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On the Question of Crown-Gall Tumor Initiation by DNA of Bacteriophage PS8

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DNA of *Agrobacterium tumefaciens* bacteriophage PS8 was isolated by using several procedures. Whole phage and phage DNA were tested for tumor-inducing ability on 10 species of plants with various additions to assist such activity. The reported tumorigenicity of phage PS8 DNA could not be confirmed, and no evidence to implicate phage PS8 involvement in tumor initiation was obtained.

Bacteriophage PS8, which infects several strains of *Agrobacterium tumefaciens*, was originally isolated from sunflower crown-gall tissue cultivated in tissue culture for many years in the apparent absence of the tumor-inducing bacterium (10). This result, plus those of Tourneur and Morel (12), who isolated *Agrobacterium* phage from several crown-gall tissues growing in sterile culture but none from cultures of normal, habituated or virus tumor tissues, is suggestive of phage involvement in crown-gall formation. Leff and Beardsley (4) reported that DNA isolated from phage PS8 could induce tumorous proliferations, albeit sporadically, on pinto bean leaves, sunflower stems, and tobacco stems. It was desirable to confirm this report of tumor-inducing ability by DNA from phage PS8 in order to study the crown-gall tumor initiation process in detail. Our experiments, however, provide no evidence for tumorigenic activity on the part of PS8 or of DNA isolated from this phage.

MATERIALS AND METHODS

A sample of PS8 phage obtained from R.E. Beardsley and J. Leff was received by G. T. H. The virus was propagated on *A. tumefaciens* strain B6-806 (10), a phage-sensitive strain isolated by R. E. Beardsley from a heavily ultraviolet (UV)-irradiated culture of *A. tumefaciens* strain B6. A virus sample from the initial lysate provided the phage stock for experiments carried out by R. Beiderbeck and J. A. Lippincott. Efforts to confirm the results of Leff and Beardsley (4) were begun independently in the two laboratories which will hereafter be referred to as New York (G. T. H.) and Evanston (R. B. and J. A. L.).

Phage preparation and titer (Evanston). *A. tumefaciens* strain B6-806 was grown in a liquid medium consisting of 1% tryptone (Difco) plus 0.25% NaCl at 30 C using a bubbler tube for aeration. For mass production of phage, overnight cultures of B6-806 containing 1.5×10^8 to 4.2×10^8 cells per ml were infected with PS8 at a multiplicity of 0.4 to 0.5. After 6 to 8 hr of incubation at 30 C with aeration, cultures were made 0.5 M with respect to NaCl and centrifuged for 10 min at 3,000 $\times$ *g* to remove bacterial debris. Polyethylene glycol 6000 (PEG) was then added to a final concentration of 7% (w/v), and the mixture was held at 4 C overnight (13). Precipitated phage were collected by sedimentation at 12,000 $\times$ *g* for 10 min at 4 C and suspended in the dilution medium (PD buffer) described by Beardsley (1). Recovery of plaque-forming units (PFU) in the sediment was 95% or greater and, typically, about 6.5×10^9 PFU were obtained per ml of initial culture.

The titer of PS8 phage preparations was determined with strain B6-806 as host, by using the standard double-agar-layer technique and the medium given above. Plaques were counted after 24 hr of incubation at 30 C.

Phage preparation and titer (New York). Phage PS8 was propagated on *A. tumefaciens* strain B6-806 grown in nutrient broth (Difco) containing 0.1% yeast extract and 0.5% sucrose. Phage titers were determined using phage assay-base-agar medium plus phage assay-overlay-agar medium (Fisher). For batch preparations of PS8, 1-liter flasks containing 300 ml of liquid medium were inoculated with sufficient B6-806 to give an initial concentration of about 10^7 organisms per ml and grown for 14 to 16 hr with shaking at 27 C. These cultures were inoculated with PS8 at a multiplicity of infection of 0.1 to 0.5, and 6 hr later the cultures were centrifuged at 5,000 $\times$ *g* for 20 min at 4 C to remove whole bacteria and cellular debris. Phage yields of 10^{10} to 10^{11} PFU/ml were commonly obtained. PS8 suspensions were further concentrated by centrifugation at 75,000 $\times$ *g* for 90 min in a refrigerated ultracentrifuge and

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resuspending the phage in small portions of 0.1 standard saline citrate buffer (SSC = 0.15 M NaCl + 0.015 M sodium citrate, pH 7.0).

