CHARACTERISTICS OF A BACTERIOPHAGE ISOLATED FOR *PSEUDOMONAS TABACI*

A Thesis

Presented to

The Faculty of the School of Sciences and Mathematics

Morehead State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science in Biology

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by

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July 1971

ABSTRACT OF THESIS

CHARACTERISTICS OF A BACTERIOPHAGE ISOLATED FOR PSEUDOMONAS TABACI

This study was undertaken to determine some of the basic properties of a Pseudomonas tabaci bacteriophage which was isolated in order that further study may indicate its potential as a biological pesticide. Phage suspensions were isolated from sewage filtrates and the soft-agar layer method of plaque assay was used to determine the number of infective bacteriophages in the various suspensions. The plaques of freshly-isolated phages possessed clear centers surrounded by a narrow, granular halo and ranged in diameter from 0.5 mm to 1.5 mm. The host range of this phage was found to include Pseudomonas putida but not a second strain of P. tabaci. The one-step growth curve lasted about 67 min and showed a burst size of 24 phages per cell. Ultraviolet light caused a 62% inactivation within 1 min, although 5% remained viable after 6 min of exposure. The thermal death point was found to be 90 C. Phage remained viable after storage in dried preparations for 5 wk but not 6 wk. Phages were completely inactivated when held for 1 hr at pH 4.0 and pH 11.0 but remained partially infective from pH 5.0 to pH 10.0. Phage particles were stable for longer periods between pH 6.8 and pH 7.0. The organic solvents, chloroform and ether, were not shown to exert a significant effect on the infectivity of the phage. Quantities of tap water up to 1 liter were not sufficient to remove phage particles, naturally occurring or artificially introduced, from the top 3 cm of a column of loam soil.

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7/19/71
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Accepted by the faculty of the School of Sciences and Mathematics, Morehead State University, in partial fulfillment of the requirements for the Master of Science degree.

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ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr. Daryl S. Bates, for his encouragement and patience throughout this research. I am grateful to Larry Crewes and Paul Love who obtained the loam soil used in experimentation and to Dr. Paul Cain for his assistance in analyzing that soil. For the assistantship that made this research possible, I am indebted to Morehead State University.

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INTRODUCTION

Various attempts have been made to use bacteriophages as control agents for pathogenic bacteria (40). Partially successful experiments involving treatment with phages have been reported with Agrobacter tumifaciens (40), Erwinia carotovora (40), Pseudomonas solanacearum (40), Pseudomonas tabaci (25), and Xanthomonas stewartii (40). The growing concern over chemical pesticides that often destroy far more than the pests for which they are intended, emphasizes the need for the development of pesticides with a high degree of specificity. Such specificities are properties that have been found to be characteristic of many of the bacteriophages.

The system chosen for this study, which could serve as a basis for the work mentioned above, consists of a virulent strain of Pseudomonas tabaci, the causative agent of wildfire in tobacco, and a phage for that bacterium which was isolated from municipal sewage filtrates. This phage was isolated as a part of this study and was subsequently characterized regarding plaque morphology, host range, growth cycle, and stability in the presence of various physical and chemical agents and physiochemical factors.

The purpose of this study was to determine some of the basic properties of this phage in order that further study may indicate the potential of this phage as a biological pesticide.

LITERATURE REVIEW

Bacterial viruses were first isolated by Twort (10, 45) in 1915. Two years later, d'Herelle (27) independently isolated viruses of dysentery bacilli and named them bacteriophages. Early research concerning these viruses was primarily directed toward therapeutic aspects (10). More fundamental research began about 1940 when Ellis and Delbruck (22) developed a method of studying the viral growth cycle in order to further study the conclusions of d'Herelle's experiments. Since then, bacteriophages have been studied extensively and much information has been gathered concerning the structure and reproductive mechanisms of certain types.

Characterization of a newly-isolated phage frequently involves determination of structure by means of electronmicrographs, experimentation to find the manner of replication, observation of plaque morphology, and determination of the extent of the host range. Studies of these characteristic properties involve the choice of appropriate isolation and purification procedures and assay methods. Additional characterization pertaining to the effect of various physical and chemical agents on extracellular viruses and to methods of storage is necessary for various reasons.

Structure

The basic structure of all bacteriophages has been found to consist of either a single- or double-stranded nucleic acid core

surrounded by a protein coat (10, 26, 36). Special terminology, originally developed by Lwoff et al. (36), has been adopted for the morphological units. Accordingly, the protein coat (capsid) is composed of identical subunits (26) (capsomeres) and the complete infective particle is known as a virion. Six basic morphological types of bacteriophages have been described (10, 26). Of the five types possessing hexagonal heads, the first has a tail that is longer than the head and is associated with a sheath. The second type of phage has a long tail without a sheath, and the third has a non-contractile tail shorter than the head. The fourth type lacks a tail but has enlarged apical capsomeres, and the fifth lacks a tail as well as conspicuous capsomeres. The sixth morphological type consists of a long, flexible filament.

The type of nucleic acid has not been found to vary within the morphological types (26), e.g. the phages possessing hexagonal heads, long tails, and contractile sheaths all contain double-stranded DNA (10).

Replication

The mechanism of replication has been studied in considerable detail for the T-group of coliphages (26). The process was divided into the following four stages by d'Herelle (1): adsorption, penetration of the phage particle into the host cell, intracellular synthesis and assembly of viral parts, and release of progeny upon lysis of the host cell. The first of these stages involves the

adsorption of the phage particles to the host cell wall for the majority of known phages although some phages have been shown to infect via the pili (10). The second stage for phages that become irreversibly adsorbed to the cell wall, was found to be the penetration of the phage nucleic acid into the cell. Hershey and Chase (30) used radioactive T2-coliphages to obtain evidence that it was the nucleic acid alone which penetrated the bacterium. During the third stage of replication, the phage nucleic acid has been shown to direct the synthesis of viral components by the host cell (26). The final stage for the phages studied includes the assembly and accumulation of phage particles within the host cell. After maturation and assembly, the progeny phage are released by lysis of cells. The host cell lysis in the case of the T-group of coliphages was shown to result from the action of a phage-directed enzyme which alters the permeability of the cell membrane so that excess water enters and the cell bursts (26).

Plaque Morphology

The clear areas observed in agar plates containing sensitive host bacteria were named plaques (1). The size and morphology of plaques have been shown to be characteristic features of certain phage-bacterium complexes. For example, the plaques produced by different phages isolated for a strain of P. tabaci (25) and by mutants of a phage isolated for a strain of P. aeruginosa (7) varied in turbidity from clear to opaque, in diameter (from 0.5 mm

to 1.5 mm for the P. tabaci phages), and in the size of a halo, if present (7, 26). Bartell and Orr (5, 6, 7) suggested that the halo produced by P. aeruginosa phages is the result of the diffusion of a phage-directed polysaccharide depolymerase.

Host Specificity

The specificity exhibited by phages for certain strains of bacteria has been shown to depend upon the presence of specific receptor sites on the cell walls of the sensitive bacteria (26). Some types of phages are highly specific and have been shown to attack only strains of a single species, while other types are known to infect bacteria belonging to different genera (1, 24). The host range has been useful in the identification of phage strains, for example in the separation of the closely-related strains T2, T4, and T6 (1). The characteristic range of host species has been observed to vary, however, due to phage mutation or changes in the phage susceptibility of the indicator bacteria (1). A practical application of the host specificity of phages is known as phage typing. The procedure involves the use of phages highly adapted to single hosts to help distinguish nearly identical bacteria and has been useful in medical diagnosis and in industry (24).

