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Title HOST RANGE, SEROLOGICAL AND ELECTRON MICROSCOPE STUDIES OF PHAGES OF AGROBACTERIUM TUMEFACIENS (SMITH AND TOWNSEND) CONN

Abstract approved (Major Professor)

Phages of crown gall bacteria were among the first to be isolated and certain properties of these phages have been studied by a number of investigators. However, several important characteristics have not been studied in detail. The present investigation was made in order to determine the major sources of phages of A. tumefaciens and to compare the host range pattern, morphology and serological relationships among phages from different sources.

Thirty-one phage isolates were obtained from sewage, soil and crown gall tissues. Phages were prevalent in sewage, but scarce in soil and gall tissue. Prevalence of these phages in sewage, where A. tumefaciens is not expected to occur, poses some interesting questions regarding host range of the phages and possible

relationships between crown gall bacteria and bacteria present in sewage.

Plaques of the phages were always round and clear with a sharp margin, but the size varied. There appeared to be four different strains of phage based on plaque size. The most common plaque type was 1.0-2.5 mm in diameter. One isolate produced small plaques, 0.25-0.50 mm, and six isolates produced large ones, 3.5-5.0 mm. One isolate produced plaques which were 2.5-3.5 mm in diameter. The size of the plaques did not correlate with source of isolation.

Forty-one strains of A. tumefaciens and a number of other bacteria associated with plants were tested for host specificity of the phage isolates. The phages were very specific as they lysed only certain strains of A. tumefaciens. Eight strains of A. tumefaciens and all the other bacteria tested including representatives of three other species of Agrobacterium were not lysed. Among the 31 phage isolates, there were 26 different strains based on host range patterns within A. tumefaciens. In some instances, the phages were closely related in host range, but most were entirely different. The source of isolation and plaque size of the phages did not correlate with the host range.

All 31 phage isolates were used as antigens to stimulate production of antibodies in rabbits. Each of the antisera produced was

tested against all of the phage isolates by the Ouchterlony agar double-diffusion method. The phages could be divided into five serological groups based on the reactions which occurred. The phages in each group contained at least two antigens, and all five groups were more or less interrelated. The host bacterium on which the phages were reproduced appeared to be correlated with serological properties of phage. Host specificity seemed to be related to serological properties in some cases but not in the others. Source of isolation and plaque size were not correlated with serological properties.

Seven phage isolates with representatives from each source of isolation were observed with the electron microscope. All of the phage particles were similar in shape but were somewhat varied in size. The heads were polyhydral, 42 mμ in width and 80-92 mμ in length. The tails were 8 mμ in width and 90-125 mμ in length. Phage morphology did not appear to be related to source of isolation, plaque size, host range or serological properties.

HOST RANGE, SEROLOGICAL AND ELECTRON MICROSCOPE STUDIES OF PHAGES OF AGROBACTERIUM TUMEFACIENS (SMITH AND TOWNSEND) CONN

by

PAIROJ BUANGSUWON

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INTRODUCTION

The crown gall disease of plants, caused by Agrobacterium tumefaciens (Smith and Townsend) Conn 1942, is typical of a group of diseases in which the major effects on the host are hyperplasia and hypertrophy. According to Walker (34) the causal organism was first isolated in Italy by Cavara in 1897 from grape (Vitis vinifera L.). Inoculation of his isolates into grape plants reproduced the disease. In the United States, E. F. Smith and C. O. Townsend (32) demonstrated the pathogenicity of this organism in a wide range of plants and described it as Bacterium tumefaciens. The disease has since become one of the most extensively studied bacterial diseases of plants.

Originally described as Bacterium tumefaciens Smith and Townsend (1907), the species was subsequently transferred to Pseudomonas by Stevens (1913), Bacillus by Holland (1920), Phytomonas by Bergey (1923) and finally to Agrobacterium by Conn (1942).

The first study of bacteriophage from A. tumefaciens was reported by G. H. Coon and J. E. Kotila (12). Since then, several

microbiologists and plant pathologists have worked with this phage, but up to the present, no investigation has been undertaken in which many isolates of the phage from different sources have been characterized and compared with each other. Particularly lacking have been studies of the serological relationships among many isolates of the phage, host specificity within strains of the species A. tumefaciens or the morphology of the phages from different sources.

The purposes of this study were (a) to ascertain the sources of phages of crown-gall bacteria, (b) to study host range of the phages within the species A. tumefaciens and also in different species and genera of bacteria, especially those which are associated with plants, (c) to find the serological relationship among isolates of phage obtained from different sources, and (d) to reveal the morphology of the phages by electron microscopy.

LITERATURE REVIEW

According to Adams (2), the first bacteriophage was reported in 1915 by Twort in England, but the word "bacteriophage" was introduced by d'Herelle in 1918. The work of d'Herelle (13) with various animal pathogenic bacteria established the existence of a transmissible lytic principle effective against certain bacterial strains. He believed his experiments showed the existence of an ultra-microscopic parasite living as an obligate parasite on bacteria.

The work of d'Herelle (13) stimulated much study of bacteriophages, particularly those attacking bacteria pathogenic to man and animals. On a comparative basis, much less has been published on the bacteriophages of plant pathogenic bacteria.

Gerretsen, Sack, Sohngen and Gryns (16) first worked with bacteriophages of bacteria associated with plants. They successfully isolated phages from nodules of various leguminous plants. These phages were specific for the bacteria of the legume concerned. Phages were also obtained from roots and stems but not from leaves of the legumes, from garden and field soil but not from heath or forest soil. The phages were found to withstand desiccation, as well as heating at from 60 to 65^{o}C for 15 minutes, and they were at least eight times as resistant to ultra-violet light as were the bacteria upon which they were effective.

Mallmann and Hemstreet (23) also demonstrated the d'Herelle phenomenon using an organism obtained from a plant source. They obtained what they call an "inhibitory substance" from cabbage which had been rotted by fluorescent organisms. They were able to demonstrate marked inhibition of growth with extremely high dilutions of the filtrate, but they were not able to demonstrate the actual lysis. The activity of this inhibitory substance was increased upon successive transplantings and filtrations and showed inhibition even at a dilution of 1:100,000,000,000. These workers further tested the "inhibitory substance" against Erwinia carotovora (L. R. Jones) Holland 1923, causal organism of soft rot of vegetables and Erwinia atroseptica (van Hall) Jennison 1923, the potato black leg organism. The first organism was inhibited when large amounts of filtrate were used, but the latter was not inhibited at all. The work of Mallmann and Hamstreet opened up a very interesting area of experimentation in plant pathology.

In the following year, Coons and Kotila (12) first reported the association of phage with Agrobacterium tumefaciens. Polyvalent bacteriophages were isolated for several plant pathogenic bacteria: Erwinia carotovora, E. atroseptica, and Agrobacterium tumefaciens. The phages were obtained from soil, river water and rotted carrot. Activity of these phages varied with the strains of the organism tested. The phage filtrate from A. tumefaciens produced lysis in dilutions as

high as 1:100,000,000. Activity of each phage was greatest at the temperature most favorable for growth of the susceptible organism. The potency of the phage filtrate decreased after a period of five and a half months storage in a sealed flask. These phages caused clumping, precipitation, swelling and decreased motility of the host organisms. Infection of potato and carrot slices with Erwinia carotovora and with E. atroseptica was prevented partially or completely by phage application.

Strains of crown gall bacteriophage have been reported from various sources. Israilsky (17, 18) isolated a phage for A. tumefaciens from a gall on sugar beet. The host specificity of this phage was limited to three of the nine strains of bacteria tested. Phage activity was obtained at all temperatures that permitted bacterial growth. The phage at the titer of 1.0×10^{11} particles per ml in meat-peptone broth was inactivated in ten minutes at 70°C. Israilsky also confirmed Coons' report on clumping and precipitation of the organism during the clearing of the broth culture. When stems and seeds were treated with the phage filtrate prior to inoculating with A. tumefaciens, the percentage infection was reduced appreciably. Two phage-resistant cultures of the crown gall organism were isolated from broth cultures to which the phage had been added. These strains also remained resistant at higher titers of the phage.

Brown and Quirk (9) isolated phages from bacterial culture

filtrates of Erwinia carotovora and A. tumefaciens and from extracts of normal carrot tissue, rotted carrot, Ricinus tumor tissues, and sugar beet tumors. When A. tumefaciens was treated with high dilutions of the filtrates from tumors and rotten carrots, galls on Ricinus plants were induced more rapidly and were larger than those on the controls. The essential factor causing the disease of crown gall was described as a filterable "proto-bacterium" form of A. tumefaciens developed in the presence of the phage. However, these investigators failed to demonstrate the presence of an infectious filterable form of the crown gall organism.

Sugar beet gall tissue and 7-day old broth culture of crown gall bacteria obtained from raspberry plants and soil samples were also found to be sources of phage against A. tumefaciens by Muncie and Patel (25, 26). Seven different species of plant pathogens; Xanthomonas pruni (E. F. Smith) Dowson, 1939; X. beticola (Smith et. al., 1911) Savulescu, 1947; X. citri (Hasse) Dowson, 1939; X. vesicatoria (Doidge, 1920) Dowson, 1939; X. phaseoli (Smith, 1897) Dowson, 1939; Erwinia carotovora (Jones, 1901) Holland, 1920; E. atroseptica (van Hall, 1902) Jennison, 1932; in addition to 17 strains of A. tumefaciens isolated from apple, rose, raspberry, geranium, peach, walnut, weeping willow, Western sand cherry and Incense cedar were used in host specificity studies. Lysis occurred only in the bacterial cultures from which the phage was isolated. The phage

from an old culture was intensively studied and found completely inactivated at 85°C in ten minutes. Lysis of the host organism also was inhibited on bile crystal violet agar which was an excellent medium for growth of the crown gall organism. Potency of this phage was enhanced by additions of a broth culture of the original host bacterium to the stock filtrate followed by successive filtrations through Berkefeld filters. Growth of A. tumefaciens was inhibited for 72 - 120 hours at higher dilutions after inoculation with phage, but growth became abundant afterwards. Injection of the phage filtrate into tomato plants that were artificially inoculated with A. tumefaciens did not prevent gall formation.

Chester (11) isolated phages against A. tumefaciens from sugar beet (Beta vulgaris, Linn.) and from Pelargonium zonale Willd. (Geranium zonale, Linn.). In 60 tests, phage was isolated from 40% of the crown galls on Pelargonium stems and from 30% of the healthy tissues surrounding crown galls, but none was obtained from the non-infected plants. In another 60 tests on sugar beet roots, the phage was isolated from 75% of the galls, from 40% of the healthy tissues surrounding the galls and from 30% of the plants that were not infected by A. tumefaciens. The tests with Pelargonium sp. showed that the phage moved outward from the points of infection into surrounding healthy tissues and that phage from the soil penetrated the healthy sugar beet root tissues. However, in both cases the distance

traversed was limited to a few centimeters or less.

In 1937, Kent (19) isolated phages from crown gall tissue on tomato, sugar beet and Paris-daisy and from healthy portions of galled tomato plants. The phage from tomato gall tissue was characterized as to certain physical and chemical properties, and protection of the plant by phage was evaluated. This phage resisted a temperature treatment of 90°C for ten minutes but was destroyed after a treatment of 95°C for the same length of time. The longevity of this phage at 5°C was 304 days in one test and over 25 months in another test. Lytic action was absent when the phage filtrate was dried in a vacuum oven at 30 inches of Hg with a temperature of 73°C and when the sterile filtrate was allowed to dry slowly at room temperature. Purification of the phage by using organic solvents such as ether, chloroform, acetone or butyl alcohol and by use of ammonium sulfate precipitation failed.

The phage was not resistant to acid or alkali and was destroyed by nitric acid concentrations stronger than 1:3,000 and by N/32 sodium hydroxide after one hour exposure. The adsorption of phage to the host bacteria was slow and incomplete. Faint lysis and increase of titer were observed after 6 - 8 hours. Phage introduced into the plant by the hypodermic needle method prior to or at the time of inoculation with the pathogen did not protect the plant. Neither artificially introduced nor naturally existing phage seemed to have

therapeutic value.

Twenty phages were isolated from raw sewage by Zajic (35). Attempts to isolate phage from soil, gall tissue, tomato plants, cultures of A. tumefaciens and river water were unsuccessful. On the basis of plaque morphology and host specificity, two phages, III and XI, were chosen for additional characterization. Phage III produced plaques ranging from 3.0 to 4.5 mm. in diameter and lysed 8 of 10 strains of crown gall bacteria. Phage XI produced smaller plaques the diameter ranging from 1.0 to 2.0 mm. and only lysed 3 of the 10 strains. These two phages also differed in stability, pH, optimum storage temperature and thermal inactivation point. Phage III was more sensitive than phage XI to 1% hydrogen peroxide, 75% and 95% ethyl alcohol, 2.5% phenol, 1:3,000 nitric acid, 1:1,000 mercuric chloride, 10% sodium citrate, 27.3% urea and to aniline blue, congo red and safranin - 0. The addition of specific antibiotics to the assay medium partially or completely inhibited phage action against the antibiotic-resistant strains. The adsorption rates, latent periods and the burst sizes of phages III and XI were studied.

Temperate phage for A. tumefaciens was induced by Beardsley (5, 6). He later (1960) isolated two omega phages obtained from a lysate produced by ultra-violet induction. Centrifugation and filtration through Selas 0 3 filter gave a phage with a titer of 5.0×10^8 to 3.0×10^9 particles per ml. The omega phage formed three

different types of plaques when plated directly on the indicator strains. Differences of these two phages were also observed in rate of adsorption, thermal inactivation, burst size and rise period, but they were identical in latent period and host specificity.

