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Partial Characterization of Bacteriophages of *Pseudomonas syringae* pv. *tomato*

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Introduction

Since the first major outbreak of bacterial speck in 1978, this disease has been a major problem for tomato growers in certain regions of North America. This disease, characterized by small brown to black lesions, is caused by *Pseudomonas syringae* pv. *tomato* (Jones et. al., 1991). While bacterial speck is not a threat to the plant itself, it reduces the marketable value of the fruit by decreasing its aesthetic appeal to the consumer. The taxonomic status of *P. tomato* is uncertain at present. It is difficult to distinguish morphologically and physiologically from other pathovars of the species, particularly *Pseudomonas syringae* pv. *syringae*. *P. syringae* pv. *syringae* has also been characterized from necrotic lesions on infected tomato fruit (Jones et. al., 1981). *P. tomato* is also of interest because it has been used as a model in plant pathogen studies with *Arabidopsis thaliana*, to gain a better understanding of the molecular basis of bacterial disease resistance (Whalen and Staskawicz, 1990). A comprehensive fine structure genetic analysis of *P. tomato* would provide useful information for these areas of interest.

Transducing bacteriophages are useful genetic tools for analyzing closely linked bacterial genes. Bacteriophages are classified as either virulent, whereby infected cells lyse with liberation of new bacteriophage particles, or temperate, in which case the infected bacteria either lyse with liberation of new bacteriophage progeny or become lysogenic (Hayes, 1968). In the lysogenic state, the bacteriophage genome, now called a prophage, replicates in tandem with the bacterial chromosome either by establishing a plasmid form or by integrating into the chromosome. Temperate bacteriophages capable of establishing lysogeny probably account for most transduction-mediated gene flow among bacteria in nature and provide systems that are most easily studied (Gerhardt et. al., 1981).

The objectives of this study were to enrich for and characterize bacteriophages of *P. tomato* suitable for transduction analysis. The characterization and analysis of bacteriophage isolates is useful for taxonomic purposes because they offer valuable methods to distinguish closely related bacterial species (Cuppels, 1983). These methods include bacteriophage sensitivity tests and fine structure mapping by transduction. Bacteriophage sensitivity patterns have been used to show that the cherry and plum pathotypes of *P. syringae* pv. *morsprunorum* are two distinct homogenous groups (Crosse and Garrett, 1963).

Materials and Methods

In preliminary tests, 44 strains of *P. tomato* were grown in NBY media to determine the presence of temperate bacteriophages and to determine which strains would be suitable hosts for bacteriophage propagation and enrichment (see Table 1). NBY media was used for all bacterial strains (Vidaver, 1967). NBY soft agar (0.7 g/100 ml) was used to create overlays for bacteriophage enrichment and titer (Adams, 1959). Media components were obtained from Fisher Scientific Co. (St. Louis, MO). In the initial survey for temperate bacteriophages, the bacterial strains tested were grown to stationary phase in NBY broth at 28°C and diluted in bacteriophage buffer to an optical density of 0.15 at 600 nm. The bacteriophage buffer consisted of 10 mM Tris-HCl and 10 mM $MgSO_4$, pH 7.5. NBY thick agar plates containing 35-40 ml of freshly prepared NBY agar were overlaid with 3 ml of molten NBY soft agar at 50°C containing approximately 1 x 10^7 cells per ml of the bacterial strain to be tested. Following overnight incubation of these plates at 28°C (16-20 hours), 5 ml of bacteriophage buffer was added to the surface of each overlay and the plates were incubated at room temperature for 2 hours. The bacteriophage buffer was drawn off each plate with a sterile Pasteur pipet and placed into 30 ml Oak Ridge centrifuge tubes, which were centrifuged at 10,000 rpm at 4°C for 10 to 30 minutes. The supernatants were removed and chloroform was added to each of these preparations (5% v/v). These supernatant solutions were tested immediately for bacteriophages or stored at 4°C for one to two days and then tested.

To determine the presence of bacteriophages, 5-10 µl of each supernatant was spotted onto overlays of all 44 *P. tomato* strains in a 44 x 44 cross. Bacteriophages in plaques that formed were propagated by removing the section of the soft agar containing the plaque by suction using a sterile Pasteur pipet and resuspending the agar plug in 0.1 to 0.5 ml of bacteriophage buffer containing chloroform (5% v/v). This suspension was incubated at

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Table 1. *Pseudomonas syringae* pv. *tomato* strains tested for phage production.