Isolation and Purification

Bacteriophages are fairly common in nature and have been isolated from various sources including sewage, soil, and infected

tissue. Phages of certain of the enteric bacteria have been isolated from sewage (10, 24), by mixing the host bacteria with municipal sewage filtrates, incubating appropriately, and filtering the fluid after centrifugation. Fulton (25) in 1950 isolated a phage of P. tabaci from macerated infected tobacco leaf tissue. In 1958, phages for a species of the genus Pseudomonas were isolated from soil in which the host organism was found (17).

Purification of a phage type after isolation commonly involves plaque purification procedures (1, 10) which improve the probable homogeneity of the phage suspension. Further purification has consisted of alternating cycles of low and high-speed centrifugation (11) and rate zonal centrifugation when the preparation of large volume lysates were desired (4, 18).

Assay Methods

Following isolation procedures, the number of phage particles present in a given suspension has been estimated with the use of the electron microscope (26). Various studies have shown, however, that every phage particle is not infective (26). The titer of infective phages in a suspension was determined in 1917 by d'Herelle (27). This plaque titration procedure involved the spreading of a mixture of an appropriate dilution of a phage suspension and a suspension of susceptible bacteria over the surface of an agar plate. The number of plaques observed following incubation indicated the number of virulent phage particles on the plate since single phage particles

have been shown to produce plaques (1).

Effects of Physical and Chemical Agents and Physiochemical Factors

The effects of various agents have been shown to be characteristic for certain phages. One of these effects is the inactivation of viruses which has been indicated by the disappearance of various biological activities (13). Loss of infectivity, one of the most important and most sensitive of the biological activities, has been detected by standard plaque assays following exposure to an agent (26). In this review, the inactivating treatments and agents are divided into the following categories: physical agents, physiochemical factors, and chemical agents.

Physical Agents

The physical agents of inactivation include mechanical treatments and ionizing and nonionizing radiations. Whereas the observed results of mechanical treatments such as sonic irradiation and surface inactivation have been fragmentation (13), ejection of the nucleic acid (13), and protein denaturation (13); the primary target for radiation inactivation by x-rays, ultraviolet rays (UV), and radioactive phosphorus has been demonstrated to be the nucleic acid (13).

Sonic Irradiation. Sonic inactivation has been shown to be the result of rapid shifts in pressure created as sound waves pass through a liquid. The formation and subsequent collapse of gas bubbles during the pressure changes produce shearing forces. Oster (13)

found that these forces cause the fracture of the rod-shaped tobacco mosaic virus (TMV), and the ejection of the nucleic acid from the T-group of phages was the result of such forces observed by Anderson et al. in 1948 (13). The degree of sensitivity to and the type of reaction occurring after sonic irradiation have been found to be characteristic for some of the animal viruses and for the coliphages (13).

Surface Inactivation. Protein is often denatured by the surface tension at liquid/gas or liquid/liquid interfaces. This reaction has been suggested to be the cause of the gradual loss of infectivity observed by Campbell-Renton (13) for phage particles in a suspension through which air was bubbled. This treatment similarly effected a few of the animal viruses, including the influenza virus (13). Differences in the rate of inactivation and in the degree of resistance to inactivation have been observed with members of the T-group of coliphages (13).

Ionizing and Nonionizing Radiations. Inactivation by various types of radiation usually proceeds exponentially (13, 26). This implies that a biological unit, such as a virus particle, contains a sensitive volume within which a single-hit (e.g. an ionization or the absorption of a quantity of UV) can inactivate the unit (13, 26). X-rays have inactivated viral particles indirectly by causing the production of toxins, and directly by a single-hit process (26). UV and radioactive phosphorus have exhibited only direct inactivation of viruses by means of a single-hit process (13).

X-rays

Absorption of energy from x-rays by a liquid medium causes the formation of peroxides, free radicals, and other materials which have been demonstrated to be toxic to certain viruses (26). The site of direct x-ray inactivation was reported to be the nucleic acid by Alexander and Stacey in 1956 (13) on the basis of physical measurements of irradiated nucleic acids. Further experimentation demonstrated that x-irradiation caused scission of one or both strands of a molecule of nucleic acid (26). The linear decrease in the titer of a virus suspension exposed to the direct effects of x-irradiation was taken to indicate a single-hit process. The rate of decrease has been found to be a characteristic property of many of the animal viruses and several of the bacteriophages (13, 26).

Ultraviolet light

In contrast to x-rays, UV has inactivated various viruses by direct effects only. Thus far, inactivation has been characteristically exponential for viruses exposed to increasing dosages (13, 26). However, at low survival values, the presence of a few relatively resistant particles have caused a decrease in the rate of inactivation (13). The rate of UV inactivation is usually constant and is a characteristic property of a virus (13). The most efficient inactivation of virus particles has been found to occur at about 2600 Å (13). This wavelength coincides with the absorption maximum for nucleic acids or nucleoproteins and indicates that it is the nucleic acid which determines the sensitivity of a virion to UV (13, 26). Irradiation

of nucleic acids at this wavelength has been observed to cause the formation of photoproducts (nucleotide dimers) which may interfere with replication (26). In visible light, this inactivation has been reversed by a mechanism known as photoreactivation, a process occurring in infected cells which contain an enzyme capable of splitting the nucleotide dimers (26).

Radioactive phosphorus

Inactivation of virus particles due to the incorporation of radioactive phosphorus (P^{32}) has been found to be similar to inactivation by x-rays and UV in that the release of considerable energy during the decay process has been associated with damage to the nucleic acid. The energy released during the decay of a P^{32} atom and its transmutation to an atom of S^{32} are known to be sufficient to cause scission of the strand containing the new sulfur atom (26). The first experiments with P^{32}, carried out with phage T2 by Hershey et al. (30) demonstrated that inactivation is strictly exponential and that only one out of 10 P^{32} disintigrations results in inactivation. Experiments have indicated that the efficiency of P^{32} inactivation is lower because scission of both strands and subsequent loss of infectivity occur in only 10% of the disintigrations (26). Viruses exposed to the effects of P^{32} have exhibited various degrees of resistance (13).

Physiochemical Factors

Study of the effects of physical agents has provided information

on the structure and function of virus particles (1). Studies concerning the stability of virus particles under the influence of physiochemical factors including high and low temperatures, dessication, changes in pH, and osmotic shock, have provided additional information which has been helpful in obtaining optimum laboratory production of virus and in minimizing losses in titer.

Heat Inactivation. Temperature sensitivity is a characteristic property of viruses. The rate of inactivation and the thermal death point (16, 24, 42) (the temperature at which all organisms within a given suspension are killed or inactivated within 10 min) of many viruses have been found to vary greatly and determination of these properties is necessary for effective sterilization procedures. Studies of thermal inactivation have lead to the conclusion that when certain sites on a virion receive energy above a critical threshold, they are altered in such a way that the virion is inactivated resulting in observed losses of titer (26). This heat-inactivation has indicated that these sites are equivalent to viral proteins or to capsomeres (26).

Freezing. In contrast to the high temperatures used for sterilization, low temperatures are often used to minimize losses of virus during storage although the susceptibility of several viruses to freezing has been found to vary. Studies of animal cells and bacteria have indicated that damage caused by freezing and thawing results either from high salt concentrations created as the water is crystallized

out, or from enzymatic activities which may continue if the storage temperature is not sufficiently low (13). Inactivation during storage has been prevented by storing at temperatures below -40 C (13).