The most recent and intensive studies of phages of A. tumefaciens were performed by Chen (10). Six phages were isolated from raw sewage, but attempts to isolate phages from soil, water and gall tissues on rose and tomato plants were unsuccessful. These six phages were divided into two groups on the basis of host range pattern, the monovalent phage type that lysed only the homologous host bacterium and the polyvalent phage type that lysed species of the genus Agrobacterium other than their original hosts. None of these phages lysed any strain of Rhizobium sp. or Agrobacterium rubi, A. stellulatum, A. gypsophilum, Pseudomonas tabaci, Ps. solanacearum, Ps. phaseolicola, Xanthomonas phaseoli, Erwinia carotovora and Escherichia coli no. 281. In the study of stability in the diluents, it was found that the phage was most stable in nutrient broth and least in distilled water. The average plaque sizes of phages varied with the type of phage and the concentration of agar used in the medium. Increasing agar concentration not only decreased the average size of plaques but also decreased the average number of plaques per plate.

When phages were stored at temperatures of 4^{o}, 10^{o}, 20^{o},

25°, and 37°C for about 14 weeks, it was found that the higher the temperature of storage, the fewer the number of particles of phages which remained viable. The concentration of phage particles in the filtrates varied with the type of filter and the amount of lysate used. The Seitz type filter was more satisfactory than the sintered glass filter. When 20, 50, 100 and 200 ml lysates were used separately for filtration, comparative concentration of phage particles in the filtrates per ml as compared to the original concentration, were 0.2%, 33%, 84% and 73%, respectively.

The effect of high frequency sound on the phages varied with the strain of phage tested. In three cases, 31%, 6% and 4% of the phage particles were left after 60 minutes treatment. The thermal inactivation point for these phages was 80°C for a period of ten minutes when nutrient broth was the substrate. The resistance of some phages to heat differed with kind of substrate used, the greatest resistance occurring in nutrient broth. The optimum pH of these phages was between 6.9 - 9.8. When the phage filtrate was dried by vacuum evaporation and by lyophilization, 18 - 34% and 52%, respectively, of the phage particles remained viable. After 30 days of freeze-drying, phage filtrate showed 8.6% of its original viability at 4°C but only 1.9% at 25° - 28°C.

For serological study, three of the phages were used as antigens for preparation of antiphage sera from rabbits. The final

dilutions giving 90 - 100% neutralization of the antibodies were 1:700, 1:1,000 and 1:1,000, respectively. The neutralized phage particles could be reactivated with sonic vibration. Sonic treatment gave the highest reactivation of neutralized phages at 10 minutes.

Two isolates of phage were studied for latent period and burst size. The latent period was 50 - 55 minutes for one phage and 65 - 70 minutes for the other one, but the burst size of the latter was about seven times greater than the former, 454 compared with 62.

Chen (10) also studied the effect of phage upon the development of crown gall disease in vivo. Prior to testing the influence of phage filtrate on crown gall formation, the filtrate was treated with extracts from tomato stem tissue and tomato gall tissue to determine whether these two extracts had any effect on the phage. The tomato stem tissue extract caused some inhibitory effect on the phage, and the gall extract caused a slight inhibition. At the end of a 24-hour incubation period in these extracts at room temperature, the phage survivals were 17% in normal tomato stem extract and 33% in tomato crown gall extract as compared to 36% in the nutrient broth control.

Mixtures of phages and homologous bacteria were inoculated into tomato plants at 1, 1.5, 3, 6, 12 and 24 hours intervals. After six weeks, the average gall weights were 0.99, 0.84, 0.81, 0.80, 0.74 and 0.44 gm, respectively. Relative gall sizes were 100%, 85%, 82%, 81%, 75% and 44%.

Filtrates of A. tumefaciens strain A6, tomato gall tissue extract and phage of A. tumefaciens strain A6, each was dried by a Rinco Evaporator apparatus. The dried materials were then dissolved in 5 ml of sterilized distilled water and inoculated to tomato plants with sterile micropipettes. After six weeks, no galls were observed on the tomato stems except those inoculated with the virulent strain A6 bacteria. When phage was sprayed on crown gall tissue in order to study the therapeutic effect, there was no significant effect. Relatively gall size was 98% when sprayed with phage as compared to 100% when sprayed with distilled water.

Roslycky, Allen and McCoy (29, 30) studied the phages of A. radiobacter. They found that some of their phages could lyse some strains of A. tumefaciens, but the antisera prepared from phages of A. radiobacter did not neutralize the phages of A. tumefaciens.

MATERIALS AND METHODS

Forty one strains of Agrobacterium tumefaciens from different sources were used as hosts in this experiment. Brief histories of these strains appear in Table 1.

Potato dextrose agar slants at about pH 7 were used to maintain the cultures of A. tumefaciens. Since nutrient broth has been shown to be the best medium for survival of phages of A. tumefaciens, it was used both in culturing the bacterium and maintaining phage cultures. Sub-cultures of the bacterium were made by transferring one part of a 24-hour nutrient broth culture to nine parts of fresh sterile nutrient broth then shaking 24 hours at room temperature.

The formulas of the media used in the investigation were as follows:

Nutrient broth

Beef extract	3.0 gm
Peptone	5.0 gm
Distilled water	1.0 liter

pH about 7.0

Yeast extract peptone dextrose broth

Yeast extract	5.0 gm
Peptone	5.0 gm
Dextrose	10.0 gm
Distilled water	1.0 liter

pH 6.8 - 7.0

Basic medium for Rhizobium

K_2HPO_4	1.0 gm
KH_2PO_4	1.0 gm
$MgSO_4$	.36 gm
$CaSO_4$	.13 gm
Yeast extract	1.0 gm
Mannitol	3.0 gm
$FeCl_3$(4 gm/100 ml H_2O)	3.0 ml
$CoCl_2$-$6H_2O$ (600 ppm)	0.04 ml
Distilled water	1.0 liter

adjust pH 6.5 - 7.0

In each case the agar medium was prepared by adding 20 gm of shredded agar per liter of broth.

Table 1. Brief histories of strains of Agrobacterium tumefaciens.

STRAIN	SECURED FROM	REMARK	PATHOGENICITY*
4-21	I. W. Deep	isolated from cherry trees in 1952.	Non-virulent
5-22	"	" "	"
RG	"	isolated from rose plant in 1958.	"
BR 3	"	isolate selected from 5-22 culture as being bacteriophage resistant.	"
4-32	"	isolated from cherry trees in 1952.	Virulent
5-14	"	" "	"
ACH	"	received from Peter Ark.	"
CG 1	"	" "	"
M_1-2	P. Buangsuwon	isolated from diseased cherry trees in 1963.	Non-virulent
M_6-1	"	" "	"
M_{14}-5	"	" "	"
M_{16}-1	"	" "	"
M_{19}-1	"	" "	"
M_{24}-5	"	" "	"
M_{12}-3	"	" "	Virulent
PS-1	"	isolated from bryophyllum galls in 1964.	"

Table 1. (Continued)

STRAIN	SECURED FROM	REMARK	PATHOGEN-ICITY*
ICPB-TT2	M. P. Starr	received from A. C. Braun, Princeton, N. J., 1941.	Virulent
ICPB-TT4 (Hofer SCA-2)	"	received from A. W. Hofer, Geneva, N. Y., July, 1943.	"
ICPB-TT10 (William 5. GlyFe)	"	from the collection of I. M. Lewis, received from O. B. Williams, Austin, Tex., 1945.	"
ICPB-TT101 (ATCC 6408)	"	received prior to 1941.	"
ICPB-TT102 (Cornell brown peach)	"	history unknown prior to 1942.	"
I-CK-9	R. S. Dickey	from soil in New York.	"
II-A-3	"	" "	"
CG	"	from pear gall in California.	Non-virulent
C-58	"	from cherry gall in New York.	Virulent
KB-1	"	" "	"
KB-3	"	" "	"
CY-8-1	"	from chrysanthemum in New York.	"
CY-8-2	"	" "	"

Table 1. (Concluded)

STRAIN	SECURED FROM	REMARK	PATHOGEN-ICITY*
PB-1	R. S. Dickey	from peony in New York.	Virulent
PB-2	"	" "	Non-virulent
Eu-4	"	from Euonymous in New York.	Virulent
Eu-7	"	" "	"
Eu-9	"	" "	"
R-1	"	from rose in Arizona.	Non-virulent
R-10	"	" "	Virulent
R-12	"	" "	"
R-R-4	"	from rose in New York.	"
TR-1-1	"	from rose in Texas.	"
TR-6-5	"	" "	"
TR-11-3	"	" "	"

* Tested on bryophyllum plants, obtained results at the end of six weeks period.

EXPERIMENTS AND RESULTS

Lysogenicity Tests

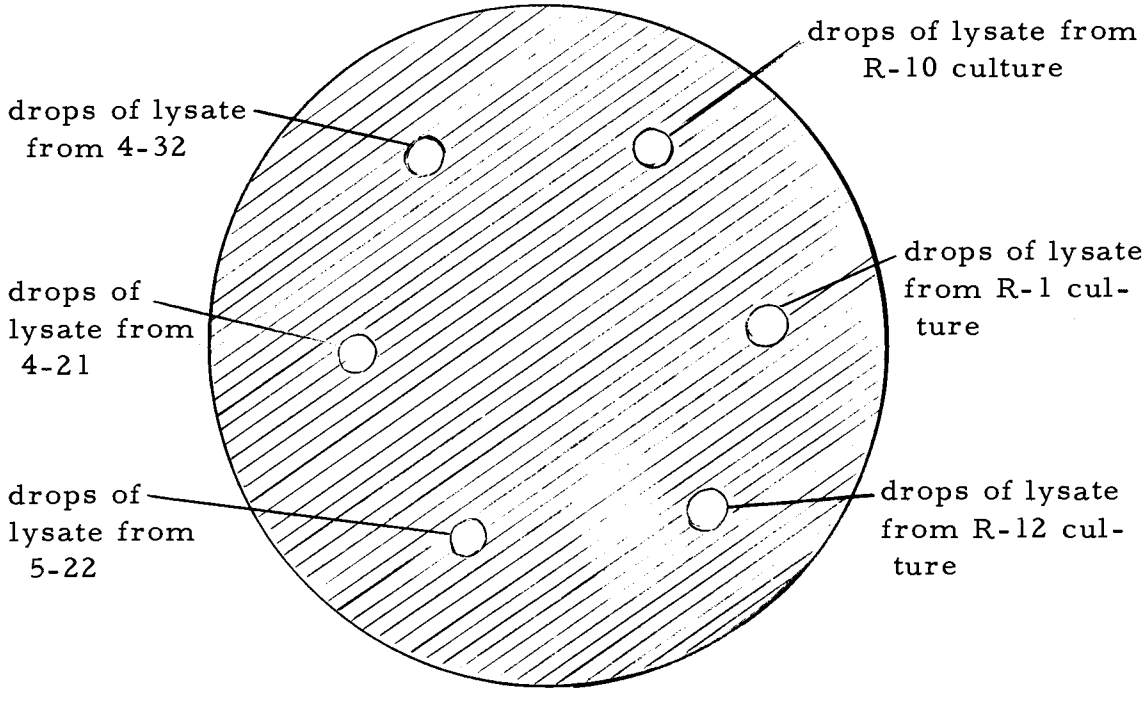
It has been known for a long time that filtrates of bacterial cultures may contain phages which lyse indicator strains of the same bacterial species or of related species. This kind of phage may be carried by the bacterial cells or the bacterial cell may be able to produce the phage from prophage. This phenomenon is known as lysogeny and has been found to be widespread in nature. Lysogenic strains of bacteria may release phage spontaneously along with cell division or they may be induced to release phages by various treatments such as irradiation with ultra-violet light.

Lysogenicity of Strains of A. tumefaciens.

Brown and Quirk (9) and Muncie and Patel (25, 26) reported that they could isolate phages from old cultures of crown gall bacteria. This suggests that the bacterial strains were lysogenic though the phenomenon was poorly understood at that time. Beardsley (5, 6) induced phage production from a culture of A. tumefaciens by using ultra-violet light.

In the present investigation, tests were made using all 41 strains of the bacterium to determine whether there is spontaneous production of phages. Two methods were applied in the investigation.

In the first test, each of the 41 strains of A. tumefaciens was grown in shake culture in nutrient broth for seven days at room temperature. Then they were centrifuged and decanted. A few drops of chloroform were added to the decants to kill all the bacterial cells. Nutrient agar plates were inoculated with the indicator strain of the bacterium by spraying a 24-hour bacterial suspension onto the surface. The plates were kept 24 hours at room temperature to let bacterial cells grow over the surface, and a few drops of decant from each of six cultures were dropped onto the plate as indicated below.



Nutrient agar layer seeded with strain 5-14

In this way, seven plates of each indicator strain were used to test the decant from each of the 41 bacterial isolates. All of the plates were incubated at room temperature for 24 hours and then examined for the presence of plaques. The experiment was replicated three times.

In the second test, lysates of all 41 strains of A. tumefaciens were prepared by the same procedure as in the first test, but instead of dropping the lysate onto the agar layer seeded with indicator strain, each lysate was tested against all 41 strains by the double-agar technique described by Adams (2).