Strains*	Source†	Geographical Origin
10862	Denny	Canada
30555	Denny	Australia
31861	Denny	Australia
AV80	Denny	Nebraska
B19	Denny	California
B88	Denny	Georgia
B117	Denny	Georgia
B118	Denny	Florida
B120	Denny	Delaware
B121	Denny	New Jersey
B122	Denny	California
B125	Denny	Canada
B181	Denny	Georgia
B191	Denny	Georgia
CNBP1323	Denny	France
JL1031	Denny	California
JL1053	Denny	California
JL1060	Denny	California
JL1075	Denny	California
JL1105	Denny	California
JL1120	Denny	California
NCPPB880	Denny	Yugoslavia
NCPPB2424	Denny	Switzerland
PDDCC3357	Denny	New Zealand
PT14	Denny	California
PT21	Denny	California
PT30	Denny	California
RG4	Denny	Venezuela
T4B1	Denny	Canada
487	Cuppels	Greece
CNBP1318	Cuppels	Switzerland
DC84-1	Cuppels	Canada
DC92-10	Cuppels	Canada
DC3000	Cuppels	England
PDDCC3647	Cuppels	Australia
PST6	Cuppels	Canada
PST95	Cuppels	South Africa
SCB1	Cuppels	Canada
SM78-1	Cuppels	Georgia
T6D1	Cuppels	Canada
OK-1	Bender	Oklahoma
PT12	Bender	Oklahoma
PT17	Bender	Oklahoma
PT23.2	Bender	Oklahoma

* Strain numbers used are those devised by the original suppliers of the cultures (Denny, 1988). Abbreviations are CNBP, Collection National de Bactéries Phytopathogènes, Angers, France; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; PDDCC, Plant Diseases Division Culture Collection, Auckland, NZ.

† Sources of strains include the following: Bender, C. Bender, Oklahoma State University, USA; Cuppels, D. A. Cuppels, Agriculture Canada, Ontario, Canada; Denny, T. P. Denny, University of Georgia, USA.

room temperature for 2 hours. Serial dilutions in bacteriophage buffer were then spotted (5-10 µl) onto an overlay of the host strains B19, PST6 or PDDCC3647. Isolated plaques were picked and the entire procedure repeated two more times to ensure purified bacteriophage preparations. Purified bacteriophage isolates were titered by a spot titering method in which 10 µl aliquots of serial dilutions of bacteriophages in bacteriophage buffer were spotted onto soft agar overlays containing one of the three bacterial hosts. A suitable dilution for enrichment was one that produced overlapping plaques (~1,000 plaques per plate). The enrichment plates were incubated overnight (16-20 hours) and overlaid with 5 ml of bacteriophage buffer. The enriched bacteriophage preparations were centrifuged and the supernatants were filtered into 1.5 ml Eppendorf tubes through 0.2 µm Supor Acrodisc 32 filters (Gelman, Ann Arbor, MI). Titers of the enriched bacteriophage isolates were determined by the spot method or by the plate method in which 0.1 ml of appropriate bacteriophage dilutions in bacteriophage buffer were added to 3 ml of molten soft agar at 50°C containing the appropriate density of host cells (1×10^7 per ml). The tube was then mixed and poured over an NBY agar thick plate. Following overnight incubation at 28°C, the plates were checked and the bacteriophage titer determined by counting plaques and multiplying by the plating factor and dilutions. High titer bacteriophage preparations were stored at 4°C.

Results and Discussion

Preliminary attempts to show bacteriophage production in broth cultures were unsuccessful. However, using the plate method described in the Materials and Methods section, over 50% of strains gave evidence of plaque for-

Table 2. Source, titer, and plaque morphology of *P. tomato* bacteriophages.