Dessication. Dried biological preparations have been shown to resemble frozen ones in that both are dessicated (13). Specimens which have been stored at room temperature when effective drying was tolerated, had to be kept at low temperatures when drying was less complete (13). Work done by Lea in 1947 (13) showed that many viruses can tolerate drying in thin films of suspensions containing a protective agent such as broth. Variations in tolerance to dessication have been reported and observation of this property is important for the long-term maintenance of many specimens.

Effects of pH. Virus stability has been noted to be influenced by pH changes. Phages are usually stable within a pH range from 5.0 to 8.0 (1). The range of tolerance, a characteristic sometimes used to differentiate related phages (25), is often more extended. For example, Fulton (25) isolated a phage of P. tabaci that was stable in nutrient broth for at least 1 hr between pH 4.0 and pH 10.5.

Osmotic Shock. Anderson demonstrated the inactivation of T-even phages by osmotic shock to be the result of the separation of the phage DNA from the protein coat and tail (13). Under favorable conditions, 98-99% of these phage particles were inactivated. The degree of sensitivity was not found to be identical for all of the T-even coliphages.

Chemical Agents

A wide variety of chemical agents has been shown to be capable of inactivating viruses. These include protein-denaturing agents such as urea; oxidizing agents which cause dehydrogenation, deamination, or the opening of ring structures that may result in denaturation; and various lytic enzymes (13). Of these chemical agents, the effects of formaldehyde and the organic solvents are frequently studied.

Formaldehyde. Formaldehyde has been found to react primarily with viral protein and therefore has been useful in the production of vaccines (26). Inactivation by this agent has been found to proceed exponentially at first followed by a decreasing rate as exposure to the chemical continues (13). The rate of inactivation has not been found to be consistent for all viruses tested.

Organic Solvents. Most organic solvents denature proteins, particularly at elevated temperatures (13). Those solvents which act on lipids are of primary interest. Many viruses, including the majority of the phages tested, were found to be resistant to the effects of organic solvents such as ether and chloroform. Chloroform has been used to eliminate bacteria from phage stocks (1, 6, 15, 17) and to destroy the host bacteria in growth curve experiments so that only the unadsorbed phage remain (22). Other viruses including certain animal viruses have not exhibited resistance to ether (26). Electron microscope studies of the effect of these agents upon susceptible viruses have indicated a disruption of the lipid-containing

membrane external to the protein coat (26).

The Host Bacterium

Pseudomonas tabaci, the causative agent of wildfire in tobacco (Nicotiana tabacum) (11), was the host bacterium employed in this study. The genus to which this organism belongs contains organisms that are among the most common and widely distributed of the bacteria. The various members occur mainly as scavengers in sewage, in marine waters, heavy brines, fresh waters, in the soil, and in decomposing organic matter. Members of this species are characteristically short to medium, gram-negative, non-spore-forming rods that are motile by means of polar flagella (11).

P. tabaci, found to exist in soils and to be transported by water to tobacco leaves, has been observed to penetrate plants through stomata or through wounds and therein cause infections manifested by the development of necrotic lesions (2). Studies have indicated that these lesions result either from enzyme (bacterial) induced tissue maceration or from bacterial toxins (2).

Bacteriophages of Pseudomonas

There is extensive literature on phages attacking the members of the genus Pseudomonas. Until recently, this work was confined to Pseudomonas aeruginosa and the plant pathogens but phages for Pseudomonas fluorescens and Pseudomonas putida have been isolated and studied (31). Phages have been isolated from lysogenic bacterial

strains, from sewage, or from soil. All known morphological types of phages have been isolated for the genus except tailless phages with large capsomeres (31). In general, the host range seems to be restricted to a single species although some phages have been observed to infect a range of species. Host ranges of phages isolated from natural environments sometimes depend on the species used for isolation; in one study, those isolated from a sewage sample on P. aeruginosa plated only on that species, but others isolated on P. putida and P. fluorescens not only plated on those two species but also on P. aeruginosa and some of the soil pseudomonads (31).

Studies of phages attacking saprophytic or phytopathogenic Pseudomonas species have been primarily concerned with phage-typing of the host (41). Novikova (38, 39) in Russia and Fulton (25) studied, with limited success, phages isolated for the wildfire organism P. tabaci, as a possible means of biocontrol for field diseases. Characterization of phages for members of the genus Pseudomonas with few exceptions has consisted of electron micrographs and observations of plaque morphology. Fulton (1950) (25) further compared two phages isolated from wildfire lesions regarding thermal stability, pH tolerance, and host range, and in 1968, Olsen et al. did a comparative study of phages attacking psychrophilic and mesophilic pseudomonads (41). Extensive characterization of phages isolated for P. tabaci, as has been done for the T-group of coliphages, has otherwise been neglected.

MATERIALS AND METHODS

Bacterial strains.

Pseudomonas tabaci (ATCC 17914), obtained from the MSU culture collection, was the host bacterium used throughout this study. Additional cultures, used in the host range experiment, were *Escherichia coli* B (ATCC 11303), *Pseudomonas aeruginosa* (ATCC 15442), *Pseudomonas fluorescens* (Midwest Culture Service, Terre Haute, Indiana), *Pseudomonas putida* (ATCC 12633), *Pseudomonas septica* (ATCC 19878), and *Pseudomonas tabaci* (ATCC 11527). These cultures were also obtained from the culture collection maintained in this laboratory. All strains were maintained on BABNA slants and stored at 4 C.

Bacteriophages.

Bacteriophages active on *P. tabaci* (ATCC 17914) were isolated from sewage filtrates obtained from the Municipal Sewage Plant of Morehead, Kentucky. The filtrates were prepared by centrifugation of raw sewage in an International centrifuge (Model HT) at 10,000 rpm for 15 min and passed through a sintered glass filter. Bacteriophage suspensions were obtained by adding 1 ml of the filtrate to a 250 ml Erlenmeyer flask containing 30 ml of broth and 1 ml of an 18 hr broth culture of the host bacterium. Following incubation for 24 hr at 26 C, the contents of the flask were centrifuged and passed through a Millipore filter to yield a stock preparation of

bacteriophage free of bacterial cells. The phages were propagated by transfer of 1 ml of a stock suspension to a 250 ml Erlenmeyer flask containing 30 ml broth and 1 ml of an 18 hr broth culture of P. tabaci. This culture was incubated at 26 C for 24 hr, centrifuged and filtered as before to remove unlysed cells, and stored as a broth suspension at 4 C.

Media.

The medium (BABNA) used for the maintenance of bacterial strains consisted of 2% blood agar base (BBL), 0.25% peptone (Difco), 0.15% beef extract (BBL), and 1.55% agar (Difco). The broth medium used for growth of bacterial strains and for maintenance and propagation of the bacteriophage was composed of 1% dextrose (Difco), 1% peptone (Difco), and 0.3% beef extract (BBL).

Bottom agar was prepared from phage broth by the addition of 1.5% agar (Difco). Soft agar consisted of 7g of agar (Difco) per 1,000 ml distilled water. The base layer of agar plates consisted of approximately 20 ml of medium, and the soft-agar tubes contained 2.5 ml of medium.

Assay procedures.

Tests for the presence of bacteriophage in filtrates were made by spreading, with a sterile cotton swab, a small quantity of an 18-24 hr broth bacterial culture over the surface of an agar plate followed by the application of two loopsful of the suspension over

the central portion of the area covered by the culture. After incubation for 24 hr at 26 C, the presence of phage was indicated by a clear area on the agar surrounded by confluent bacterial growth.