Result of Lysogenicity Test

Plaques did not develop on the plates following either of these tests. Therefore, none of the 41 tested strains was lysogenic to others by the spontaneous production method.

These results support the findings of Zajic (35) and Chen (10) who also failed to show lysogenicity among strains of A. tumefaciens used in their experiments.

Isolation of Phages of A. tumefaciens

The materials used for phage isolations were (a) raw sewage from the City of Corvallis, State of Oregon, (b) soils from different orchards, corn fields, vegetable gardens, forests, greenhouses in

several areas in the State of Oregon, (c) crown gall tissues of diseased bryophyllum plants and prune and cherry trees from the Botany Farm, Oregon State University, Corvallis, Oregon.

Methods of Phage Isolations

1. From Soil. Approximately 50 gm of soil were suspended in 100 ml of sterile distilled water, shaken vigorously for a few minutes then filtered through filter paper in order to get rid of the large particles. Five ml of filtrate were transferred to 25 ml of fresh sterile nutrient broth, and 1 ml of a 24-hour culture of the indicator bacterium was added. The mixtures were incubated at room temperature for 24 hours then centrifuged at 5,000 rpm for 30 minutes to throw down the bacterial cells. Chloroform was added to the clear broth obtained from centrifugation at the rate 0.1 ml of chloroform per 5 ml of broth in order to kill any bacteria remaining in the liquid. The mixture was shaken vigorously, and the chloroform was allowed to settle to the bottom of the container. The clear broth which was pipetted from the top was ready to be used for detecting the presence of phages by the double layer agar method described by Adams (2).

The first agar layer was prepared by pouring about 20 ml of melted 2% nutrient agar in sterile Petri dishes. The agar was allowed to solidify at room temperature and left overnight to

eliminate excess moisture. One ml of the bacterial broth culture lysate obtained by the processes mentioned above was transferred by a sterile pipette to 9 ml of nutrient broth in tubes and serial dilutions were made. One ml of each lysate at dilutions of 1:100, 1:1,000, 1:10,000 and 1:100,000 was mixed with one ml of 24-hour broth culture of the indicator strain of bacterium in tubes containing 2.5 ml of melted soft nutrient agar cooled down to 45°C. These components were thoroughly mixed by shaking then poured onto the surface of the first layer of agar and the plate immediately rotated to insure that the second layer was spread equally over the top of the first agar layer. The soft agar medium contained 0.8% agar, and the dilutions were made in a temperature bath at 45°C.

The plates were incubated at room temperature for 18 - 24 hours. When clear areas appeared on the plates, the soft agar medium from a single plaque was scraped out and transferred to a test tube which contained 9 ml of sterile nutrient broth. Two tenths ml of chloroform was added to the test tubes and shaken vigorously for a few minutes to kill all of the bacterial cells. The chloroform was allowed to settle to the bottom, and the clear broth lysate was collected. Serial dilutions of the lysate were prepared and tested again with the same indicator strain by the double-agar layer technique. This single plaque isolation was repeated at least five times until the phage was purified as indicated by uniformity of size,

shape and appearance of the plaques on the same plate. The phages then were collected and stored in nutrient broth at 4°C for further studies.

2. From Gall Tissues. About 25 gm of washed gall tissue was chopped with 75 ml of sterile distilled water in a Waring blender for five minutes and filtered through filter paper. Then the same process as described above was followed.

3. From Sewage. Ten ml of raw sewage were mixed with 1 ml of a 24-hour culture of the indicator bacterium in 25 ml of fresh sterile nutrient broth. This was incubated at room temperature for 24 hours and the mixture was centrifuged, treated with chloroform and tested for presence of phage as indicated above.

The following isolates of bacteria were used as indicator strains:

5-14	Eu-4
5-22	PB-2
4-21	KB-3
PS-1	R-10
C-58	CG

One hundred and twenty five samples of soil from different areas in Oregon, 40 samples of crown gall tissues from cherry, plum and bryophyllum plants and 10 samples of raw sewage from Corvallis, Oregon, were used in phage isolation.

Thirty-one isolates of phage of A. tumefaciens were collected

and designated as shown in Table 2. The results indicate that phages of A. tumefaciens are present in all three sources, but they appear to be much more prevalent in sewage than in soil or associated with galls. Twenty phage isolates were obtained from only 10 samples of raw sewage while only 7 isolates were recovered from 125 soil samples and 4 isolates from 40 samples of crown gall tissues. The comparative scarcity of phages in soil and in diseased tissues may explain why Zajic (35) and Chen (10) failed to obtain any phage from these sources. Their failure may also have been due, in part, to use of small numbers of samples and few strains of indicator bacteria.

The average diameter of the plaques was between 1.0 - 2.0 mm for most of the phage isolates while one isolate had an average plaque size of 0.25 to 0.50 mm, and a few phage isolates produced plaques which were 3.5 to 5.0 mm in diameter.

The plaque morphology of phage isolates did not show any relationship to the source of the collection as it varied among isolates from the same source.

Host Specificity of Phages of A. tumefaciens

As is true of plant and animal viruses, bacteriophages vary in their degree of host specificity. Host range information is a useful characteristic for identification of phage strains, for showing

Table 2. Origin and plaque characteristics of phages of A. tumefaciens.

ISOLATE	ORIGIN	PLAQUE MORPHOLOGY	
	From Soil:		
SL 1 (5-14)	vegetable garden, Corvallis, Oregon, enriched by strain 5-14.	1.0-2.0 mm	clear round with definite edge.
SL 2 (5-14)	corn field, Corvallis, Oregon, enriched by strain 5-14.	1.5-2.0 mm	" "
SL 3 (5-14)	corn field, Staton, Oregon, enriched by strain 5-14.	1.0-2.0 mm	" "
SL 4 (PS-1)	cherry orchard, Corvallis, Oregon, enriched by strain PS-1.	3.5-5.0 mm	" "
SL 5 (PS-1)	" "	3.5-5.0 mm	" "
SL 6 (PS-1)	" "	3.5-5.0 mm	" "
SL 7 (4-21)	cherry orchard, Corvallis, Oregon, enriched by strain 4-21.	1.0-2.0 mm	" "
	From Gall Tissue:		
GP 1 (5-22)	cherry gall, Botany Farm, O.S.U., enriched by strain 5-22.	1.5-2.0 mm	" "
GP 2 (5-22)	" "	1.5-2.0 mm	" "
GP 3 (4-21)	cherry gall, Botany Farm, O.S.U., enriched by strain 4-21.	4.0-5.0 mm	" "
GP 4 (PS-1)	cherry gall, Botany Farm, O.S.U., enriched by strain PS-1.	1.0-2.0 mm	" "

Table 2. (Continued)

ISOLATE	ORIGIN	PLAQUE MORPHOLOGY	
	From Sewage		
SW 1 (5-22)	raw sewage, Corvallis, Oregon, enriched by strain 5-22.	1.0-2.0 mm	clear, round with definite edge.
SW 2 (5-22)	" "	1.0-2.0 mm	" "
SW 3 (5-22)	" "	1.0-2.0 mm	" "
SW 4 (5-22)	" "	1.5-2.5 mm	" "
SW 5 (5-14)	raw sewage, Corvallis, Oregon, enriched by strain 5-14.	1.5-2.5 mm	" "
SW 6 (5-14)	" "	1.0-2.0 mm	" "
SW 7 (5-14)	" "	1.0-1.5 mm	" "
SW 8 (5-14)	" "	1.0-2.0 mm	" "
SW 9 (PB-2)	raw sewage, Corvallis, Oregon, enriched by strain PB-2.	1.0-1.5 mm	" "
SW 10 (PB-2)	" "	1.0-1.5 mm	" "
SW 11 (PB-2)	" "	1.0-2.0 mm	" "
SW 12 (PS-1)	raw sewage, Corvallis, Oregon, enriched by strain PS-1.	4.0-5.0 mm	" "
SW 13 (PS-1)	" "	2.5-3.5 mm	" "
SW 14 (C-58)	raw sewage, Corvallis, Oregon, enriched by strain C-58.	0.25-0.5 mm	" "
SW 15 (Eu-4)	raw sewage, Corvallis, Oregon, enriched by strain Eu-4.	1.0-2.0 mm	" "
SW 16 (Eu-4)	" "	1.0-2.0 mm	" "
SW 17 (Eu-4)	" "	1.5-2.5 mm	" "

Table 2. (Concluded)

ISOLATE	ORIGIN	PLAQUE MORPHOLOGY
	From Sewage, (continued)	
SW 18 (Eu-4)	raw sewage, Corvallis, Oregon, enriched by strain Eu-4.	1.0-2.0 mm clear, round with definite edge.
SW 19 (Eu-4)	" "	1.0-1.5 mm " "
SW 20 (Eu-4)	" "	1.0-2.0 mm " "

the relationship of one bacterium to another and for the recognition and rapid identification of the host bacteria (33).

Some phages are known to be highly specific in attacking only certain strains of a single species of bacteria while other phages attack bacteria of different species; some even attack bacteria in different genera.

The host ranges of the 31 phages were studied by plaque formation using the double agar layer method described earlier. The media used in these studies were (1) nutrient broth and nutrient agar for strains of A. tumefaciens, (2) yeast extract-peptone-dextrose broth and agar for other bacteria except Rhizobium sp., and (3) basic medium broth and agar for strains of Rhizobium sp. Formulas are given in the methods section.

The 41 strains of A. tumefaciens designated in Table 1 were tested against each of the 31 phage isolates. Several other species and genera of bacteria were also tested. These bacteria were obtained from different sources, and brief histories appear in Table 3. The results of host specificity tests are shown in Table 4.

The phages could infect from 2 to 14 strains of A. tumefaciens. Eight of the 41 strains of crown gall bacteria tested were not infected by any of the phage isolates. Six of these were avirulent, and one of them, BR-3, was selected from culture 5-22 as a bacteriophage resistant isolate. Culture 5-22 was lysed by 12 phage isolates.

Table 3. Bacteria used in host specificity tests.

Organism	Source
Erwinia	
E. amylovora EA 135	Obtained from R. N. Goodman, Dept. of Hort., University of Missouri, Columbia, Missouri.
Rx-S-1	" " " " (resistant to 1,000 ppm streptomycin)
E. aroideae	" " " "
E. ananas	" " " "
Agrobacterium	
A. radiobacter R 1001	Obtained from R. J. Boyd, Dept. of Plant Pathology, University of Wisconsin, Madison, Wisconsin.
S 192	" " " "
A. rhizogenes 132-6	Obtained from R. S. Dickey, Dept. of Plant Pathology, Cornell University, Ithaca, N. Y.
136-A-1	" " " "
ATCC 13332	Obtained from R. J. Boyd, Dept. of Plant Pathology, University of Wisconsin, Madison, Wisconsin.
A. rubi ATCC 13335	" " " "
Pseudomonas	
Ps. savastonoi PS 170	Obtained from M. P. Starr, Curator, International Collection of Phytopathogenic Bacteria, University of California, Davis, Cal.
PS 118	" " " "
Ps. morsprunorum PM 122	" " " "

Table 3. (Continued)

Organism	Source
Pseudomonas (continued)	
Ps. tabaci PT-1	Obtained from M. P. Starr, Curator, International Collection of Phytopathogenic Bacteria, University of California, Davis, Cal.
Ps. alliicola PA 10	" " " "
Ps. solanacearum	" " " "
Xanthomonas	
X. malvacearum	Obtained from I. W. Deep, Dept. of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon.
X. phaseoli	" " " "
X. juglandis	" " " "
X. campestris	" " " "
Rhizobium	
R. meliloti Effective F 29	Obtained from H. J. Evans, Dept. of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon.
Parasitic DOal	" " " "
3 DOal 26	" " " "
R. phaseoli K 11	" " " "
P 23	Obtained from E. A. Schwinghamer, Dept. of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon.
R. leguminosarum L 1	" " " "
L 2	" " " "
L 4	" " " "

Table 3. (Concluded)

Organism	Source
Rhizobium (continued)	
R. leguminosarum	
L 5	Obtained from E. A. Schwinghamer, Dept. of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon.
L 6	" " " "
L 7	" " " "
C 56	Obtained from H. J. Evans, Dept. of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon.
R. trifolii	
P 29	" " " "
162-K 4	" " " "
T 1	Obtained from E. A. Schwinghamer, Dept. of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon.
T 3	" " " "
T 4	" " " "
T 23	" " " "
T 40	" " " "
TA 1	" " " "

Table 4. Host specificity tests of isolates of phages upon strains of A. tumefaciens.