Isolation of phage DNA (Evanston). Phage precipitated with PEG was suspended in PD buffer to a titer of 7×10^{11} to 9×10^{11} PFU/ml and passed through a membrane filter (0.22-μm pore size; Millipore Corp.) to remove bacteria. The filtered phage was again precipitated with PEG and suspended to a titer of about 10^{12} in 1 × SSC buffer or in tris(hydroxymethyl)aminoethane (Tris-buffer (0.01 M at pH 8 containing 0.1 M NaCl). DNA was isolated from phage in 1 × SSC by a procedure based on that of Marmur (8). Phage was shaken with an equal volume of chloroform-butanol (4:1, v/v) plus 0.2% sodium dodecyl sulfate. The detergent was omitted in some experiments, and the aqueous phase, after centrifugation, was extracted twice with an equal volume of chloroform-butanol. The aqueous phase was removed, and the DNA was precipitated with 2.5 volumes of ethanol. After washing with ethanol, the DNA was dissolved in the Tris buffer described above. A phenol procedure for isolation of DNA (3) was also used. Phage suspended in Tris buffer (*see above*) was extracted three times with an equal volume of freshly distilled phenol saturated with the same buffer. The aqueous phase containing the DNA was extracted four times with ether to remove the phenol, and the DNA was precipitated and washed with ethanol as in the previous procedure. In each procedure, only gentle shaking was used and only a minimum of pipetting using large orifice pipettes, was involved. DNA prepared by both procedures was readily spooled in 70% ethanol and showed the following relative absorbancy relations, respectively, at 260, 280, and 230 nm: phenol DNA, 1:0.465:0.436; Marmur, DNA, 1:0.465:0.547. DNA concentrations were calculated from absorbancy measurements at 260 nm. Sterility of DNA preparations was checked by plating 0.1-ml samples of the preparations on nutrient broth-yeast extract-sucrose agar (5). All preparations contained less than 200 colony-forming bacteria per ml, which is well below the threshold required for tumor formation by *Agrobacterium* in the tumor bioassays in which these preparations were tested. Thirteen DNA preparations were checked for tumorigenicity. Eight were prepared by the Marmur method, and five were prepared by the phenol method.

Isolation of phage DNA (New York). DNA, freshly prepared by Leff in Beardsley's laboratory at Manhattan College by means of a modified Marmur's procedure (8), was utilized in one of the experiments reported. Sterility tests and plant inoculations were then carried out at New York University. In other cases, DNA was prepared at N.Y.U. by a procedure described by Marmur (8) in which phage DNA was deproteinized by shaking the phage three to four times with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) in the presence of perchlorate plus sodium dodecyl sulfate. The DNA was then precipitated with ethanol and dissolved in 1 × SSC. DNA was also prepared by a phenol procedure which has been described (9). DNA isolated from another *Agrobacterium* phage by this phenol procedure retains biological activity as evidenced by its ability to transfect (9). Concentrations of DNA were estimated from absorbancy determinations at 260 nm. Portions of all preparations were streaked on nutrient agar plates, and only those which were sterile were used for the bioassay. Four DNA preparations in all were tested; two were prepared by the Marmur method and two by the phenol method. One of these gave colonies on nutrient agar plates which appeared similar to those of strain B6-806, and this contamination was removed by further extraction with chloroform followed by alcohol precipitation.