The soft-agar layer method described by Adams (1) was used to determine the infective titer of bacteriophage suspensions. A 0.5 ml portion of a 26 C, 18-24 hr broth culture of P. tabaci transferred to a tube of melted soft agar served to form the bacterial lawn. Phage inoculations of the soft agar consisted of 0.1 ml samples of phage dilutions.

Host range.

To determine whether the phage isolated on P. tabaci (ATCC 17914) could attack other bacterial strains, portions of 24 hr broth cultures of E. coli B, P. aeruginosa, P. fluorescens, P. putida, P. septica, and P. tabaci (ATCC 11527) were spread over the surface of duplicate agar plates with cotton swabs. Two loopsful of the phage suspension were placed on the central portion of the area covered by each culture.

One-step growth curve.

Nine dilution blanks containing 2.7 ml of broth, the tube containing P. tabaci at a concentration of 1.2×10^7 cells/ml, and the tube containing a broth suspension of phage at a concentration of 1×10^6 infective particles (plaque-forming units - PFU) per ml were held at room temperature while 22 tubes of soft agar were melted and maintained in a 40 C water bath (Thelco, Model 83).

One ml of P. tabaci and 1 ml of the phage suspension were then transferred aseptically by means of 1 ml pipettes to a sterile tube labelled "adsorption" and 1 ml of phage was similarly transferred to a sterile tube labelled "input" which contained 1 ml of broth. The contents of both tubes were thoroughly mixed on a Super-Mixer (Lab-Line Instruments, Inc.). After a 20 min period allowed for adsorption, a 1 ml pipette was used to transfer 0.3 ml from the adsorption tube to a 2.7 ml dilution blank. The resultant mixture was used to prepare serial 10-fold dilutions from 10^{-1} to 10^{-5}. Two 0.1 ml samples withdrawn from the 10^{-5} dilution were placed in separate tubes of melted soft agar each of which had previously been inoculated with 0.5 ml of the host culture. The contents of each tube were mixed and poured over the surface of agar plates labelled 1. About the same time, 1 ml was removed from the adsorption tube and passed through a Millipore filter. Serial 10-fold dilutions were made from 10^{-1} to 10^{-5}. Two 0.1 ml samples were withdrawn and placed in tubes of melted soft agar previously inoculated with 0.5 ml of culture. The contents were mixed and poured over agar plates labelled "unadsorbed" phage.

Serial dilutions were made from 10^{-1} to 10^{-5} of a sample taken from the "input" tube. Two 0.1 ml samples were withdrawn from the 10^{-5} dilution and placed in melted agar as described above. The contents were mixed and poured over agar plates labelled "input" phage.

At 12 min and 17 min after the 20 min adsorption period, two 0.1 ml samples of the 10^{-5} dilution of the adsorption tube were transferred to tubes of melted agar previously inoculated with culture. The contents of each tube were mixed and poured over the surface of agar plates labelled 2 (12 min) and 3 (17 min).

At 37, 57, 67, and 77 min after the adsorption period, 0.3 ml from the 10^{-5} dilution of the adsorption tube was transferred to a 2.7 ml dilution blank to make a 10^{-6} dilution from which two 0.1 ml samples were transferred to tubes of melted agar as before. The contents of each tube were mixed and poured over the surface of agar plates labelled 4 (37 min), 5 (57 min), 6 (67 min), and 7 (77 min).

Serial 10-fold dilutions from 10^{-1} to 10^{-5} of the P. tabaci culture were prepared using the 2.7 ml dilution blanks. By means of a 1 ml pipette, two 0.1 ml samples were transferred to tubes of melted soft agar. The contents were mixed and poured over the surface of agar plates labelled "culture".

Ultraviolet inactivation.

Stock phage suspensions were diluted in 9.9 and 0.9 ml broth dilution blanks to prepare 10 ml at a 10^{-5} dilution. The 10 ml of the 10^{-5} dilution were transferred to the bottom half of a Petri dish and were exposed with shaking to the short wave (2537 Å) ultraviolet light (UV) that issued from a Mineralight ultraviolet lamp (UVSL-25, Ultra-Violet Products Inc.) placed 14 cm from the sample. Samples of 0.1 ml

were withdrawn at 1,2,3,4,5, and 6 min and assayed by the soft-agar layer method described above.

Thermal inactivation.

One-tenth ml samples from a stock phage suspension were transferred to 0.9 ml broth dilution blanks to make 10^{-1} dilutions that contained 2.0, 3.3, or 3.6 x 10^5 PFU/ml. Each tube was placed in a water bath for 10 min at different temperatures and then was rapidly cooled in an ice bath. The temperatures used ranged from 40 C to 90 C at 5 degree intervals. Triplicate samples were withdrawn from the cooled suspensions and assayed by the soft-agar layer method described above.

Storage at room temperature.

Two drops of a stock phage suspension containing 1.6 x 10^7 PFU/ml were placed on slides that had been presterilized in Petri dishes. In addition, 0.2 g loam soil was mixed with 10 ml of the same suspension and 2 drops of this suspension were similarly placed on presterilized slides. After the film had dried on all the slides at room temperature, the Petri dishes containing the slides were placed in a 26 C incubator. At weekly intervals, one slide of each type was removed and the dried suspension was washed with 5 ml of broth into a 250 ml Erlenmeyer flask containing 1 ml of host culture and 30 ml of broth. Care was taken to avoid contamination except possibly that from the air. Following incubation for 24 hr at 26 C, the contents of each flask were

centrifuged and the filtrates were passed through Millipore filters into sterile test tubes. Two loopsful of each filtrate obtained were spread over the central portions of duplicate plates which had been swabbed with P. tabaci.

Stability in solutions of various pH values.

One ml samples of stock phage suspensions were diluted to concentrations of 30.0 and 140.0 PFU/ml. The diluted phage suspensions were transferred aseptically to tubes containing 1 ml quantities of broth previously adjusted to pH 4, 5, and 6 (with HCl) and to pH 8, 9, 10, and 11 (with NaOH). The contents of each tube were mixed and all tubes were held for 1 hr at room temperature. Triplicate samples were withdrawn from each tube and assayed by the soft-agar layer method described previously. Survival values were derived from comparisons to the control (pH 7.0).

Stability in organic solvents.

The organic solvents chloroform (J. T. Baker Co.) and ethyl ether (J. T. Baker Co.) were supplied by the MSU chemistry department and were used to treat suspensions of the phage. One ml samples of diluted phage suspensions were transferred to tubes containing either 1 ml of ether or 1 ml of chloroform. The contents of each tube were mixed and held at room temperature for 40 min. Two layers were formed in each tube during the 40 min period; the chloroform layer appeared below the layer of phage suspension and the ether layer appeared above the phage suspension. A sterile,

disposable 1 cc syringe was used to transfer the phage-containing layers to sterile test tubes from which 0.1 ml samples were removed by 1 ml pipettes and assayed by the soft-agar layer method described previously.

Percolation through loam soil.

Glass columns composed of four (10 cm x 34 cm) glass plates were supported by wooden frames constructed to permit observation of the percolation of the P. tabaci phage down through 30 cm of loam soil. The central opening of the wooden frame over which a column was placed, was covered with wire mesh and 4 layers of cheesecloth to support the soil and allow passage of water into a beaker situated directly beneath the opening in the frame.