Strains of A. tumefaciens	Phage Isolate																														
	From Soil							From Galls				From Sewage																			
	1	2	3	4	5	6	7	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
4-21	+		+				+	+	+	+							+		+					+	+	+	+		+		
5-22		+		+		+		+	+	+	+	+	+	+	+		+														
4-32		+										+	+					+	+					+							
5-14	+	+	+	+	+		+	+	+		+			+	+	+	+	+	+	+	+	+	+			+	+		+	+	
C-58			+					+	+	+								+							+	+	+		+		
*KB-1																															
KB-3			+				+			+			+			+				+	+		+			+	+		+		+
TT 102							+																								
CG								+	+	+						+															
RR-4		+														+			+												
R-1		+		+												+		+		+	+										
R-10	+																	+													
R-12				+				+	+	+								+													
TR-1-1		+																								+	+		+		
*TR-6-5																															
TR-11-3				+																											
RG	+							+	+	+						+														+
CY-8-1																		+													
CY-8-2			+																												
PB-1																		+	+												
PB-2			+			+				+	+			+																	
Eu-4					+						+							+								+	+	+	+	+	+
Eu-7				+				+	+	+																+	+		+		+
Eu-9	+			+				+	+	+																					

Table 4. (Continued)

Strains of A. tumefaciens	Phage Isolate																															
	From Soil							From Galls				From Sewage																					
	1	2	3	4	5	6	7	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
ACH						+										+				+	+					+	+		+		
CG 1	+					+																				+	+		+		+
TT 2	+						+												+							+	+		+		
TT 4			+													+			+												
TT 10			+	+															+							+	+		+		
TT 101				+										+	+					+	+										
PS-1	+	+	+	+	+	+		+	+		+			+	+		+						+	+	+			+		+	
M_{12}-3		+														+				+	+					+	+		+		+
M_{14}-5							+																									
M_{24}-5							+																			+	+		+		+
I-CK-9							+																									
*BR 3																																
*II-A-3																																
*M_1-2																																
*M_6-1																																
*M_{16}-1																																
*M_{19}-1																																

* The strain was not lysed by any of the phage isolates.

Of the 41 strains of A. tumefaciens:

phage	SL 1	lysed	8	strains	phage	SW 6	lysed	4	strains
"	SL 2	"	8	"	"	SW 7	"	9	"
"	SL 3	"	9	"	"	SW 8	"	9	"
"	SL 4	"	10	"	"	SW 9	"	7	"
"	SL 5	"	3	"	"	SW 10	"	7	"
"	SL 6	"	5	"	"	SW 11	"	2	"
"	SL 7	"	8	"	"	SW 12	"	3	"
"	GP 1	"	10	"	"	SW 13	"	3	"
"	GP 2	"	10	"	"	SW 14	"	4	"
"	GP 3	"	10	"	"	SW 15	"	14	"
"	GP 4	"	5	"	"	SW 16	"	14	"
"	SW 1	"	2	"	"	SW 17	"	2	"
"	SW 2	"	4	"	"	SW 18	"	14	"
"	SW 3	"	4	"	"	SW 19	"	3	"
"	SW 4	"	4	"	"	SW 20	"	8	"
"	SW 5	"	10	"					

Of the 31 isolates of phage:

strain	4-21	was lysed by	13	isolates
	5-22	"	12	"
	4-32	"	6	"
	5-14	"	23	"
	C-58	"	9	"
	KB-3	"	12	"
	TT 102	"	1	"
	CG	"	4	"
	RR-4	"	3	"
	R-1	"	6	"
	R-10	"	2	"
	R-12	"	5	"
	TT 2	"	6	"
	TT 4	"	3	"
	TT 10	"	6	"
	TT 101	"	5	"
	PS-1	"	17	"
	M_{12}-3	"	8	"
	M_{14}-5	"	1	"
	M_{24}-5	"	5	"
	I-CK-9	"	1	"

Of the 31 isolates of phage: (continued)

strain	TR-1-1	was lysed by	4 isolates
	TR-11-3	"	1 "
	RG	"	6 "
	CY-8-1	"	1 "
	CY-8-1	"	1 "
	PB-1	"	2 "
	PB-2	"	15 "
	Eu-4	"	9 "
	Eu-7	"	8 "
	Eu-9	"	5 "
	ACH	"	7 "
	CG-1	"	6 "
	KB-1	"	0 "
	TR-6-5	"	0 "
	BR-3	"	0 "
	II-A-3	"	0 "
	M_1-2	"	0 "
	M_6-1	"	0 "
	M_{16}-1	"	0 "
	M_{19}-1	"	0 "

Six strains of the bacterium were each lysed by only one strain of phage. Culture 5-14 was the most susceptible organism. It was lysed by 23 of the phage isolates.

There were four groups of phages which were identical in their host range. Phages GP 1 and GP 2 both could lyse the same ten strains of A. tumefaciens. These two phages were obtained from different gall tissues but were enriched by the same strain of crown gall bacterium, 5-22. Phages SW 9 and SW 10 both lysed the same seven strains of host bacteria. They were obtained from sewage enriched by the same strain of crown gall bacteria, PB-2. Phages

SW 3 and SW 4 were both obtained from sewage using strain 5-22, and they lysed the same four strains. Phages SW 15, SW 16 and SW 18 lysed the same 14 strains of A. tumefaciens. They were isolated from sewage enriched by the same strain of bacteria, Eu-4. No other two isolates of phage showed the same host range. Therefore, of 31 isolates of phage there were 26 different strains as determined by host range within A. tumefaciens.

The phages were very specific to A. tumefaciens. None of them could lyse any strain of Rhizobium meliloti, R. phaseoli, R. leguminosarum, R. trifolii, Erwinia amylovora, E. aroideae, E. ananas, Agrobacterium radiobacter, A. rhizogenes, A. rubi, Pseudomonas savastonoi, Ps. morsprunorum, Ps. tabaci, Ps. alliicola, Ps. solanacearum, Xanthomonas malvacearum, X. phaseoli, X. juglandis and X. campestris.

Serological Studies of Phages of A. tumefaciens

Bordet and Ciuca (7) first demonstrated that injection of rabbits with a phage suspension stimulates the production of phage-neutralizing antibodies. Their antisera also contained agglutinins for the host bacterium. The injection of host bacteria, however, failed to stimulate the production of phage-neutralizing antibodies. Because antigenic relationships are correlated with morphological and biological similarities of phages (Delbrück, 14), the ability of

antisera to neutralize or react with specific bacteriophage particles has proven useful in the differentiation and classification of various phages into groups.

The method used in this investigation was a modification of the method described by Adams (2).

Preparation of the Antigen

The phage suspensions to be injected into rabbits were obtained from plates prepared by the double agar layer method. A few drops of a 1:100,000 dilution from the phage stock culture were added to 1 ml of 24-hour broth culture of host bacterium in 2.5 ml of melted soft nutrient agar, then poured onto the surface of the first layer of nutrient agar which had already been prepared. After 24 hours incubation at room temperature, plaques appeared all over the soft agar layer. Six ml of physiological saline solution consisting of 0.85% sodium chloride in 0.15 M phosphate buffer were added to each plate. The soft agar layer was scraped out by using a clean glass rod. The mixture of soft agar and saline solution was centrifuged for 30 minutes at 5,000 rpm to separate phage particles from bacterial cells and the agar debris. The supernatant was then collected and centrifuged once more by the Spinco Ultra-centrifuge at 38,000 rpm using rotor no. 40 for 90 minutes. A light brown precipitate appeared at the bottom of the ultra centrifuge tubes. The

supernatant was discarded, and three ml of saline and 0.2 ml of chloroform were added to the precipitate. The precipitate was resuspended and centrifuged again at low speed (5,000 rpm) for 15 minutes. The suspension was drawn out by a sterile pipette and kept at 5^{o}C in a sterile glass tube. The phage suspension was tested for sterility by dropping 0.5 ml into 9.0 ml of fresh, steriled nutrient broth and incubating at 27^{o}C and 35^{o}C for 24 hours. When the broth showed no sign of microbial growth, the suspension was ready to be used as antigen. The concentration of phage particles in the suspension prepared in this way was found well above 10^{11} particles per ml.

Production of the Antisera

Healthy rabbits weighing about five pounds each were used for production of antisera. Because of the large number of phage isolates used as antigens, a single rabbit was used for each phage isolate.

The rabbits were injected five times using 2.5 ml disposable plastic syringes with #25 needles.

First injection,	2.0 ml	of antigen	applied intravenously.		
Second	"	2.0	"	applied sub-cutaneously, 3 days after the first injection.	
Third	"	2.0	"	"	" 7
Fourth	"	2.0	"	"	" 10
Fifth	"	2.0	"	"	" 14

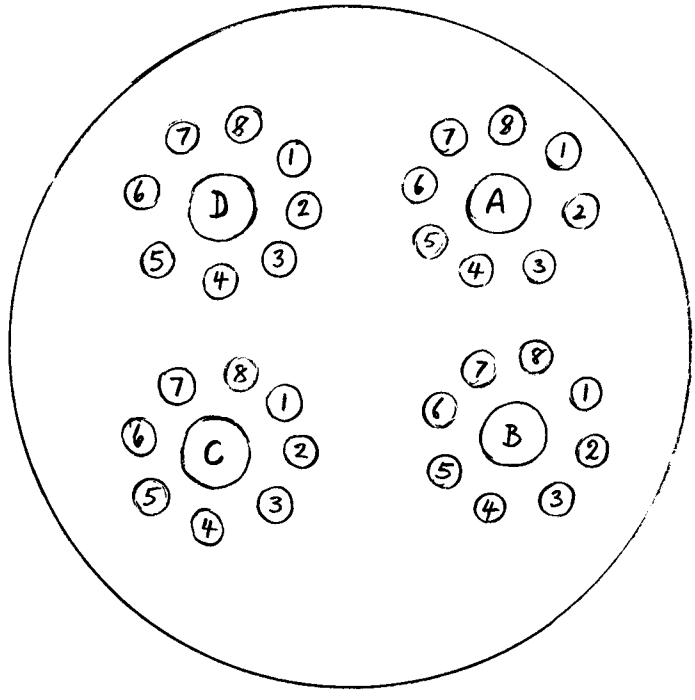
Seven days after the last injection, 10 ml of blood were collected from the ear veins of a number of rabbits, and the sera were separated out and tested for the amount of antisera by the micro-precipitin method as described by Ball (4). The titer of antiserum was too low so a series of five more injections was made. Two ml of phage suspension was injected sub-cutaneously into each rabbit at three-day intervals. Seven days after the last injection, blood samples were collected and checked again. This time, the antisera showed a sufficiently high titer, and 25 ml of blood were collected from the ear vein of each rabbit. The blood was allowed to clot at room temperature for two hours and was placed in the cold room ($5^{o}C$) overnight. The serum was removed by a sterile pipette. Low speed centrifugation was used to clear the serum, which was then labeled and stored at $5^{o}C$ in sterile glass tubes.

Eleven of the rabbits died during the experiment, but they were replaced and the same procedure was followed.

Testing of Antisera Against the Phage Isolates

The Ouchterlony agar double-diffusion method from Ball (4) was followed in this experiment. The medium was prepared by adding 5 gm of ion agar to 1 liter of distilled water, autoclaving, cooling to $45^{o}C$ and adding 100 ml of 1:500 sodium azide solution. The medium was shaken and 15 ml were poured into each sterile plastic

plate. After the agar had solidified, 36 wells were cut in each plate using cork borers size 2 and 3 as shown in Figure 2.



Four big and 32 small wells were cut in each plate. Each big well was surrounded by eight small wells at a distance of not more than 5 mm. The big wells were filled by one of the antisera, and the different phage isolates were placed in the small wells. In this way, all 31 phage isolates were tested against one of the antisera at the same time. The last small well was used for a control by filling with a suspension of culture filtrate from the host bacterium. This suspension contained no phage and no bacterial cells and was used to show that the antibodies were induced by phages only and that materials from the bacterial cells were not acting as

antigens. All of the antisera were tested by this method, and the plates were kept at room temperature in a moist chamber. Reactions between the antisera and the phages were recorded after 24 hours and every day for a week.

Results of the Serological Studies

When phage particles and antiserum were added to wells within an agar medium, they diffused into the agar. Where phage and antiserum met in optimum proportions in the agar, a precipitation zone developed. In this test, the precipitation zone was always a straight line formed between wells of antibody and phage. These lines could be seen by passing an oblique light through the bottom of the petri-dish. The controls containing the bacterial culture showed no reaction indicating that no host material was involved in the antisera production. All results were recorded after seven days and are shown in Table 5.

The 31 phage isolates can be divided into five serological groups as shown in Table 6. The phages in the first group were obtained from three strains of A. tumefaciens, 5-14, 5-22 and PS-1. Members of the second group were obtained from strain PB-2, the third group from Eu-4, the fourth group from 4-21 and the last group was only a single isolate from strain C-58.

These results show that serological differences among phages

Table 5. Reaction of antisera and phage isolates in Ouchterlony agar double-diffusion tests.

Antiserum	From Soil							From Galls				From Sewage																		
	1	2	3	4	5	6	7	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
ASL 1	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASL 2	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASL 3	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASL 4	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASL 5	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASL 6	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASL 7							+			+																+	+	+	+	+	+
AGP 1	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
AGP 2	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
AGP 3							+			+																+	+	+	+	+	+
AGP 4	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASW 1	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASW 2	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASW 3	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASW 4	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASW 5	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASW 6	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASW 7	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASW 8	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASW 9	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+						
ASW 10	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+						
ASW 11	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+						
ASW 12	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							
ASW 13	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+				+	+							

Table 5. (Continued)

Antiserum	From Soil							From Galls				From Sewage															
	1	2	3	4	5	6	7	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
ASW 14																				+	+	+			+	+	+	+	+	+	+
ASW 15							+				+														+	+	+	+	+	+	+
ASW 16							+				+														+	+	+	+	+	+	+
ASW 17							+				+														+	+	+	+	+	+	+
ASW 18							+				+														+	+	+	+	+	+	+
ASW 19							+				+														+	+	+	+	+	+	+
ASW 20							+				+														+	+	+	+	+	+	+

Table 6. Phage grouping due to serology reaction.