Tumorigenicity tests (Evanston). The procedures used by Leff and Beardsley (4) to measure tumor induction in sunflower stems were closely followed. The roots were cut from 16- to 18-day-old seedlings of *Helianthus annuus*, and the stems were placed in various test solutions for 2 days, adding sterile distilled water as necessary to maintain the level of the liquid to about 0.5 cm on the base of the cuttings. The plants were then transferred to sterile vermiculite and wounded a day later by making two needle insertions perpendicular through the stem. Tumor formation was scored 3 weeks later.

Inoculations using whole sunflower seedlings were made by wounding the stem 1 cm below the cotyledons by pushing a 0.4-mm diameter syringe needle through the stem and injecting about 0.05 ml of solution into the wound. Tumor formation was estimated by measuring the diameter of the stem parallel to the direction of the wound at 10 to 15 days after inoculation, using a dissecting microscope with an ocular scale. Increase in diameter due to proliferation within the inoculated area was determined for each stem by expressing this diameter as a percent of the mean diameter of the same stem (= 100%), determined from measurements 1 cm above and 1 cm below the wound area. Figure 1 shows the relation obtained between concentration of tumor-forming bacteria inoculated and increase in stem diameter. For strain B6-806, 10^4 bacteria per ml, or approximately 500 bacteria per wound, are required to produce an increase in stem diameter above control level. Stem diameter is further increased in proportion to the logarithm of bacterial concentration to about 10^7 bacteria per ml. PEG at 0.5% (maximal amount to be expected in PEG precipitated phage) did not inhibit tumor formation in this system.

Other tumorigenicity tests were made using the primary leaves of *Phaseolus vulgaris* cv. "Pinto" wounded with carborundum (5) and leaves of *Kalanchoe daigremontiana* wounded with multiple needle punctures (2).

Tumorigenicity tests (New York). Roots or tubers of carrots (*Caucus carota*), beets (*Beta vulgaris*), sweet potatoes (*Ipomoea batatas*), potatoes (*Solanum tuberosum*), and parsnips (*Pastinaca sativa*) were obtained from local markets. These were washed, wiped with 70% alcohol, and soaked in 1% sodium hypochlorite (1:5 dilution of Chlorox, a commercial solution) for 20 min. They were then rinsed three times in sterile distilled water. Cylinders of tissue were removed by using a 10-mm cork borer.

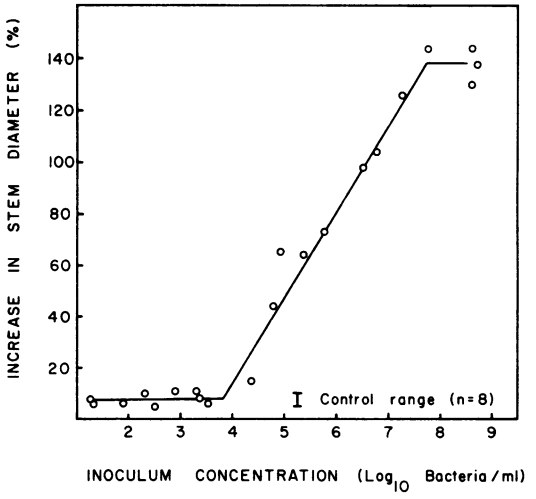

FIG. 1. *Increase in diameter of sunflower stems injected with various concentrations of Agrobacterium tumefaciens strain B6-806 over non-inoculated portions of the same stem. The data are from three experiments, each point represents the mean of 10 plants.*

Discs of tissue about 5 mm in thickness were cut from these cylinders and randomly distributed to sterile petri dishes containing a 5-mm layer of 1% water-agar. Aseptic techniques were employed throughout these procedures. The discs were inoculated by applying one drop of test material (ca. 0.05 ml) to each disc. The inoculated tissue discs were placed in a temperature-regulated chamber at 27 C under cool, white fluorescent light and scored for tumor formation 35 days after inoculation.