Columns of loosely packed loam soil, pH 5.2, received either 10 ml broth or 10 ml phage suspension at a concentration of 1 x 10^5 PFU/ml followed by 100 ml quantities of tap water up to 1 liter. Five minutes after each 100 ml portion had been added, a 1 g sample of soil was taken from the top 3 cm of soil and a 1 ml sample of the liquid which had passed through the column and into the beaker was withdrawn. The soil and liquid samples were placed in separate flasks containing 30 ml of broth and 1 ml P. tabaci. Following incubation, the contents of each flask were centrifuged and filtered. Filtrates were assayed for the presence of bacteriophage as described earlier.

(Agar plates from all experiments were incubated for 24 hr at 26 C, except those of P. aeruginosa which were incubated at 37 C.)

EXPERIMENTAL DATA

Isolation of Pt-17914 Phage

The medium employed during the first unseccessful attempts to isolate phage on P. tabaci (ATCC 17914) from sewage filtrates was tryptose phosphate broth (BBL), a medium used in this laboratory for the propagation of T1 on E. coli B. Phage was first isolated on P. tabaci from sewage in December, 1970 in a modification of the medium used by Fulton (25) to isolate phage on P. tabaci (see Materials and Methods). This phage will be hereafter referred to as Pt-17914 phage. Bacteriophages were not isolated for P. tabaci (ATCC 11527) in several attempts using similar procedures.

A method was described by Smith (44) for the isolation of virus from soil samples by shaking 10 g of soil in 12 ml of potassium phosphate buffer, pH 7.6. Phage was not recovered from loam soil samples (obtained in Rowan Co., Kentucky) using this method. However, when broth was added to a column of the loam soil (pH 5.2) and washed down with 1 liter of tap water, phage was isolated on P. tabaci (ATCC 17914).

Characterization of Pt-17914 Phage

Plaque morphology. Pt-17914 phage isolated from sewage formed

plaques ranging from 0.5 mm to 1.5 mm in diameter. The plaques possessed clear centers surrounded by narrow, granular halos (Fig. 1).

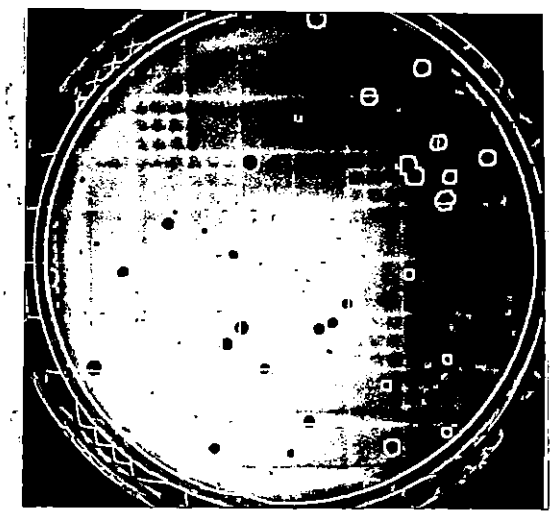

Fig. 1. Plaques formed by Pt-17914 phage.

Host range. The host range of Pt-17914 phage did not include any of the bacteria tested except for P. putida (Table 1). A second strain of P. tabaci (ATCC 11527) was also resistant.

One-step growth cycle. The one-step growth curve of Pt-17914 phage is presented in Fig. 2 and was constructed from the results contained in Table 2. Host cells from an 18 hr culture were infected with phage at a multiplicity of infection (MOI) of 0.57. The latent period extended for about 15 min and the log phase for 40 min with the burst finishing approximately 67 min after the

adsorption period. The burst size was 24 phages per cell (see Discussion for calculations).

Table 1. Host range of Pt-17914 phage.[a]

Host organism	Lysis
Escherichia coli	-[b]
Pseudomonas aeruginosa	-
Pseudomonas fluorescens	-
Pseudomonas putida	+
Pseudomonas tabaci (ATCC 11527)	-

a. Phage isolated on P. tabaci (ATCC 17914).
b. Symbols: + = lysis on duplicate agar plates;
- = no lysis.

Ultraviolet inactivation. The results of exposing 10 ml of broth containing phage at various concentrations to UV are listed in Table 3. The percentage of phage remaining infective (Fig. 3) was found to decrease sharply during the first minute of exposure and to continue decreasing until only 5% of the original suspension remained viable at the end of 6 min. Phage surviving the irradiation produced 3 types of plaques: plaques, 2 mm in diameter, with clear centers surrounded by narrow halos; plaques, 1 mm in diameter, with clear centers surrounded by moderate halos; and plaques, 0.5 mm in diameter, with clear centers surrounded by large halos.

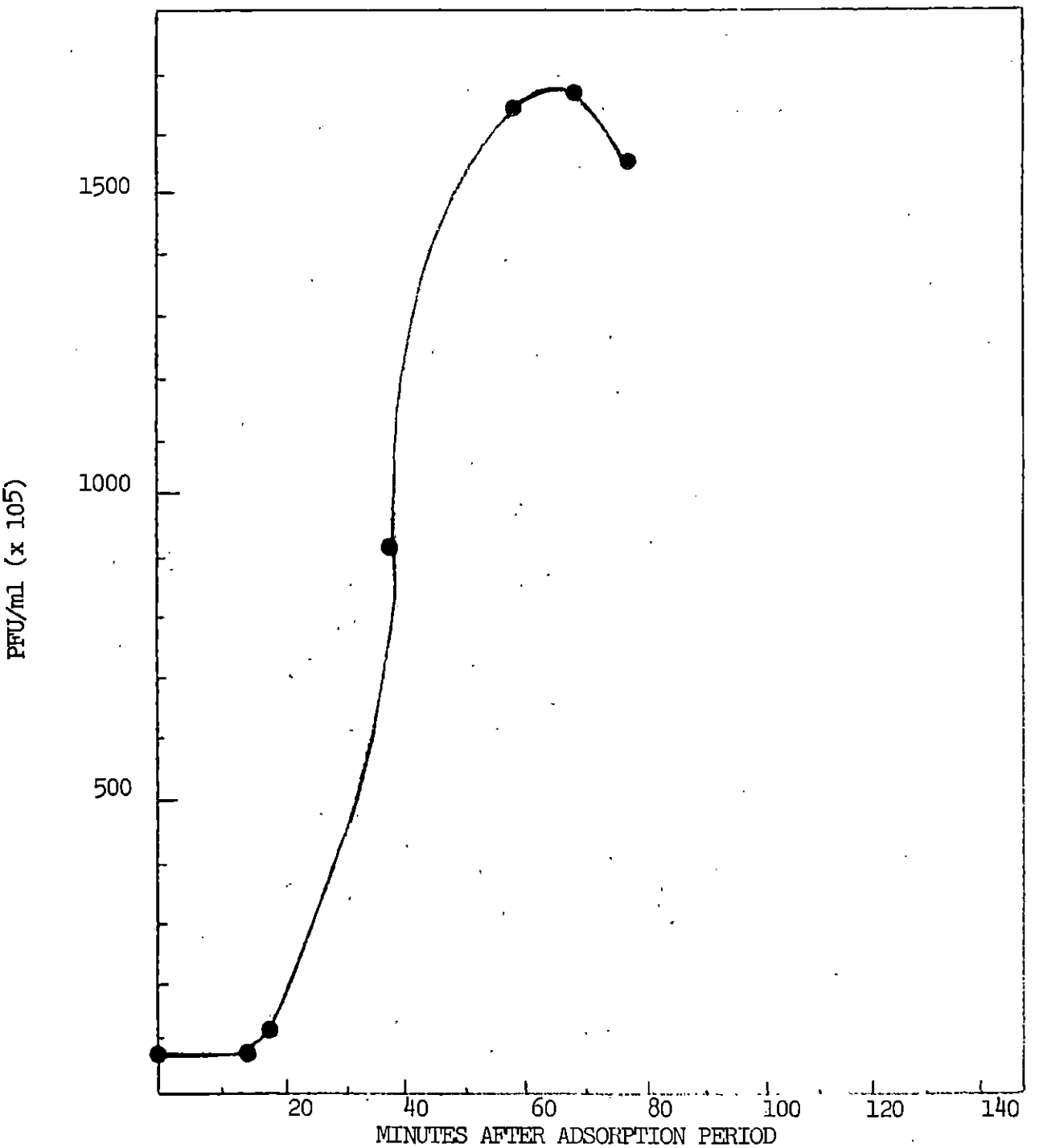

Fig. 2. One-step growth curve of Pt-17914 phage.