Group of Phage	ASL							AGP				ASW																		
	1	2	3	4	5	6	7	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	+	+	+	+	+	+		+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+							
2																				+	+	+			+						
3							+			+															+	+	+	+	+	+	+
4							+			+																+	+	+	+	+	+
5																				+	+	+			+	+	+	+	+	+	+

Group 1 represents phages SL 1, SL 2, SL 3, SL 4, SL 5, SL 6, GP 1, GP 2, GP 4, SW 1, SW 2, SW 3, SW 5, SW 6, SW 7, SW 8, SW 12, SW 13

Group 2: phages SW 9, SW 10 and SW 11

Group 3: phages SW 15, SW 16, SW 17, SW 18, SW 19 and SW 20

Group 4: phages SL 7 and GP 3

Group 5: phage SW 14

of A. tumefaciens exist. However, the serological group seems to be correlated primarily with the strain of bacterium used in isolation of the phage. Source of phage did not influence serological grouping.

Electron Microscope Study of Phages of A. tumefaciens

According to Adams (2), the electron microscope was first applied to the study of phages by Ruska in 1940. Since then many phages have been examined. Bradley and Kay (8) studied 22 different bacteriophages and divided them into different morphological groups. For Agrobacterium sp. only phages of A. radiobacter have been studied with the electron microscope (Roslycky et al., 30). No one has used this technique in the study of phages of A. tumefaciens.

The purposes of the electron microscope studies in this investigation were to reveal the morphology of the phages of A. tumefaciens, to compare the structure of phages collected from different sources and to study the relationship between morphology and other characteristics of the phages.

Preparation of the Supporting Film

Copper screens were used in the experiment. Two kinds of supporting film were applied to the screens by the following techniques described by Pease (27).

(A) Collodion film. A container filled with distilled water was used. Dust was cleaned off the surface with a wet paper towel. When the water surface was calm, three drops of collodion dissolved in 0. 5% amyl acetate were dropped onto the surface. After five minutes, copper grids were placed on the film, rough side up. The film and grids were picked up by pushing a glass slide down through the water. After draining off the water by leaning the slide against a support over a paper towel the film was dried overnight in a desiccator. The grids were turned over on a clean glass slide and a carbon film evaporated on the under side of the grid in a vacuum chamber. This supporting film was used for shadow casting.

(B) Formvar film. A cleaned, dry slide was dipped in formvar, polyvinyl formal in dichloroethane and allowed to dry. The edge of the slide was rubbed with a razor blade. The slide was gently immersed in distilled water to float off the film. Copper grids were placed, rough side up, on the film and retrieved with a glass slide. Excess water was drained off and the grids were allowed to dry overnight in a desiccator. This formvar film was used for the negative staining technique.

Preparation of the Phage Suspension

Phage suspensions were prepared from plates made by the double layer agar method as described earlier except that deionized

water was used instead of physiological saline to resuspend the phage.

Electron Micrography of Phages of A. tumefaciens

The RCA EMU-3D electron microscope in the Dept. of Plant Pathology, University of California, Berkeley, California, was used in this study. Because of the very small size and poor contrast of the phage particles, two techniques, negative staining and shadow casting, were used to increase the contrast and make the phage particles visible in the electron microscope.

A. Negative Staining Technique

Phage particles were stained by sodium phosphotungstate prepared by adjusting a 1% (W/V) solution of phosphotungstic acid to pH 7.2 using sodium hydroxide. The staining solution was added to the phage suspension at a ratio of 1:1 and mixed thoroughly for 30 seconds by sucking and releasing with a small dropper. A tiny drop of the mixture was then placed on the formvar film. After one minute the excess liquid was blotted with a small piece of filter paper, and the film was ready for observation with the electron microscope.

B. Shadow Casting Technique

A small drop of the mixture of phage suspension and a 1:2,000 dilution of 264 mμ polystyrene latex balls was placed on the carbon coated collodion film. After one minute, the excess liquid was blotted with a small piece of filter paper. The grid was allowed to

dry overnight and was shadow-cast in the vacuum shadow-caster with a platinum-palladium wire using the method described by Pease (27).

Result of Electron Microscope Study of Phages of A. tumefaciens

Seven phages were observed with the electron microscope. Three of the phages came from soil, three from sewage and one from gall tissue. These phages were SL 4 (PS-1), SL 6 (PS-1), SL 7 (4-21), SW 7 (5-14), SW 12 (PS-1), SW 14 (C-58), and GP 1 (5-22). Pictures of the phages are shown on the following pages.

All of the phages were similar in shape. The heads were regular polyhydra with the length equal to about twice the width. Tails were long and slender without noticeable terminal knobs or tail fibres. The sizes of the phage particles varied a little as shown in Table 7.

The sizes of phage particles observed in this study are only rough figures as measured from the few particles seen in the pictures. The heads of the particles were about the same width, but the length varied from 80 to 92 mμ. The pictures show an obvious difference in head length of phage SL 4 (PS-1) and SL 6 (PS-1). The difference in length of the tails is doubtful as the tails might have been broken during preparation of the phage suspension, and the pictures are not clear enough to accurately determine the end of the

Table 7. Size of phage particles from different sources.

Phage Isolate	Source	Size of Particle in mμ	
		Head	Tail
SL 4 (PS-1)	Soil	42 x 80	8 x 90
SL 6 (PS-1)	"	42 x 92	8 x 104
SL 7 (4-21)	"	42 x 90	8 x 125
SW 7 (5-14)	Sewage	42 x 90	8 x 108
SW 12 (PS-1)	"	42 x 84	8 x 110
SW 14 (C-58)	"	42 x 84	8 x 125
GP 1 (5-22)	Gall tissue	42 x 80	8 x 90

tails of most particles. Although the size seemed to vary somewhat, there was no correlation between size and source of the phage isolate.

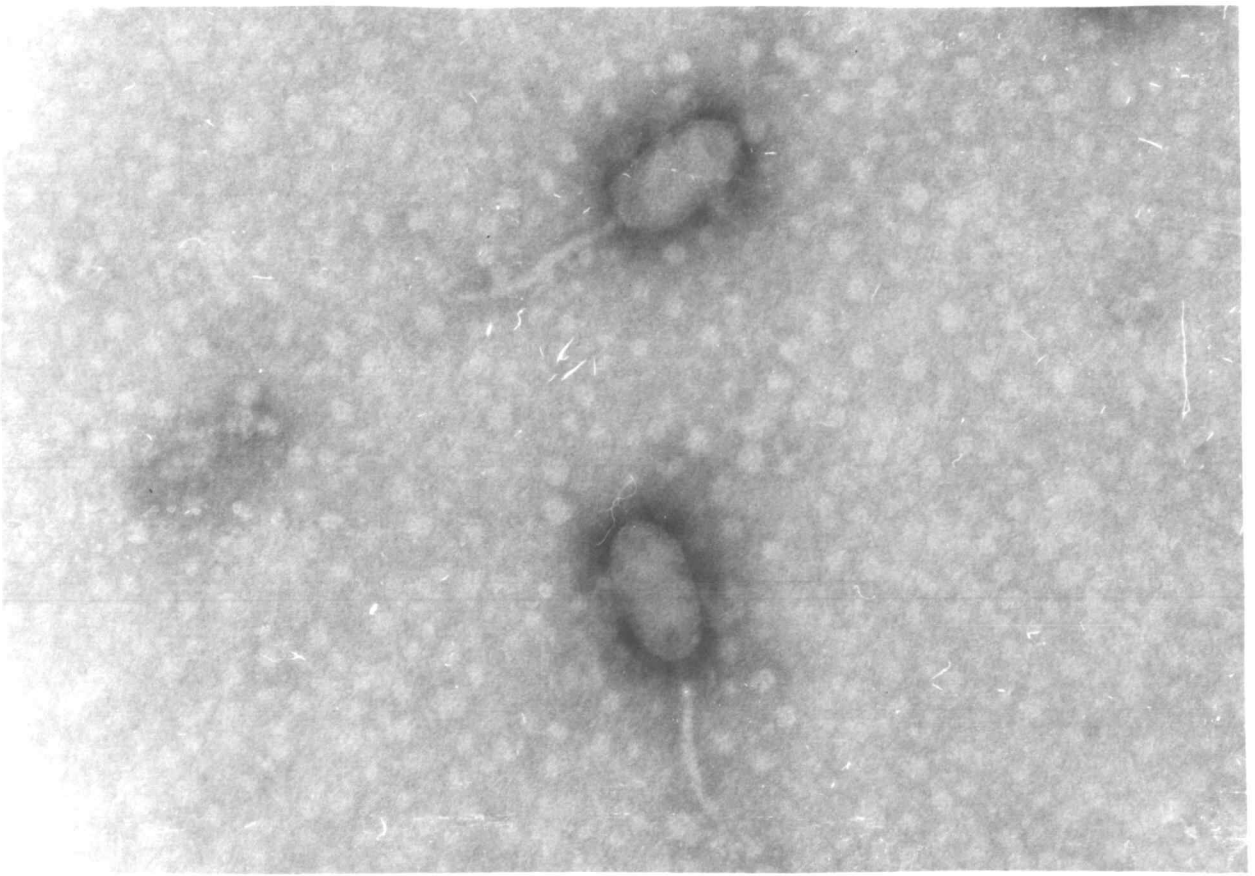
Changing of Phage Morphology

It was found during this study that after five days of resuspension in distilled water, several phage isolates were changed in shape from polyhydra with long tail to almost round particles. Picture 8 shows the particles in the SL 6 phage suspension after five days. This may be compared to normal SL 6 phage in picture 2. An experiment was set up to determine whether the phage particles remain virulent after the change in morphology. The suspension of phage SL 6 and the supporting film were prepared the same way as

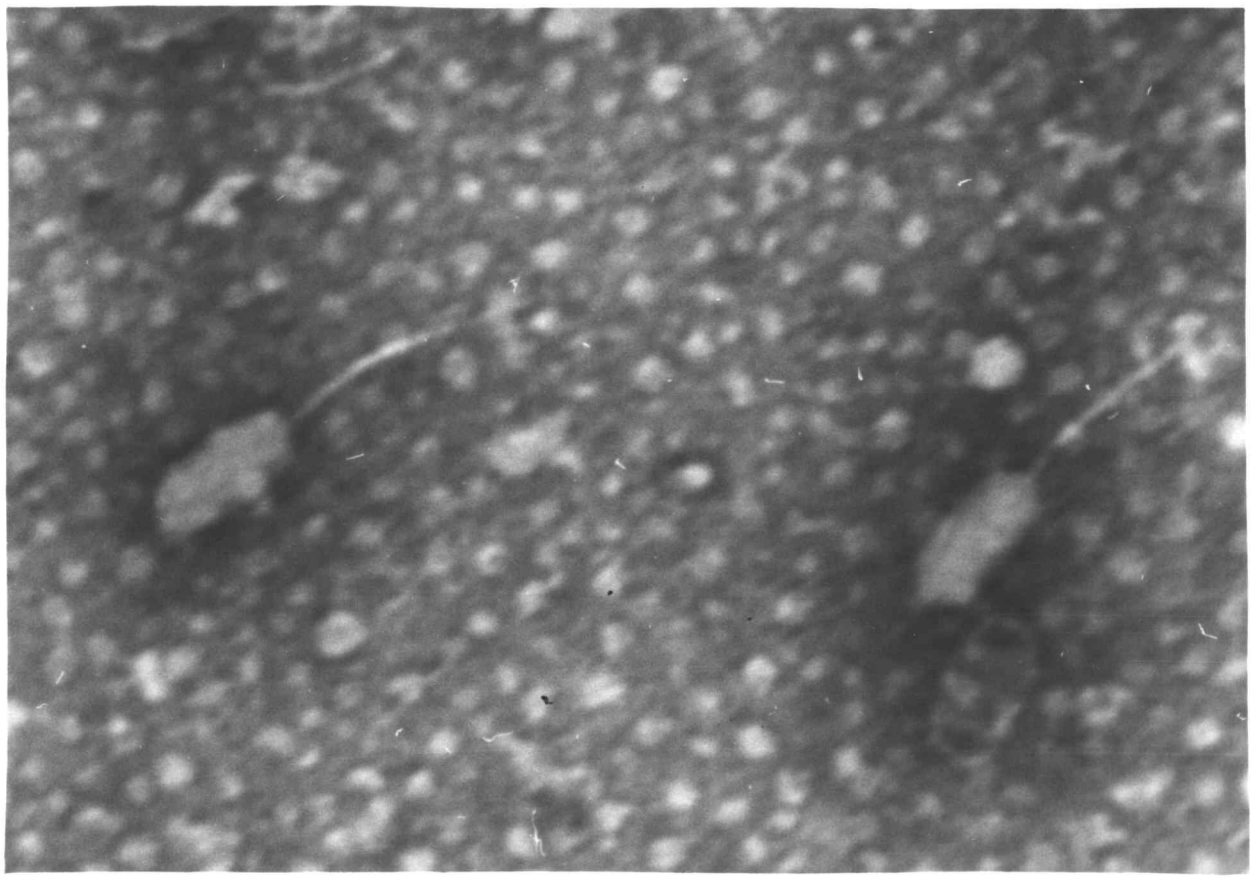
described previously and the suspension was kept at 5°C.

Every day for a week a small drop from the container was applied to a grid and shadow cast. At the same time, the infectivity of the phage was tested against strain PS-1 of A. tumefaciens at different concentrations by the double agar layer technique.

