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

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Abstract.—Bacteriophages from supernatants of the plant pathogenic bacteria *Pseudomonas syringae* pv. *tomato* (*P. tomato*) were isolated, enriched, and purified by density block centrifugation in cesium chloride (CsCl) step gradients. The DNA from purified phage was isolated and digested with the restriction endonucleases *Eco*R1 or *Hin*dIII. Three different DNA fingerprint patterns were determined indicating 3 unique phage isolates. Genome sizes of the phage ranged from 40 to 52 kilobases (kB). Buoyant densities of phage particles in CsCl varied from 1.36 to 1.51 g/ml. Electron microscopy revealed a single morphological type with an elongated polyhedral head and a long tail indicating the family Siphoviridae.

Key words:— Bacteriophages, *Pseudomonas syringae* pv. *tomato,* DNA, endonucleases, *Eco*R1, *Hin*dIII, Siphoviridae.

Introduction

Bacterial speck, first described and identified in Taiwan and the United States in 1933, caused a major outbreak in 1978, and the disease has become a major problem for tomato growers in certain regions of North America. Characterized by small brown to black lesions on various tomato tissues, this disease is caused by the bacterial pathogen *Pseudomonas tomato* (Jones et al. 1991). Bacterial speck is not significantly harmful to the plant itself, but severe spotting on the fruit can reduce marketable yield.

The taxonomic status of *P. tomato* is unclear. It is difficult to distinguish morphologically and physiologically from other pathovars of the species, particularly *Pseudomonas syringae* pv. *syringae* (*P. syringae*). Pathovars of *P. syringae* cause disease on different plant hosts, but *P. syringae* has also been characterized from necrotic lesions on infected tomato fruit (Jones et al. 1981).

Pseudomonas tomato is also of interest as it has been used as a model pathogen in studies with *Arabidopsis thaliana*, a common lawn weed of early spring that has proven amenable to molecular genetic manipulations. Researchers are studying *P. tomato* infections of *A. thaliana* to gain a better understanding of the molecular basis of bacterial disease and bacterial-disease resistance (Whalen and Staskawicz 1990). The genomes of *A. thaliana* and *P. tomato* strain DC3000 have been sequenced; however, there is limited knowledge of *P. tomato* temperate phage (Cuppels 1983, Minor et al. 1996).

Temperate phages capable of lysogeny probably account for most transduction-mediated gene flow among bacteria in nature and provide genetic systems that are most easily studied (Gerhardt et al. 1984, Kidambi et al. 1993). Genomic sequencing of a number of plant pathogenic bacteria has revealed that a substantial portion of these genomes contain mobile genetic elements, including up to 7% prophage sequences (Buell et al. 2003, Simpson et al. 2000). The role of phage in the disease process caused by these bacterial pathogens is unclear at present. Studies have recently been performed to investigate the suitability of phage in controlling bacterial plant diseases (Flaherty et al. 2000, Flaherty et al. 2001, Obradovic et al. 2004). Further genetic knowledge of phage used in these studies would be beneficial and might lead to a more efficient use of this disease-control strategy.

The objectives of this study were to enrich for, then characterize bacteriophages of *P. tomato* by electron microscopy and restriction endonuclease digestion. This approach should reveal unique phage isolates for further transduction and possible DNA sequencing studies. The information from these studies should clarify the role these phage play in host-pathogen relationships between *P. tomato* strains and appropriate host plants.

Materials and Methods

Bacterial Strains and Media.—Three *P. tomato* strains, B19, Pst6, and PDDCC3647, which had proven to be suitable hosts for phage propagation and enrichment (Minor et al. 1996), were used for the isolation and characterization of phages in this study. NBY media was used for all bacterial strains (Vidaver 1967). NBY soft agar (0.7g/100 ml) was used to create overlays for phage enrichment and titer (Adams 1959). Media components as well as all reagents for phage enrichment and DNA isolation were obtained from Fisher (St. Louis, MO).