Table 2. One-step growth experiment of Pt-17914 phage.[a]

Plate	Avg. PFU/plate	PFU/ml (x 10^5)
1 (0 min)	66	66
2 (12 min)	65.5	65.5
3 (17 min)	108.5	108.5
4 (37 min)	91.5	915.0
5 (57 min)	163.0	1630.0
6 (67 min)	167.0	1670.0
7 (77 min)	155.0	1550.0
Input (0 min)	99.5	99.5
Unadsorbed (0 min)	31.0	31.0

a. P. tabaci concentration was 1.2 x 10^5/ml.

Table 3. Survival of Pt-17914 phage after 1 to 6 min exposure to 2537 Å UV.

	Experiment Number										
	1[a]		2[b]		3[c]		4[d]		5[e]		
Min	PFU/ plate	% Surv.	PFU/ plate	% Surv.	PFU/ plate	% Surv.	PFU/ plate	% Surv.	PFU/ plate	% Surv.	Mean % Surv.
0	100		330		140		90		310		
1	32	32	130	39	63	50	40	44	100	31	37.4
2	14	14	78	24	45	31	24	27	56	18	22.2
3	8	8	50	15	23	16	21	24	43	14	15.4
4	5	5	31	9	20	14	14	16	29	9	10.4
5	1	1	28	8	13.5	9	8	9	20	6	6.9
6	0	0	18	5	7.5	5	8	9	16	5	5.2

a. Average of 1 replicate.
b. Average of 3 replicates.
c. Average of 2 replicates.
d. Average of 2 replicates.
e. Average of 6 replicates.

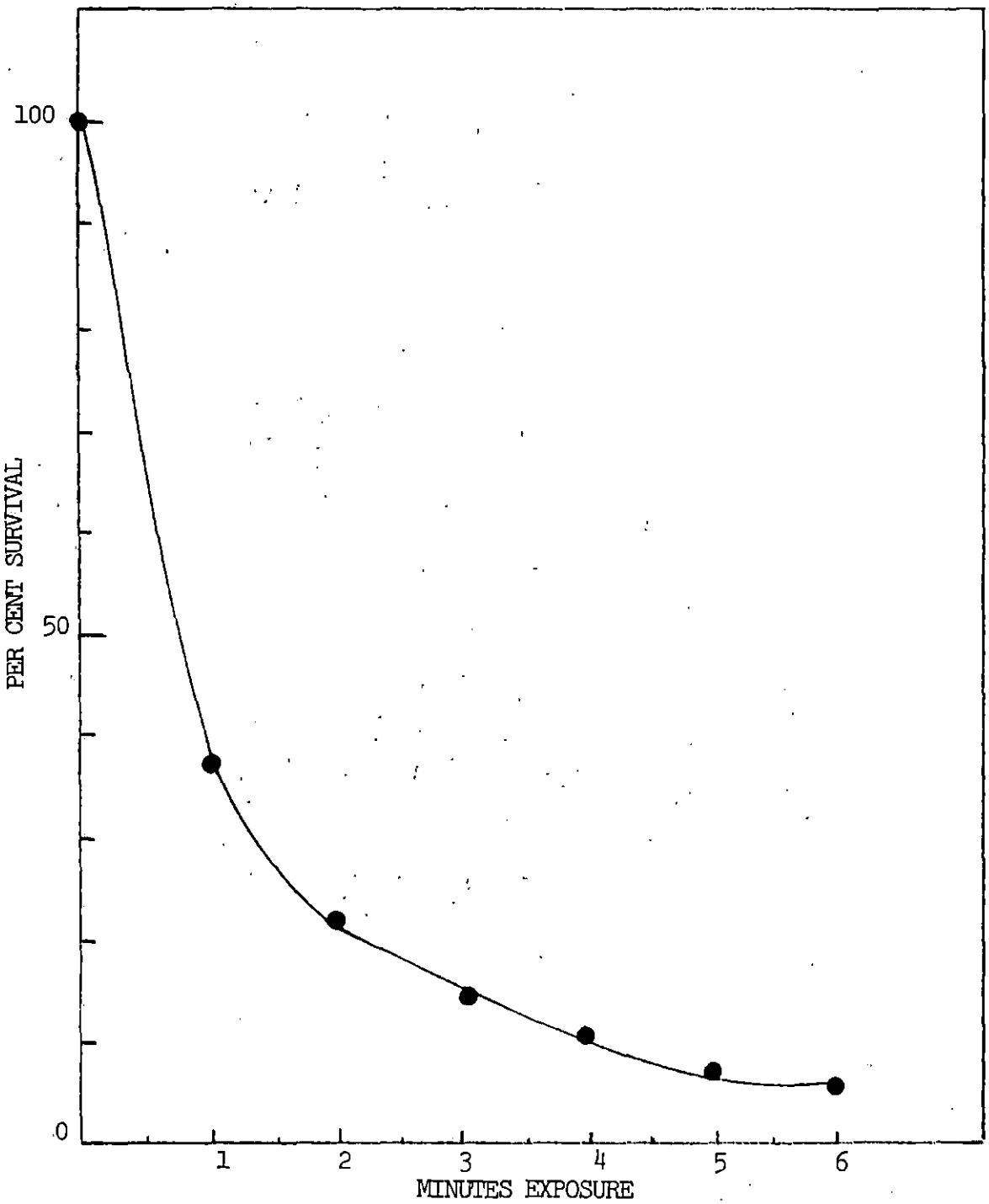

Fig. 3. Inactivation of Pt-17914 phage by 2537 Å UV.

Thermal inactivation. Table 4 lists the results obtained when the Pt-17914 phage was exposed to various temperatures. Although less than 1% of the phage in suspension remained viable when exposed to 60 C for 10 min, the thermal death point, 90 C, was found to be 30 C higher. Plaque mutants (plaques with small centers and large halos) were observed at temperatures above 55 C.

Table 4. Stability of Pt-17914 phage at various temperatures.[a]

Exp. No.	PFU/ml Control	Temperature C	No. Replicates	PFU/plate
1	3.6×10^5	40	3	complete lysis
		60	3	994
		80	3	100
		90	3	0
2	3.3×10^5	55	6	complete lysis
		60	6	970
3	2.02×10^5	85	9	76
		90	9	0

a. 10 min exposures.

Storage of dried phage suspensions. The presence of phage dried in broth films after storage for 5 wk at room temperature was indicated by central lysis on duplicate plates (Fig. 4) and presence of phage in a soil film was indicated by lysis on one of duplicate plates. Viable phage was not recovered from either dried broth suspensions or dried soil suspensions at the end of 6 wk of storage.