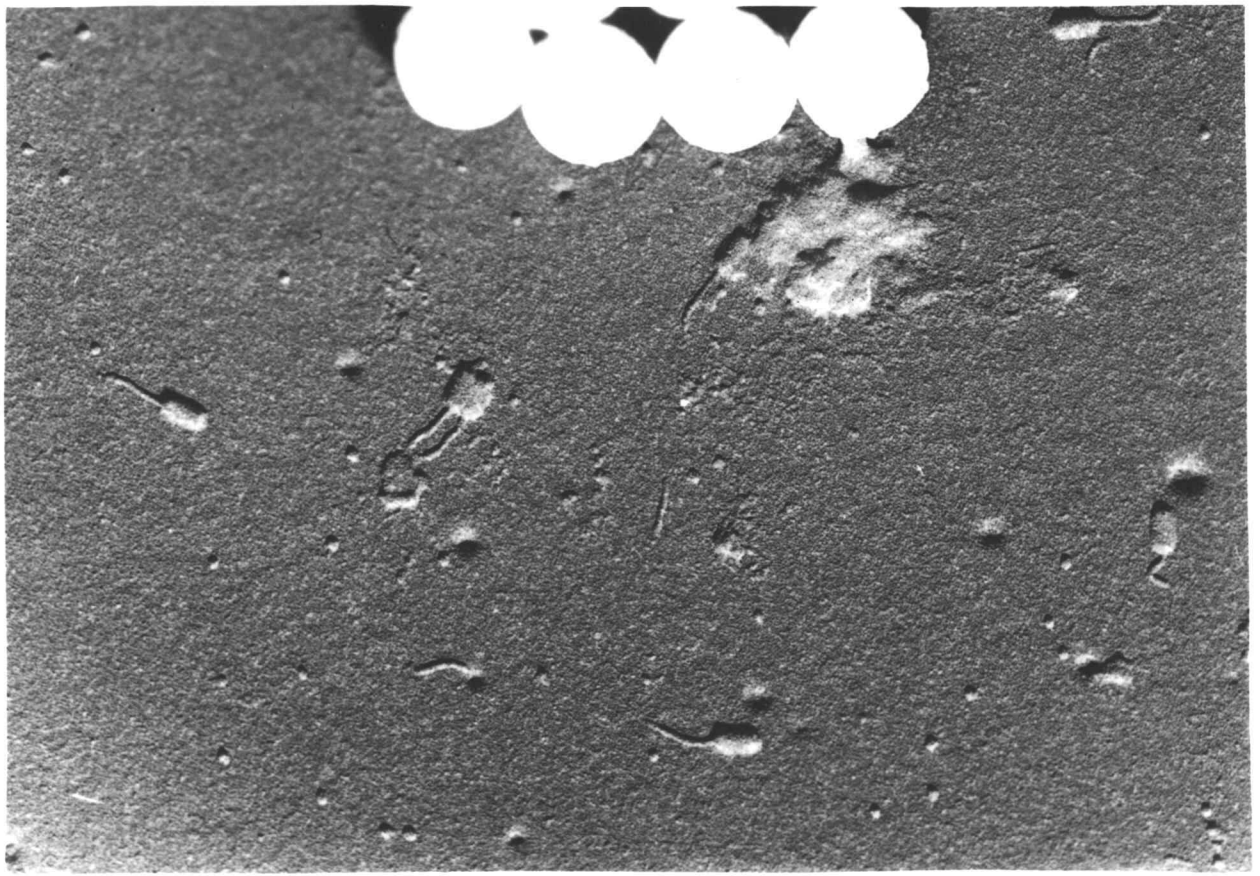
After seven days in distilled water, the phage was still as active as on the first day, but unfortunately, the electron microscope was broken down during this time, and pictures could not be taken. Therefore, the question as to the virulence of these round phage particles still needs to be resolved.



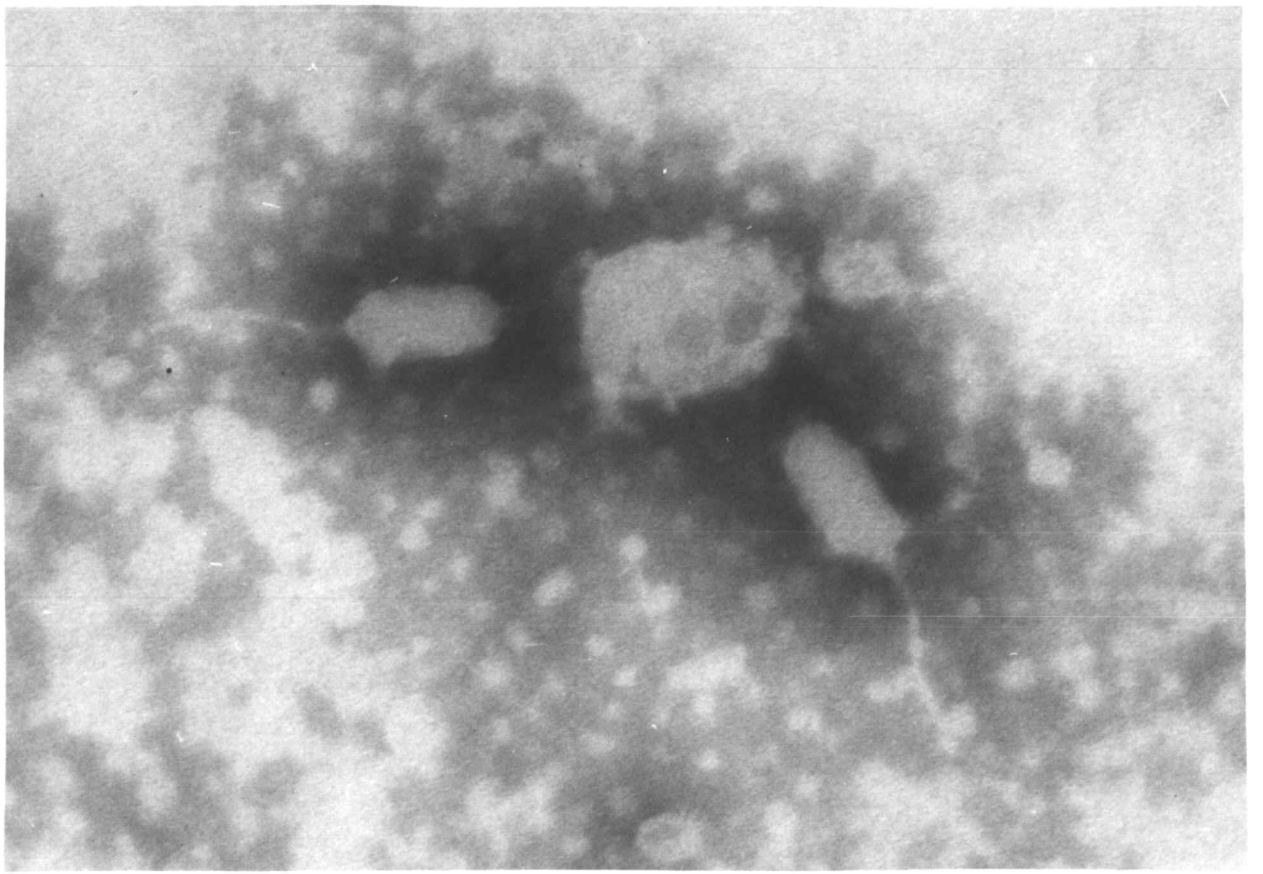
Picture 1. Phage SL 4 (PS-1). Negative stained, X 240,000.



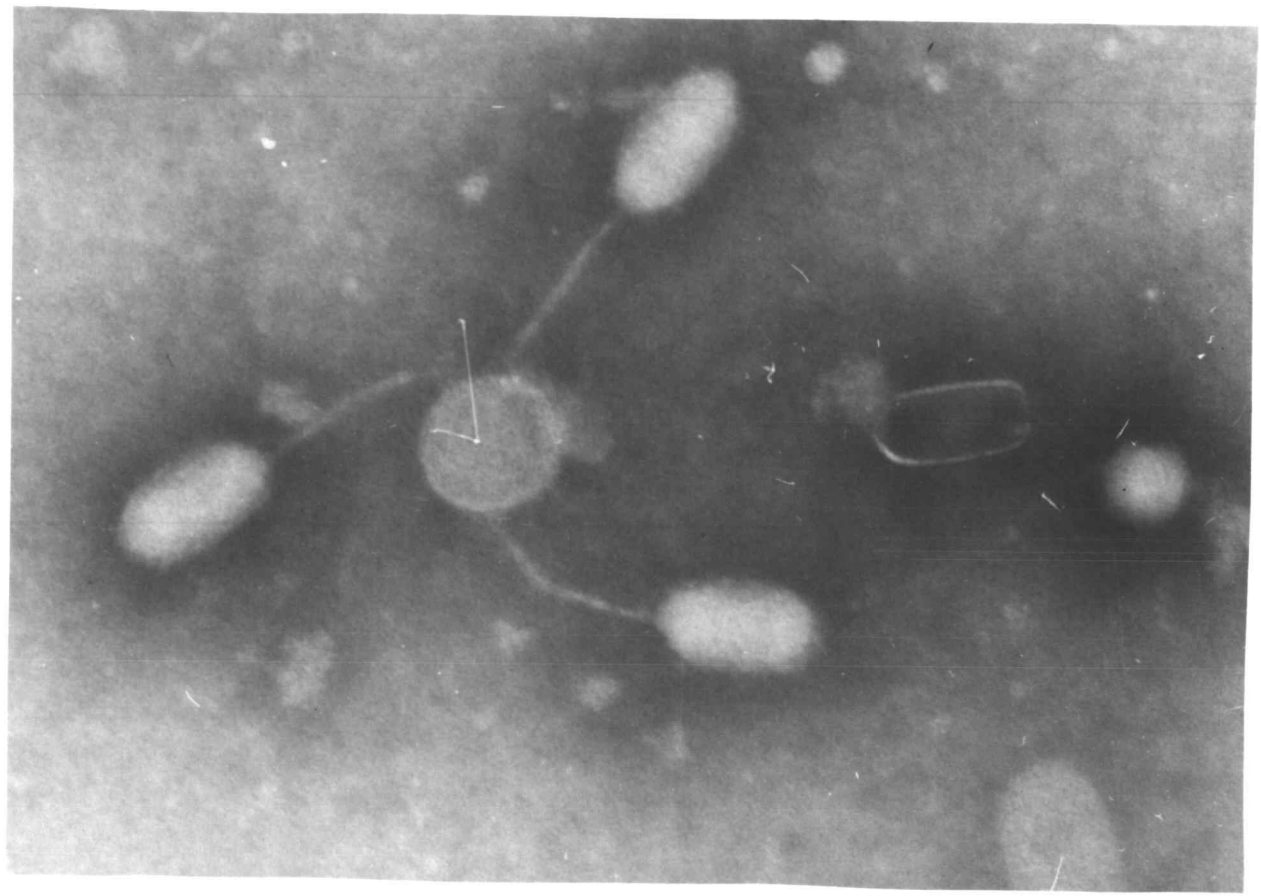
Picture 2. Phage SL 6 (PS-1). Negative stained, X 240,000.



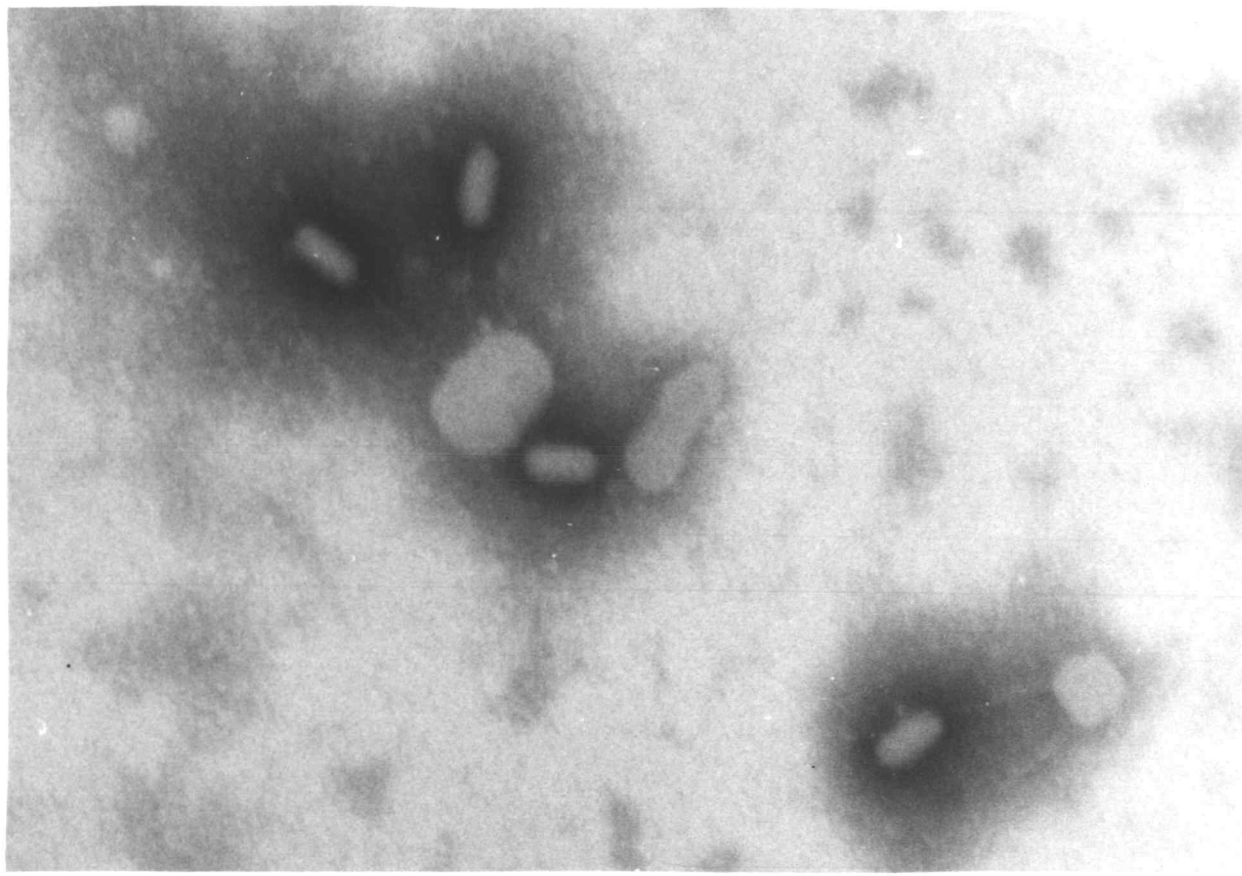
Picture 3. Phage SL 7 (4-21). Shadow casting, X70,000.