Other tests employed seedlings of Alaska pea (*Pisum sativum*), periwinkle (*Vinca rosea*), sunflower (*Helianthus annuus*), and pinto beans (*Phaseolus vulgaris*). Stems of the first three were inoculated by passing a dissecting needle perpendicular through the center of the stem and applying a drop (ca. 0.05 ml) of test material to the wound. Primary pinto bean leaves were inoculated by wounding with a holder containing 300 no. 1 insect pins and flooding the surface of the leaf with 0.1 ml of the inoculum. Tumor formation was scored after 8 days in the case of the bean leaves and after 3 weeks on stem-inoculated plants.

RESULTS

Phage characteristics. Three plaque types —clear, turbid, and halo—were observed in the original PS8 phage sample when plated on strain B6-806. Three successive isolations for each plaque type resulted in temporary enrichment of each particular type, although the other types were still present in substantial number, indicating each had the potential for generating the other two types at high frequency. One of these clear plaques, PS8C, was the progenator of the phage used in the investigations reported for Evanston, whereas the subcultures of the original PS8 phage inoculum were used for those in New York. Phage PS8C formed plaques equally well at 30 and 34.5 C, showed a latent period of 1.75 hr, and gave average single burst sizes of 120 to 220. The phage, after precipitation with PEG, showed a 260:280 nm absorbancy ratio of 1.53 which is similar to the ratios reported for phage purified in $CsCl_2$ (13), and which suggests that there was no large contamination of the phage with nucleic acid or protein. The nucleic acid isolated from these preparations gave a positive diphenylamine test, indicating it to be a DNA phage, and only a negligible reaction in the orcinol test, indicating that the phage prepared in this manner did not contain significant amounts of ribosomes.

Tumorigenicity tests (Evanston). PS8C phage DNA prepared by the Marmur method was tested on sunflower seedlings at concentrations of 50 to 60 μg/ml in four experiments. None of a total of 55 sunflower seedlings treated with DNA according to the procedures described by Leff and Beardsley (4) showed visible tumor formation, and measurements of stem diameter at wound sites where the DNA was introduced were not significantly above controls. Similarly, PS8C DNA prepared by the Marmur method in three experiments in which wounded *Kalanchoe* leaves were flooded with 75 to 290 μg of DNA per ml resulted in no tumors 3 weeks after inoculation at 1,147 wound sites. Sixty pinto bean leaves inoculated with phenol-prepared PS8C DNA at 40 to 120 μg of DNA per ml also showed no tumors 10 days after inoculation in two experiments.

Further exploration of the tumorigenicity of phage PS8C was made with intact sunflower seedlings and a single stem inoculation. This procedure, using the same host reported to give best results (4), was readily quantifiable and eliminated contamination problems associated with the use of nonsterile cuttings soaking for 2 days in DNA solutions. Table 1 shows the effect of whole PS8C phage, or of phage treated under mild conditions to release DNA, on the growth of wounded sunflower stems relative to that obtained with mixtures of the phage with cells of B6-806. The phage alone failed to promote wound growth beyond that shown by controls (Fig. 1), and the same phage added with B6-806 had no significant effect on the outgrowth induced by the bacterium, indicating that there was nothing inhibitory for growth in the phage preparations. Sodium docecyl sulfate and sodium dodecyl sulfate plus Pronase

treatment, which reduced the plaque-forming ability of PS8C preparations by 90 to 99%, probably with release of the intact viral nucleic acid, did not give rise to growth-promoting activity on the part of PS8. Similar amounts of SDS or SDS plus Pronase did not affect tumor formation by the host bacterium.

Polyornithine promotes tobacco mosaic virus uptake by plant protoplasts (11). Inoculation of this polypeptide at concentrations of 6.5 and 7 μg/ml with 1.2×10^{11} PFU per ml of PS8 phage to stems of 100 sunflower seedlings, however, failed to promote stem growth beyond that of wounded controls. The plant-growth regulators, β-indole-3-acetic acid and kinetin, in combination, generally promote growth and development of plants. These regulators had no effect on the tumorigenicity of the phage when added together, at concentrations of 3 and 2.2 μg/ml, respectively, with 1.7×10^{11} PFU/ml of phage PS8 to 30 sunflower plants. Similarly, carnosine, an analogue of a natural crown-gall growth factor (7), applied at 0.5 mg/ml with 1.7×10^{11} PFU/ml of phage PS8 to 30 sunflower seedlings failed to provide any evidence for tumorigenicity on the part of PS8.

