^5)	14 ×
Control, no UV	2.0	2.0	
35 C	6.0	88.0	15 ×
Control, no UV	6.0	6.0	
Chloramphenicol,[a] 30 C	1.5 (× 10^6)	14.0 (× 10^6)	9.4 ×
UV control, no chloramphenicol, 30 C	1.7	18.0	11.0 ×
Chloramphenicol control, no UV, 30 C	1.7	1.6	

[a] Chloramphenicol (100 μg/ml) + EDTA (5.0 × 10^{-5} M, final concentration) was added 10 min before ultraviolet irradiation.

TABLE 3. *Immediate release of phage particles by A. tumefaciens V-1 spheroplasts*

Spheroplasts	Supernatant fluid (PFU/ml × 10^5)
Disrupted	390
Intact	9.2

and were lysed by infection from without by phage LV-1. One of the cured strains was designated *A. tumefaciens* CV-1. The ability to produce plant tumors of this cured strain was similar to the original lysogenic parent strain when tested by the pinto bean leaf assay (9).

DISCUSSION

Temperature influences the synthesis of phage LV-1, carried in *A. tumefaciens* V-1, in a number of ways. Incubation at 35 C in comparison to 30 C suppresses the spontaneous release of phage particles. The ratio of free phage to viable bacterial cells drops from 1 per 100 to 1 per 1,000 at the higher temperature (Fig. 1). However, the bacteria are able to synthesize phage at 35 C if induced by UV irradiation or thermal shock. If the bacteria are shifted to 30 C after incubation at 35 C, an increase in free phage particles follows a 90-min lag period. This is the normal latent period for phage synthesis when *A. tumefaciens* B-6 is infected with phage LV-1 from without. This indicates that the block is in the initial steps of synthesis rather than after partial synthesis has occurred. The higher temperature, 35 C, favors the prophage state. A still higher temperature, 42 C, induces phage synthesis (Fig. 5 and 6). Heat induction is limited to a very specific temperature; slightly higher or lower temperatures do not induce phage synthesis (Table 1). The effect of heat induction may be upon attachment of the prophage or upon the repressor (or both).

The first increase in PFU following induction occurred very rapidly; samples taken immediately after irradiation contained at least a 10-fold rise in PFU. The time interval is too short for de novo synthesis and must represent completely synthesized phage particles within the cell population which are released by UV irradiation. This increase is a characteristic of the phage LV-1. *A. tumefaciens* B-6, artificially lysogenized by this virus, also produced an increase in PFU immediately after UV irradiation, which was later followed by a second increase after the normal latent period.

Bacterial cells converted to spheroplasts with lysozyme, when disrupted by osmotic shock, immediately released phage particles (Table 3). Under similar conditions, this did not occur in *E. coli* K-12 (λ). No final step dependent upon protein synthesis was required for the immediate release of phage particles; chloramphenicol, added 10 min before UV irradiation at a concentration which inhibited bacterial growth and phage synthesis, did not prevent the release of these particles (Table 2). The bacterial cell population is synthesizing phage but not releasing the particles; the action of the UV irradiation causes these cells to lyse, perhaps prematurely, resulting in an immediate increase of free phage. This phenomenon has not been reported in other lysogenic bacteria.

The pattern of phage release by this lysogenic strain seems to parallel the changes in tumor production observed by Lippincott with a tumorgenic strain, *A. tumefaciens* B-6(10).

Lippincott demonstrated that elevated temperatures inhibit tumor initiation in the first 9 to 10 hr after infection. Plants held at 32 C showed fewer tumors on pinto bean leaves; decreasing the temperature by 5 C for 2 hr resulted in definite increases in the number of tumors initiated (10). Earlier, Braun had also shown that the initiation of crown-gall tumors is a temperature-sensitive process (3). An increase in the incubation temperature to 35 C suppresses spontaneous phage synthesis but not bacterial growth.

UV irradiation induces changes in the infectivity of *A. tumefaciens*. Heberlein and Lippincott (4) found that irradiation doses which gave a 60% loss of viability resulted in an increase in tumor formation after 1.75 hr of incubation in the dark at 27 C. An immediate enhancement of

A. tumefaciens infectivity also occurred immediately after UV irradiation. The authors state that this UV irradiation promotion of infectivity is similar to the induction of prophage and bacteriocins. The increased infectivity after irradiation appears to be due to an actual increase in the number of bacteria that will subsequently initiate tumors. This concept agrees with our finding that, after UV irradiation, there is an increase in the number of bacteria that can initiate vegetative virus synthesis. The increase in vegetative phage synthesis may also result in an increase in the tumor-inducing principle, either as a by-product of synthesis of phage or the release of an internal cell component when the cell lyses.

Stonier, McSharry, and Speitel (12) have demonstrated that the free phage particle does not initiate tumor production or rid the plant of crown-gall bacteria. Beardsley (2), in an earlier paper, reports on a lysogenic strain, *A. tumefaciens* B-6, which carried the phage *omega*. He also found that bacteria-free lysates containing *omega* do not induce crown-gall tumors in susceptible tissues (6).

A. tumefaciens V-1, when cured of the phage LV-1, retains the same level of tumor formation on pinto bean leaves; therefore, this particular phage is not directly associated with pathogenicity. Other prophage may be related to tumorgenic ability.

Evidence indicates that the phage particle itself is not the tumor-inducing principle, but it may be an associated metabolic product. The substance perhaps is unstable and, therefore, not demonstrable in lysates. The possibility exists that the production of the tumorgenic substance is enhanced under conditions which also favor phage synthesis.

ACKNOWLEDGMENTS

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