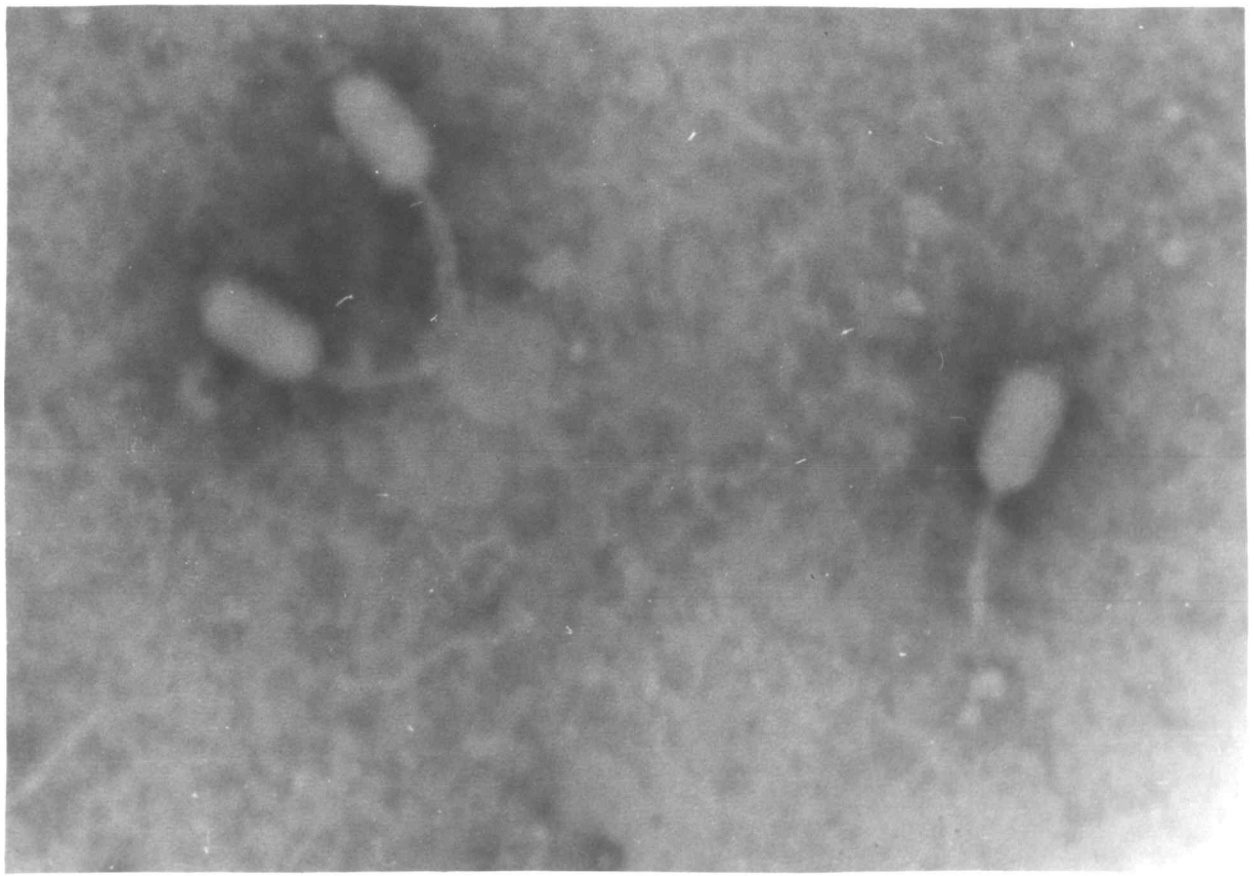
Picture 4. Phage SW 7 (5-14). Negative stained, X240,000.



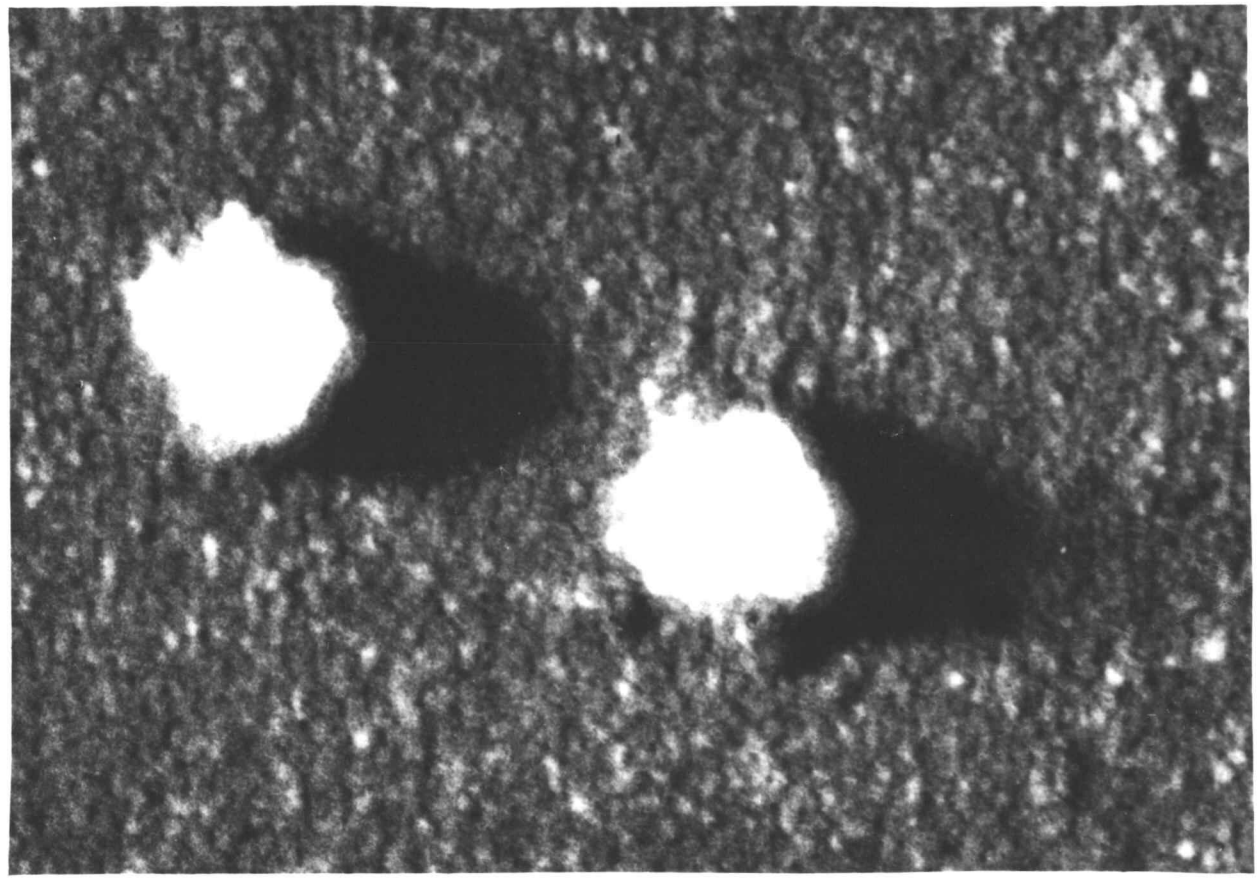
Picture 5. Phage SW 12 (PS-1). Negative stained, X240,000.



Picture 6. Phage SW 14 (C-58). Negative stained, X 120,000.



Picture 7. Phage GP 1 (5-22). Negative stained, X 220,000.



Picture 8. Phage SL 6 (PS-1), 5 days in distilled water. Shadow casting, X 600,000.

DISCUSSION

Lysogenicity of strains of A. tumefaciens

Some bacterial cells are able to carry phage particles without any lytic action, and the phages may be released during the growth process. This type of bacterium is called lysogenic and has been found widespread in nature. The works of two teams of early investigators may have resulted from the presence of lysogenic strains of A. tumefaciens. Brown and Quirk (9) and Muncie and Patel (25, 26) reported that they were able to isolate phage particles from pure cultures of A. tumefaciens, suggesting that the strains of A. tumefaciens used in their works were lysogenic. Some lysogenic strains will not release phage particles freely, but they can be induced by using other agents such as chemicals or irradiation. Beardsley (6) was the first to induce phage production from a culture of A. tumefaciens by using ultra-violet light.

Tests were made to determine whether any of the 41 strains of A. tumefaciens used in this investigation is lysogenic. This was necessary because once a bacterial strain has been lysogenized by a certain phage, the bacterial culture will be resistant to further lytic action by the same phage. The presence of lysogenic strains could cause confusion in the study of host specificity.

The results of tests for lysogeny in this study were all

negative, indicating that none of the strains used in this investigation released phage particles freely in the culture. No attempts were made to induce lysogeny. Zajic (35) and Chen (10) also were not able to detect lysogeny among strains of A. tumefaciens used in their investigations. This suggests that although lysogeny may occur among strains of A. tumefaciens, it is not very common.

Phage Isolation

In this investigation, phages of A. tumefaciens were obtained from sewage, soil and crown gall tissues. Coon and Kotila (12) isolated A. tumefaciens phages from soil in 1925, but since that time no reports have been made of successful isolation of these phages from soil. Zajic (35) and Chen (10) in Wisconsin reported failures to obtain A. tumefaciens phages from soil though they made many attempts.

The percentage of success in isolating phage from soil in this investigation was very low. One hundred and twenty-five soil samples were tested using ten strains of A. tumefaciens, but only seven phage isolates were collected. Four of the phages were obtained from soil in the same cherry orchard. Two isolates came from corn fields and one from a vegetable garden.

Phages could not be isolated from many samples of soil from areas where A. tumefaciens was known to be present, and they were

isolated from corn fields where the bacterium would not likely be present. Lack of correlation between presence of phage and probable presence of A. tumefaciens may suggest some relationship between the phage and other soil borne bacteria. The reports by Coon and Kotila (12) and Brown and Quirk (9) that phages were isolated from rotted plant tissues which probably did not contain A. tumefaciens may also support this idea.

Phages were isolated from crown gall tissues by Israilsky (17), Muncie and Patel (25), Chester (11) and Kent (19). Zajic (35) and Chen (10), however, were not able to isolate phage from galls of tomato and rose. In this investigation, four phage isolates were obtained from 40 gall samples using 10 indicator strains of the bacterium. This meager success in obtaining phages from diseased tissue might have been expected since the plants were infected by bacteria from the soil, and the phages appear to be scarce in the soil.

The most successful source for phage isolation in this study was sewage. Twenty phage isolates were obtained from only ten samples of raw sewage. Zajic (35) and Chen (10) also found the phages to be abundant in raw sewage. Most of the bacteria in raw sewage come from the intestinal tracts of man, and one would not expect to find crown gall bacteria present. However, this point has not been thoroughly investigated. Several investigators have indicated a high specificity of these phages to Agrobacterium species,

but prevalence of the phages in sewage suggests either a wider host range or presence of Agrobacterium species in sewage. In this connection, a recent report by Ark (3) of a serological relationship between Agrobacterium species and several species of Escherichia is very interesting.

The plaques of all phage isolates were clear and round with a definite edge. The size of the plaques produced by the same phage isolate varied even after single plaque isolations were repeated several times. The average size of plaques for each phage isolate is indicated in Table 2. Plaque size does not appear to be a stable characteristic as it varied depending on the host bacterium. However, certain tests in which the host bacterium was the same indicated that the phage isolates could be divided into four groups on the basis of plaque size as follows: very small plaques, 0.25-0.50 mm in diameter, represented by a single isolate, SW 14; small plaques, 1.0-2.0 mm and 1.5-2.5 mm in diameter, represented by SL 1, SL 2, SL 3, SL 7, GP 1, GP 2, GP 4, SW 1, SW 2, SW 3, SW 4, SW 5, SW 6, SW 7, SW 8, SW 9, SW 10, SW 11, SW 15, SW 16, SW 17, SW 18, SW 19, SW 20; medium plaque size, 2.5-3.5 mm in diameter, represented by a single isolate, SW 13; and large plaque size, 3.5-5.0 mm in diameter, represented by SL 4, SL 5, SL 6, GP 3, and SW 12. There was no correlation between plaque size and source of the collection.