Table 2 shows results obtained when several DNA preparations from phage PS8C were injected into stems of sunflower seedlings alone or with carnosine or polyornithine. As in the tests in which the cut stems were allowed to take up phage DNA, addition of phage DNA to stem wounds on intact plants had no significant effect on the proliferation of the wounded

TABLE 1. *Growth response of sunflower stems inoculated with phage PS8C, with viable cells of strain B6-806 plus phage, or with detergent or pronase plus detergent treated phage (data from two experiments)*

Additions to wounded stems	Conc of additions[a] (per ml)	No. of plants inoculated	Mean increase in diam at inoculation site (%)
None	0	98	9.7
Phage PS8C	1.1×10^{10} PFU	40	6.4
Phage PS8C	9.5×10^{10} PFU	35	7.0
PS8C treated with SDS[b]	3.0×10^{10} PFU	30	8.9
PS8C treated with SDS plus pronase[b]	3.0×10^{10} PFU	120	10.8
Pronase plus SDS[b]	0	120	10.0
Strain B6-806	1.2×10^{4} CFU	35	33.7
plus PS8C	9.5×10^{10} PFU		
Strain B6-806	1.4×10^{6} CFU	25	63.0
plus PS8C	1.5×10^{10} PFU		

[a] Abbreviations: PFU, plaque-forming units; CFU, colony-forming units.

[b] Sodium dodecyl sulfate (SDS) added to 0.15%, or SDS plus 100 μg/ml of Pronase added and preparation heated at 46 C for 30 min.

TABLE 2. *Summary of experiments on the effect of DNA from phage PS8C on sunflower stem growth*[a]

Additions to stem wounds	Concn of DNA applied (μg/ml)	No. of plants inoculated[b]	Mean increase in stem diam at wound site (%)[b]
None	0	45	9.5
Marmur procedure PS8 DNA	75 to 290	65	9.1
Marmur DNA plus carnosine (1 μg/ml)	75	30	9.0
Marmur DNA plus polyornithine (6.5 μg/ml)	107	15	12.2
None	0	50 (50)	8.4 (7.1)
Phenol procedure PS8 DNA	40 to 120	50 (50)	7.4 (7.4)
Salmon sperm DNA[c]	200	(100)	(9.7)
None	0	(100)	(10.0)

[a] Data from six experiments: three preparations of Marmur-method DNA were tested in five experiments, and three preparations of phenol-method DNA were tested in three experiments.

[b] Results in parentheses obtained at 10 days after inoculations; all others at 15 days.

[c] Mann Research Laboratories, Inc., New York, lot #E1107.

plant tissue, and none of the additions changed this response.

Tumorigenicity tests (New York). Tables 3 and 4 present results of tumorigenicity tests of phage PS8 and DNA from phage PS8 on several different plant parts. Both in vitro and in vivo tests failed to show transforming ability on the part of the virus, or on the part of its isolated nucleic acid.

Further attempts to demonstrate activity on the part of phage PS8 are given in Table 5. Again, neither the phage nor its isolated nucleic acid induced tumorous proliferation. Also, combined inoculations of phage PS8 or its DNA with a "potentially tumorigenic" strain of *A. tumefaciens*, strain IIBNV6 (6), to serve as helper failed to elicit a tumorous response. The phage had no significant effect on tumor formation by strain B6-806 when the two were combined and inoculated. Addition of PS8 DNA with highly virulent cells of *A. tumefaciens* strain B6, the latter at a level just

TABLE 3. *Response of sterile discs from several plants to phage PS8 and DNA isolated from this phage*

Additions to sterile tissue discs	Concn of additions	No. of discs inoculated/no. of discs with tumors				
		Carrot	Potato	Sweet potato	Beet	Parsnip
Strain B6	3×10^9 CFU/ml	25/24	25/5	25/23	25/25	25/25
Phage PS8	8×10^{10} PFU/ml	25/0	25/0	25/0	25/0	25/0
PS8 DNA[a]	128 μg/ml	25/0	25/0	25/0	25/0[b]	25/0

[a] DNA prepared by phenol technique; identical results obtained with 15 and 30 μg/ml of PS8 DNA.
[b] One disc formed a small amount of callus tissue similar to that occasionally found on control discs.