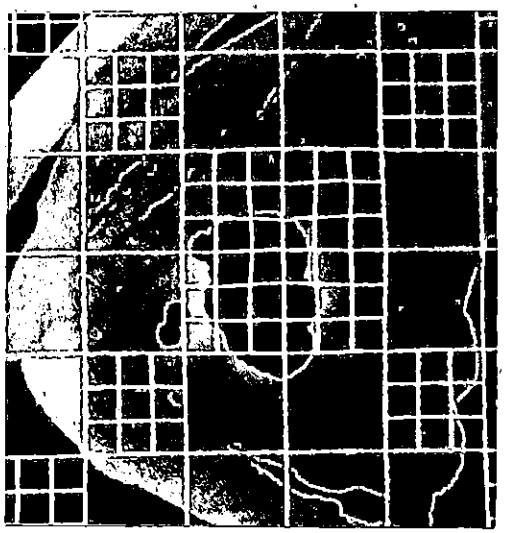

Fig. 4. Lysis indicating the presence of phage in filtrates.

Stability in solutions of various pH values. Some of the phages were found to remain viable for 1 hr when held at pH values of 5, 6, 7, 8, 9, or 10 but not at pH values of 4 or 11 (Table 5). However, total infectivity was retained only at the pH level at which phage

Table 5. Survival of Pt-17914 phage held at various pH levels for 1 hr.[a]

pH	PFU/plate Control	Mean PFU/plate	% Survival	Mean % Survival
4	30.0	0.0	0	0
5	30.0	8.9	30	30
6	3.2	2.3	66	
	140.0	112.5	80	75
8	140.0	95.0	68	68
9	140.0	76.0	46	46
10	3.2	2.0	66	
	140.0	48.0	24	38
11	30.0	0.0	0	0

a. The survival values were derived from comparisions to the control (pH 7.0).

was propagated and maintained, pH 6.8 to pH 7.0. Fig. 5 shows that the phage was somewhat less tolerant to acidic than to alkaline conditions.

Stability in organic solvents. Chloroform and ethyl ether have been found to cause little or no damage to lipid-free viruses. Only slight decreases in the infectivity of Pt-17914 phage exposed to these solvents were observed, 1% for chloroform and 8% for ether (Table 6).

Percolation through loam soil. Viable phages were recovered from all samples of soil and liquid taken from both columns receiving phage and columns receiving broth only.

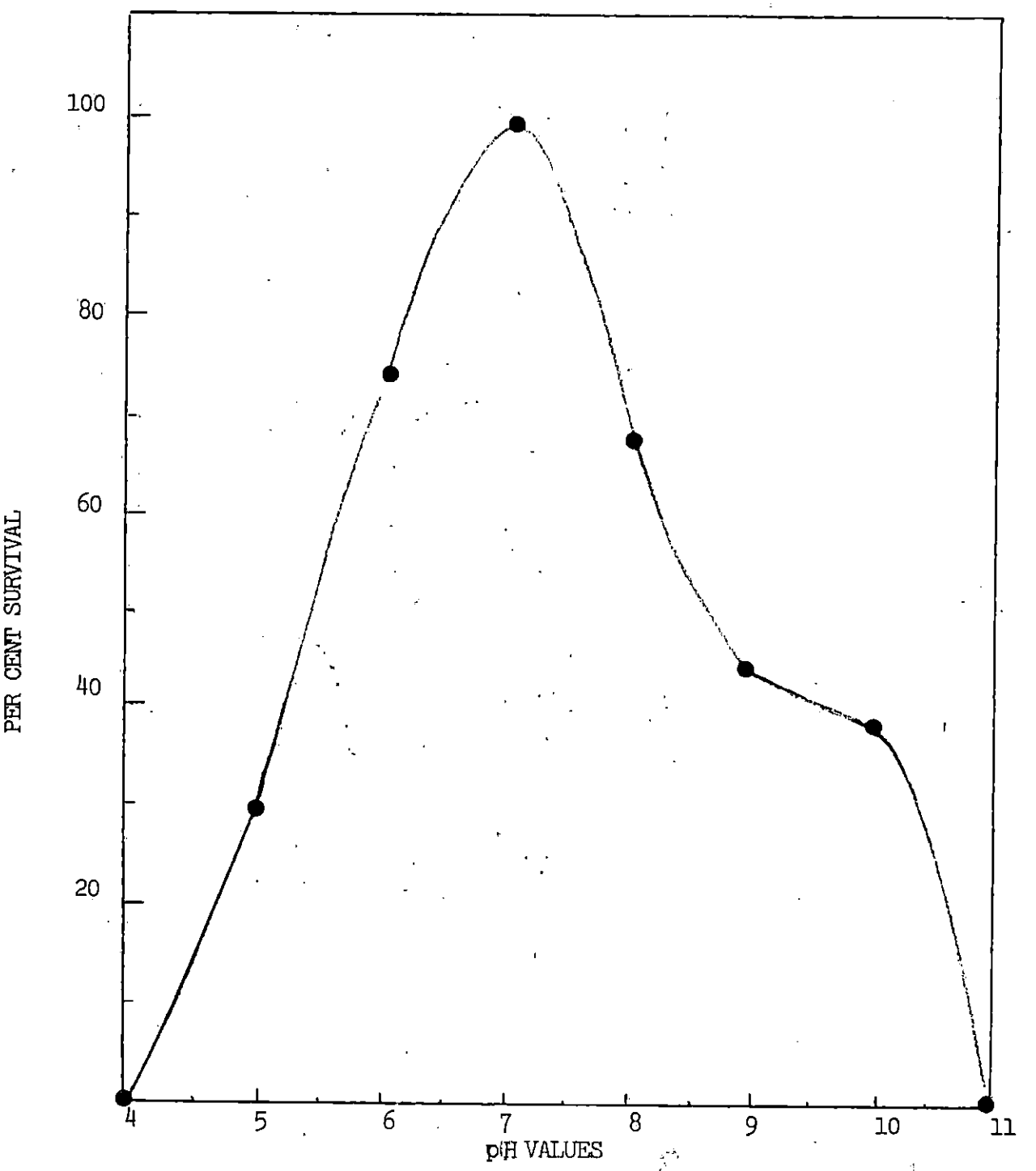

Fig. 5. Effect of pH on infectivity of Pt-17914 phage.

Table 6. Stability of Pt-17914 phage in chloroform and ether.

Solvent	PFU/plate	% Deviation	Mean % Deviation
none	202		
chloroform	182	-10	
	208	3	
	294	2	
	209	3	
	202	0	
	237	17	
	177	-11	
	175	-13	-1.0
ether	179	-11	
	177	-11	
	208	3	
	169	-16	
	182	-10	
	162	-20	
	182	-10	
	222	9	
	188	-7	-8.0

DISCUSSION

The host organism, Pseudomonas tabaci, of the phage studied is the causal organism for wildfire, a disease of tobacco which was prevalent in Kentucky until recently. After a medium suitable for phage propagation was determined, phage was readily isolated from sewage filtrates and also from soil samples.

The Pt-17914 phage was able to lyse a strain of P. putida, an inhabitant of decaying organic material, but not a second strain of P. tabaci. It is possible that specific receptor sites necessary for the attachment of Pt-17914 phage to host cells occurred in the cell walls of the two susceptible strains but not in the cell walls of the other strains tested. The plaque morphology was similar for both of the susceptible species of Pseudomonas. It is of interest that the susceptible strain of P. tabaci caused the formation of lesions when applied to tobacco leaves in laboratory experiments conducted concurrently with this study.