Host Specificity of Phages of A. tumefaciens

Host range pattern is a valuable characteristic for identifying individual phage strains. Israilsky (17) reported that his phage was able to lyse three of nine strains of A. tumefaciens tested while Muncie and Patel (26) reported their phage isolate lysed only the bacterial culture from which the phage was obtained. The phage isolates used by Zajic (35) and Chen (10) were able to react with more than the host bacteria from which they were obtained. In this investigation, each phage isolate lysed from 2 to 14 strains of the 41 cultures of A. tumefaciens tested, and 33 of the 41 bacterial strains were susceptible to one or more phage isolates.

Among 31 isolates of phage, there appeared to be 26 different strains based on host range. In four cases the host range was exactly the same for two or three isolates. Phages GP 1 and GP 2 were both obtained from galls on cherry trees using strain 5-22 of the bacterium. They lysed the same ten strains of A. tumefaciens. Phages SW 9 and SW 10 were obtained from sewage using strain PB 2, and they lysed the same seven strains. Phages SW 15, SW 16 and SW 18 were isolated from sewage using strain Eu-4. They lysed the same 14 strains of A. tumefaciens. Phages SW 3 and SW 4 were isolated from sewage using strain 5-22, and they lysed the same four strains of A. tumefaciens. These groups of phages had the same host range

and plaque size, they were obtained from the same source using the same bacterial host, and they belonged to the same serological group. Therefore, they probably represent the same strain of phage.

There seems to be little relationship between host specificity and source of phages. Phages from soil and sewage were quite variable in host range. Phages from gall tissue seemed to be related in host range, but this means little since only four isolates of phage from galls were obtained and studied.

The plaque size of the phage isolates does not correlate with host specificity as several phage isolates show the same plaque sizes but are entirely different in host range.

The phages isolated and tested in this investigation were very specific in host range. They lysed only strains of A. tumefaciens. Negative results were obtained when tests were made on one or more strains of the following bacterial species. Agrobacterium radiobacter, A. rhizogenes, A. rubi, Rhizobium meliloti, R. phaseoli, R. leguminosarum, R. trifolii, Erwinia amylovora, E. aroideae, E. ananas, Pseudomonas savastonoi, Ps. tabaci, Ps. morsprunorum, Ps. alliicola, Ps. solanacearum, Xanthomonas malvacearum, X. phaseoli, X. juglandis and X. campestris.

Chen (10) reported that the phage isolates used in his investigation lysed some strains of A. radiobacter, and Roslycky, Allen and McCoy (29) reported lysis of A. tumefaciens strains with

A. radiobacter phages. Lack of success in obtaining lysis of other species of Agrobacterium with the phages used in this investigation may have been due to use of only a few strains of other species of Agrobacterium.

Additional work should be done on the host range of A. tumefaciens phages within the family Rhizobiaceae, but considering the prevalence of these phages in sewage, tests of host range among bacteria from this source would be even more profitable.

Serological Studies of Phages of A. tumefaciens

Antisera produced from 31 different phage isolates were tested against each phage by the Ouchterlony agar double-diffusion method. The phage isolates could be divided into five groups according to the serological reaction.

Group 1 is composed of 19 phage isolates: SL 1, SL 2, SL 3, SL 4, SL 5, SL 6, GP 1, GP 2, GP 4, SW 1, SW 2, SW 3, SW 4, SW 5, SW 6, SW 7, SW 8, SW 12 and SW 13. Each of these was isolated using one of three strains of A. tumefaciens, 5-14, 5-22 and PS-1. Group 2 contains three phage isolates obtained from strain PB-2 of A. tumefaciens. They are SW 9, SW 10 and SW 11. Group 3 contains six phage isolates all of which were enriched by strain Eu-4 of A. tumefaciens. They are SW 15, SW 16, SW 17, SW 18, SW 19 and SW 20. Group 4 is composed of two phage isolates,

SL 7 and GP 3. They were obtained from strain 4-21 of A. tumefaciens. Group 5 contains a single phage isolate, SW 14. It was obtained from strain C-58 of A. tumefaciens.

The overlapping reactions which occur among these groups are shown in Tables 5 and 6.

When phages in group 1 were used as antigens, the antibodies produced reacted with phages in group 1 only, but when phages in group 2 were used as antigens, their antibodies reacted with phages in groups 1 and 5 as well as group 2. Antibodies produced from phages in group 3 reacted with phages in groups 4 and 5 as well as group 3. Antibodies produced from phages in group 4 reacted with phages in group 3 as well as group 4. Antibodies produced from the phage in group 5 reacted with phages in groups 2 and 3 as well as the homologous phage. These reactions are shown in the following diagram in which the dark block represents the homologous reaction and the striped ones show reactions with other groups.

Group of phage isolate	Antisera induced by phages in group					Type of antigens present
	1	2	3	4	5	
1						AB
2						BE
3						CDE
4						CD
5						BCE

Assuming that one type of antigen is present and induces the production of an antibody for the homologous reaction in each group, there should be a minimum of five different types of antigens. Phages in group 1 have antigen A which stimulates production of antibody As. The phages in groups 2, 3, 4 and 5 have antigens B, C, D and E which induce production of antibodies Bs, Cs, Ds, and Es, respectively. The overlapping reactions between different groups of phages as shown in the above diagram suggests that each phage has more than one kind of antigen.

Since phages in group 1 react with antibodies induced by phages in group 2, phages in group 1 must have at least two kinds of antigens, A and B. However, no antibodies were induced by phages in group 1 which will react with phages in group 2. This suggests that antigen B in group 1 failed to stimulate production of antibody Bs or that antigen B is a complex only part of which is present in phages group 1.

The antibodies produced by phages in group 2 can react with phages in groups 1, 2 and 5. Phages in this group contain at least two antigens, B and E. They do not have antigen A since they do not react with antibodies produced from phages in group 1. Antigen B stimulates the production of antibody Bs which not only reacts with the homologous phage but also with phages in group 1. Since antibodies from phages in group 2 can react with phages in group 5 and vice versa, phages in group 2 must have the homologous type antigen

present in group 5, that is, antigen E. By the same explanation, phages in group 3 have antigens C, D and E; group 4, C and D; and group 5, B, C, and E.

Therefore, 31 phage isolates can be divided into five groups according to the types of antigens present and the serological reactions.

Group 1 is composed of 19 phage isolates which contain antigens A, B

" 2 " 3 " " B, E

" 3 " 6 " " C, D, E

" 4 " 2 " " C, D

" 5 " 1 " " B, C, E.

When these five groups are viewed individually, there appear to be some groups which are serologically entirely different from others. For example, group 1 does not appear to be at all related to groups 3 or 4. Other groups such as 3 and 4 and 2 and 5 appear to be very closely related serologically. When all five groups are considered, however, they all appear to be interrelated. Group 1 is related to group 2, group 2 is related to group 5. Groups 3 and 4 are related to each other, and group 3 is related to group 5.

There appears to be a definite relationship between serological group and the strain of host bacterium used in isolation and reproduction of the phage. Groups 2, 3, 4 and 5 were each isolated using a single strain of bacterium, and although three bacteria, 5-14, 5-22

and PS-1, were used in isolating phages in group 1, these bacteria are related. PS-1 was isolated from a gall on a Bryophyllum plant which had been inoculated with strain 5-14, and 5-14 and 5-22 are virulent and avirulent strains of A. tumefaciens obtained from the same cherry gall. Perhaps the host bacterium somehow influences the types of antigens produced by the phage or perhaps there is an association in the genetic make-up of the phage between the factors of virulence to a certain host and serological grouping.

Pathogenicity of the host bacterium appears to have no effect on serological properties of the phages obtained from them since in group 1 two of the host bacteria were virulent and one was avirulent. Source of phage also shows no correlation with the serological reaction because phages from different sources belong to the same serological group as in group 1 or group 4. Plaque size of phage isolates does not appear to be correlated with serological properties. Phages in group 1 have average plaque sizes which vary from 1.0-2.0 to 3.5-5.0 mm in diameter.

Serological relations seem to be correlated with host specificity in groups 2 and 3. Each of these groups seems to have a common host range pattern. But when phages in group 1 are considered, there is no apparent relationship between host range and serological properties. In fact, two of the phages in this group, SL 1 and SW 2, have an entirely different host range. Phage GP 3 is very similar in

host pattern to phages GP 1 and GP 2 which belong to a different serological group, but the host range of GP 3 is very different from that of phage SL 7 which belongs to the same serological group. Therefore, there appears to be no consistent relationship between host range and serological properties.

Electron Microscope Study of Phages of A. tumefaciens

No reports have been made on the morphology of phages of A. tumefaciens. Roslycky, Allen and McCoy (30) studied phages of A. radiobacter and reported the particles to be almost round, approximately 60 mμ in diameter, with short, blunt tails.

In the present investigation seven phages were studied, three of them came from sewage, three from soil and one from gall tissue. These phage isolates were selected in order to compare phages from different sources as to general morphology.

All phage isolates studied were similar in size and shape though there was some variation. The heads were polyhedral, 42 mμ in width and from 80 to 92 mμ in length. The tails were 8 mμ in diameter and 90 to 125 mμ in length. The apparent variation in tail length may have been due to inaccuracies in making the measurements, but the variation in head length appears to be real. However, there was no noticeable correlation between size of particles and other factors such as source of phage, plaque size, host range or

serological group.

Morphology of the A. tumefaciens phages is quite different from morphology of A. radiobacter phages which are almost round and have a blunt tail. The A. tumefaciens phages are similar in morphology to Staphylococcus phage 6 as shown by Bradley and Kay (8). The shape and size of head of these two phages are about the same, but the tail of Staphylococcus phage is much longer with a knob at the end. It is possible, however, that the tails of the A. tumefaciens phages were broken off during high speed centrifugation. The similarity between these phages is quite interesting because Staphylococcus is a group of bacteria usually found in sewage, and A. tumefaciens phages appear to be quite prevalent in sewage. Further studies should be made to determine whether there is a relationship between these phages.

The almost round particles shown in picture 8 are believed to be the disintegrated form of phage SL 6, which is shown in picture 2. These two pictures were obtained using the same phage suspension except that in picture 2 the phage particles are seen soon after the preparation of the suspension while in picture 8 the phage particles are seen on the fifth day. These particles are particularly interesting because the shape is similar to that reported for phages of A. radiobacter.

Several characteristics of the phage isolates studied by electron micrography are summarized in Table 8.

Table 8. Some characteristics of phages of A. tumefaciens.

Phage	Source	Plaque Size mm	Particle Morphology Head mμ	Particle Morphology Tail mμ	No. of Host Range	Serological Reaction	Host Bacterium	Pathogenicity of Host Bacterium
SL 4	Soil	3.5-5.0	42 x 80	8 x 90	10	Group 1	PS-1	Virulent
SL 6	"	3.5-5.0	42 x 92	8 x 104	5	" 1	PS-1	"
SL 7	"	1.0-2.0	42 x 90	8 x 125	8	" 4	4-21	Non-virulent
SW 7	Sewage	1.0-1.5	42 x 90	8 x 108	9	" 1	5-14	Virulent
SW 12	"	4.0-5.0	42 x 84	8 x 110	3	" 3	PS-1	"
SW 14	"	0.25-0.50	42 x 84	8 x 125	4	" 5	C-58	"
GP 1	Gall	1.5-2.0	42 x 80	8 x 90	10	" 1	5-22	Non-virulent

SUMMARY

Thirty one phages of A. tumefaciens were isolated from sewage, soil and gall tissues of infected plants. Although the phages were present in all three sources, they were much more prevalent in raw sewage. The host specificity, serology and electron microscopy of these phages were studied.

Plaques of all phages were clear and round with a definite edge. Average plaque size for most phages was 1.0-2.0 or 1.5-2.5 mm in diameter, one phage isolate produced plaques which were 0.25-0.50 mm in diameter, and several produced large plaques, 3.5-5.0 mm in diameter. Size of plaques did not correlate with source of phage isolation as there was variation among phages from the same source as well as phages from different sources.

Forty-one strains of A. tumefaciens obtained from different sources were tested for lysogenicity by spontaneous production. None of these strains was lysogenic.

In addition to 41 strains of A. tumefaciens, one or more strains of a number of bacteria which are associated with plants were tested for host specificity of the phages. They were Agrobacterium radiobacter, A. rubi, A. rhizogenes, Erwinia aroideae, E. ananas, E. amylovora, Xanthomonas malvacearum, X. phaseoli, X. juglandis, X. campestris, Pseudomonas savastonoi,

Ps. tabaci, Ps. morsprunorum, Ps. alliicola, Ps. solanacearum, Rhizobium leguminosarum, R. meliloti, R. phaseoli, and R. trifolii. Phages of A. tumefaciens were very specific in host range as they lysed only strains of A. tumefaciens. The host range of most phage isolates within A. tumefaciens was quite different. There were four cases where two or more phages showed the same host range, but they were also identical in all other characteristics studied, and they probably represent the same strain of phage in each case. The source of isolation and plaque morphology of phages were not correlated with host range.

The 31 phage isolates were divided into five different serological groups, but there appeared to be interrelationships among all five groups. The serological differences seemed to be correlated with the host bacterium used in reproduction of the phage. Source of isolation and plaque size of phages did not correlate with serological group. The serological relatedness appeared to be correlated with host range of phages in some cases but not in the others.

Seven phages were observed with the electron microscope. The particles of these phages were similar in shape but slightly different in size. The heads were polyhedral, 42 mμ in width and from 80-92 mμ in length. The tails were 8 mμ in width and from 90-125 mμ in length. The difference in size was not correlated with sources of isolation, plaque size, or serological reaction.

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