TABLE 4. *Tumorigenicity of phage PS8 and DNA from phage PS8 when inoculated into wounds of plants in vivo*

Additions to wounded plants	Concn of additions	No. of plants treated/no. of plants showing tumors			
		Pea stems	Vinca stems	Sunflower stems	Bean leaves
Strain B6	1.2×10^9 CFU/ml	16/15	6/6	8/8	24/24
Buffer control	0.1 SSC	16/0	6/0	8/0	24/0
PS8 phage	8×10^{10} PFU/ml	16/0	6/0	8/0	24/0
PS8 DNA[a]	90 μg/ml	16/0	6/0	8/0	24/0

[a] DNA prepared by the Marmur method; identical results obtained with 15 and 30 μg/ml of PS8 DNA.

TABLE 5. *Response of sterile plant discs to PS8 phage or PS8 DNA applied separately or in combination with various strains of A. tumefaciens*

Additions to tissue discs	Concn of additions (per ml)	No. of discs treated/no. giving tumors		
		Carrot	Beet	Parsnip
Strain B6	1.2×10^9 CFU	30/30	15/15	15/14
Strain IIBNV6	1.1×10^9 CFU	30/0	15/0	15/0
Strain B6-806	8×10^8 CFU		25/18	15/13
PS8 phage	8×10^{10} PFU	40/0	30/0	15/0
PS8 DNA[a]	46 μg	60/0	30/0	25/0
PS8 DNA[a]	232 μg	60/0	40/0	25/0
Denatured PS8 DNA[a]	212 μg	30/0	15/0	15/0
Strain IIBNV6	5×10^8 CFU	35/0	15/0	15/0
plus PS8 phage	4×10^{10} PFU			
Strain IIBNV6	5×10^8 CFU	25/0	20/0	15/0
plus PS8 DNA[a]	116 μg			
Strain B6-806	4×10^8 CFU		25/13	15/9
plus PS8 phage	4×10^{10} PFU			
Strain B6	1.2×10^5 CFU			15/0
plus PS8 DNA[a]	116 μg			
Strain B6	1.2×10^5 CFU			15/0

[a] DNA prepared by the Marmur method.

below the sensitivity of parsnip root discs, similarly failed to result in tumorous outgrowths.

DISCUSSION

Our data fail to provide any evidence to confirm the reported tumorigenicity of bacteriophage PS8 DNA, despite the fact that DNA was prepared in collaboration with J. Leff in one experiment by the procedure reported by Leff and Beardsley (4) and by additional mild procedures. Also, many different plants and plant parts were used in our bioassays, including the sunflower seedlings and procedures which these authors have indicated to give best results. All of these tests have failed to give positive responses in our hands to either PS8 phage or to the DNA of this phage, although the same plant material readily responds to relatively low numbers of whole bacteria. By conservative estimates, the amount of phage DNA applied to each plant wound in the numerous tests reported here amounts to the equivalent of about 10^{11} phage genomes. Since the tumor bioassays employed give tumors, depending on the assay, when somewhere between 10^3 and 10^6 bacteria are applied, the sensitivity of the assays appears fully adequate to have detected tumorigenicity on the part of PS8 DNA. We conclude that the conditions for obtaining tumor formation by phage PS8 DNA are not sufficiently known to permit reasonable attempts at confirmation, and the reported tumorigenicity of such preparations must be considered doubtful until such conditions are presented.

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