At various times during this study, the host culture (P. tabaci, ATCC 17914) acquired a slimy appearance. At such times, the bacteria would not form lawns of confluent growth on agar plates. It was also observed that small resistant colonies of bacteria appeared within the plaques formed on plates which were incubated for 48 hr. Transfer of the stock culture to new slants every 3 days and incubation of plates for 24 hr or less were found to eliminate the inability of the culture to form lawns and to suppress the appearance of

resistant colonies. These observations suggest that resistant cells become predominant in older cultures and precipitate the absence of lawns and the appearance of colonies within plaques.

The growth cycle of Pt-17914 phage was found to differ from that of the T-group of coliphages. The log phase, lasting an average of 15 min for the T-even phages (26), extended to 40 min for Pt-17914 phage and the entire growth cycle lasted approximately 67 min compared with 45 min for the T-even phages. The extended log phase may be due to a difference in the length of time required for cell division by E. coli B and by P. tabaci. This is supported by the observations that lawns sufficiently heavy to permit plaque counts have been found to appear within 10 hr of incubation for E. coli B but not until approximately 22 hr of incubation for P. tabaci. The following calculations were made using the data listed in Table 2:

Ratio of bacteriophage to P. tabaci

$$\frac{\text{Input phage}}{\text{P. tabaci conc.}} = \frac{0.995 \times 10^7}{1.2 \times 10^7} = \frac{4.0}{5.0}$$

Adsorbed bacteriophage

$$\text{Input phage} - \text{unadsorbed phage} = 99.5 \times 10^5 - 31.0 \times 10^5$$
$$= 6.85 \times 10^6$$

Per cent bacteriophage adsorbed

$$\frac{\text{Adsorbed phage}}{\text{Input phage}} \times 100 = \frac{68.5 \times 10^5}{99.5 \times 10^5} = 69\%$$

Multiplicity of infection

$$\frac{\text{Adsorbed phage}}{\underline{P.\ tabaci}\ \text{conc.}} = \frac{6.85 \times 10^6}{12.0 \times 10^6} = \frac{57}{100}$$

New bacteriophage produced

Newly produced phage - unadsorbed phage =

$$167.0 \times 10^6 - 3.1 \times 10^6 = 1.64 \times 10^8$$

Burst size

$$\frac{\text{Final titer}}{\text{Initial titer}} = \frac{164.0 \times 10^6}{6.85 \times 10^6} = 24.0$$

In addition to inactivation, UV exposure may induce phage mutation. The inactivation efficiency is low (26) and inactivated T2 phage particles, although unable to reproduce themselves, are still capable of killing host cells (1). In contrast, heat generally destroys both infective and reproductive capacities. Plaque mutants of Pt-17914 phage were formed after exposure to both UV and heat. UV exposure induced the formation of 2 types of plaques other than that produced by untreated phage, whereas only 1 new type of plaque resulted from heat treatment. The number of mutant plaques was also higher following UV exposure than following heat treatment. This may be explained by differences in the manner of inactivation found to be characteristic for these agents. The formation of plaque mutants, observed to be a frequent occurrence following UV irradiation, is a plausible result

since the nucleic acid has been indicated to be the primary target. On the other hand, reactions to heat treatment other than inactivation due to protein denaturation have been infrequently observed (13).

Drying has been shown to be as harmful as freezing for some biological units (13). Viable Pt-17914 phages were not recovered from dried preparations after 5 wk storage. Since both frozen and dried biological preparations are effectively dessicated, it is suggested that the drying procedure, not dessication itself, was detrimental to Pt-17914 phage which was stored for extended periods at 4 C.

One bacteriophage isolated on P. tabaci by Fulton (25) was stable for 1 hr between pH 4.0 and pH 10.5 and another between pH 5.0 and pH 10.0. Some of the Pt-17914 phage remained viable between pH 5.0 and pH 10.0 but complete stability was observed only between pH 6.8 and pH 7.0. Differences such as these, possibly the result of slight genetic variations, may be helpful in differentiating between phages isolated on the same host bacterium but in different laboratories.

During early experiments concerned with determining the characteristics of the growth cycle, chloroform was used to kill bacterial cells in the determination of the number of unadsorbed phage. Chloroform appeared to effect some of the phage particles since the number of unadsorbed phage was found to be nearly equivalent to the number of input phage. This was of interest

because few bacteriophages containing lipid have been isolated up to this time. Subsequent experiments indicated that the sample from the adsorption tube which was withdrawn to determine the number of unadsorbed phage, was not removed quickly enough to prevent some phage replication and the number of unadsorbed phage was higher than would be expected. In addition, the effect of chloroform and ether in separate experiments was probably not significant although ether did decrease infectivity by 8%.

In order for a bacteriophage to be an effective pesticide for plant pathogens, it must be available in the top layers of soil where the host bacteria are found to occur. Many of the bacterial plant pathogens, including P. tabaci, are disseminated by water. Therefore, a phage that is easily transported by water down through the soil would be ineffective as a pesticide. Pt-17914 phage was found to be present in the top 3 cm of a column of loam soil after quantities of tap water up to 1 liter had been added. Additional studies are necessary to determine the quantity of water required to remove all viable phage particles from the soil surface.

It is hoped that this study concerning various properties of Pt-17914 phage will provide a basis for additional experimentation, as mentioned above, that may lead to the development of this or similar bacteriophages as successful biological pesticides.

SUMMARY

The objective of this study was to determine some of the basic properties of a Pseudomonas tabaci bacteriophage isolated from municipal sewage filtrates in order to provide a basis for additional study which may indicate its potential as a biological pesticide.

Tests for the presence of bacteriophage in filtrates consisted of applying small quantities of the suspension to the central area of an agar plate previously swabbed with a portion of a broth culture of P. tabaci, and observing for lysis following incubation. The number of infective particles in various phage suspensions was determined by the soft-agar layer method of plaque assay.

(1) Plaques produced by freshly-isolated Pt-17914 phage possessed clear centers surrounded by narrow, granular halos and ranged in diameter from 0.5 mm to 1.5 mm.

(2) The host range of this phage was found to include P. putida, an inhabitant of decaying organic material, but not a second strain of P. tabaci.

(3) Following a 20 min adsorption period, the one-step growth curve lasted about 67 min and showed a burst size of 24 phages per cell, when the host bacteria were infected with phage at an

MOI of 0.57.

(4) Ultraviolet light inactivated 62% of phage suspensions within 1 min although 5% remained viable after 6 min of exposure. Two types of plaque mutants were induced: plaques 1 mm in diameter with clear centers surrounded by medium halos and plaques 0.5 mm in diameter with clear centers surrounded by large halos.

(5) Less than 1% of the phage particles remained viable when held for 10 min at 60 C. The thermal death point, however, was found to occur at 90 C. A single type of plaque mutant was observed at temperatures above 55 C; plaques possessing small centers surrounded by large halos.

(6) Phage remained viable after storage in dried preparations at room temperature for 5 wk but not for 6 wk.

(7) Phages were inactivated when held for 1 hr at pH 4.0 and pH 11.0 but exhibited various degrees of resistance between pH 5.0 and pH 10.0. Phage particles remained stable for extended periods between pH 6.8 and pH 7.0. Acidic conditions were found to be more detrimental than alkaline conditions.

(8) The organic solvents, chloroform and ether, known to inactivate many of the lipid-containing viruses, exerted no appreciable effect on this phage.

(9) Viable phage was recovered from the top 3 cm of columns

of loam soil through which quantities of tap water up to 1 liter had been passed.

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