Host Strain	Phage Isolate	Source*	Titer†	Plaque Morphology‡
PST6	7	AV80	$1.5x10^{10}$	<1mm,T
PST6	9	PT30	$1.2x10^{10}$	1-2mm, T
PST6	17	PT17	$1.2x10^{10}$	1-2mm, T
PST6	18	DC92-10	$1..0x10^{10}$	1-2mm, T
B19	3	CNBP1323	$2.3x10^{10}$	2-3mm, C, H
B19	21	PST95	$4.7x10^{10}$	2-3mm, T, H
B19	27	T4B1	$6.9x10^{10}$	2-3mm, C, H
B19	31	30555	$3.0x10^{10}$	2-3mm, C, H
B19	33	B121	$5.3x10^{10}$	2-3mm, C, H
B19	36	B125	$6.1x10^{10}$	2-3mm, C, H
B19	40	10862	$4.1x10^{10}$	2-3mm, C, H
B19	43	SM78-1	$3.4x10^{10}$	2-3mm, C, H
PDDCC3647	5	JL1060	$1.6x10^{10}$	1-2mm, T
PDDCC3647	10	JL1053	$1.6x10^{10}$	1-2mm, T
PDDCC3647	14	PT23.2	$1.5x10^{10}$	<1mm, T
PDDCC3647	15	PT14	$4.8x10^{10}$	1-2mm, T
PDDCC3647	16	DC84-1	$3.0x10^{10}$	1-2mm, T
PDDCC3647	22	JL1075	$1.0x10^{10}$	1-2mm, T
PDDCC3647	23	PT21	$4.2x10^{10}$	<1mm, T
PDDCC3647	24	JL1031	$2.3x10^{10}$	<1mm, T

*Refer to Table 1

† Titers (pfu/ml) are the highest achieved from plate enrichments as determined by either spot titers or plate titers as indicated in the Material and Methods. Titers were repeated at least twice for each bacteriophage isolate with duplicate samples counted and an average determined in each instance.

‡ C = clear, H = halo and T = turbid

mation (23 of 44 strains tested). Critical steps in this procedure involved using freshly prepared media and obtaining the correct density of cells for lawn formation (1×10^7 cells per ml of overly medium).

Supernatants from 16% of the strains produced plaques on more than 11 of the 44 strains tested, while supernatants from 84% of the strains produced plaques on less than 11 of the 44 strains tested. Of the 44 strains tested, 11 strains appeared to be suitable hosts based on the number of strains yielding supernatants which produced plaques on these 11 strains. Strains B19, PST6 and PDDCC3647 were selected from this group of 11 strains for propagation and enrichment of bacteriophages since supernatants from 39 of the 44 strains formed plaques on at least one of these three hosts. The plate enrichment method using these three hosts yielded titers of $\geq 1 \times 10^{10}$ plaque forming units per ml (pfu/ml) as shown in Table 2. Titers of bacteriophage isolates remained stable ($\geq 1 \times 10^9$ pfu/ml) for more than nine months at 4°C if the preparations were filtered. Most of the bacteriophage isolates were more unstable when stored over chloroform. Plaque morphologies of bacteriophage isolates appeared to be fairly uniform for a given host, although different plaque morphologies between hosts suggest unique bacteriophage isolates (Table 2).

The results of this study indicate that a majority of *P. tomato* strains contain temperate bacteriophages. Although lysogeny has not been demonstrated for any of the strains tested, the high percentage of *P. tomato* strains harboring bacteriophages is in agreement with an earlier study (Cuppels, 1983). Studies with other *P. syringae* pathovars have also revealed a high percentage of strains containing bacteriophages (Baigent et. al., 1963; Nordeen et. al., 1983). Host ranges of the bacteriophage isolates

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obtained in this study have not been rigorously examined. Nonetheless, plaque forming ability in supernatants from strains of diverse geographical orgin was observed. Bacteriophage isolates propagated and enriched on strain B19 from California came from such disparate locations as Australia, France, South Africa and Venezuela. In contrast, strain PDDCC3647 from Australia showed good plaque formation using supernatants obtained only from California and Canada strains. The basis of these differences is not clear. It does indicate however that *P. tomato* strains which harbor bacteriophage are ubiquitous and raises the possibility that bacteriophages may play a role in gene transfer on plant surfaces (Kidambi et. al., 1994). Demonstration of transducing activity for bacteriophages isolated from supernatants of *P. syringae* pv. *syringae* strains indicates that the bacteriophage isolates obtained in this study also may be useful for this purpose (Nordeen and Currier, 1983). Further physical characterization of the bacteriophage isolates including electron microscopy of density gradient purified bacteriophage particles and restriction enzymes digests of bacteriophage DNA is in progress and should establish the identity of the bacteriophage isolates.

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