Propagation and Enrichment of Bacteriophages.—NBY thick agar plates containing 35 to 40 ml of freshly prepared NBY agar were overlaid with 3 ml of molten NBY soft agar at 50° C containing approximately 5 x 10^7 cells per ml of the appropriate propagating strain. These strains were grown to stationary phase in NBY broth at 28° C and diluted in phage buffer to an optical density of 0.15 at 600 nm. The phage buffer consisted of 10 mM Tris HCl, pH 7.5, and 10 mM $MgSO_4$. Following overnight incubation at 28° C (15 to 18 hours), 5 ml of phage buffer was added to the surface of the overlay, and the plates were incubated at room temperature for 30 minutes to 2 hours or 4° C overnight. The phage buffer was then drawn off with a sterile Pasteur pipet

and placed into 30-ml Oak Ridge centrifuge tubes. The tubes were centrifuged at 10,000 rpm at 4° C for at least 10 minutes. The supernatants were filtered through a 0.2-mm cellulose acetate filter into sterile 1.5-ml Eppendorf tubes. These phage solutions were stored at 4° C until they were used in experiments to determine phage titer.

Phage enrichments for DNA isolation were prepared using 0.35% (w/v) of the synthetic gelling agent gelrite to avoid inhibition of restriction endonucleases (Bouche 1981). The NBY thick-plate underlay contained 0.74% (w/v) gelrite. The enrichment plates were incubated overnight (15-18 hours) at 28° C and overlaid with 5 ml of phage buffer for 30 min at room temperature. The enriched bacteriophage preparations were centrifuged and filtered as previously described. Titers of the enriched bacteriophage isolates were determined by the plate-overlay method (Adams 1959). Following overnight incubation at 28° C, the plates were checked and the phage titer determined. High-titer phage isolates, >10^9 pfu (plaque forming units) per ml, were further concentrated to >10^{10} pfu per ml by polyethylene glycol 6000 precipitation (Yamamoto et al. 1970). High-titer phage stocks were purified by CsCl block density gradients (Davis et al. 1980).

Electron Microscopy.—Phages purified from CsCl gradients were dialyzed in two changes of phage buffer overnight at 4° C to remove CsCl. The phage titers were determined and samples with a titer of >10^{10} pfu per ml were spread onto grids with carbon-coated Formvar support films, negatively stained with 1% uranyl acetate, and examined with a Joel 1200EX electron microscope.

Phage DNA Extraction and Digestion with Restriction Endonucleases.—EDTA was added to purified phage at a 20-mM final concentration. The phage were treated with proteinase K at 20 mg/ml and 0.5% Sodium Dodecyl Sulphate (SDS) at 56° C for 1 hr to disrupt phage heads and digest phage proteins. An equal volume of phenol was added to extract proteins followed by an equal volume of chloroform:isoamyl alcohol (24:1 v/v) to remove remaining proteins and the phenol. The supernatant containing phage DNA was precipitated by adding a one-tenth volume of 3-M sodium acetate (pH 5.2) and 2 volumes of absolute alcohol followed by overnight incubation at -20° C. DNA was collected by centrifugation at 14,000 rpm in a microcentrifuge for 10 minutes and washed in 70% ethanol and centrifuged again at 14,000 rpm for 2 minutes.

After pellets had dried at room temperature, they were resuspended in 50 microliters of TE buffer (10 mM Tris 1 mM EDTA pH 8.0). Ten-microliter samples were run on 0.7% agarose gels to determine purity and estimate the volume needed for restriction endonuclease digestion. Approximately one microgram of phage DNA was digested in appropriate buffers containing RNase at a final concentration of 10 mg/ml to eliminate RNA, which was abundant in most samples.

Restriction endonuclease digests were incubated with 2 to 6 units of *Eco*R1 or *Hin*dIII per microgram of DNA at 37° C for 3 hours. DNA digests were electrophoresed in 0.7% agarose gels in TAE buffer at a constant voltage of 5 to 6 volts per cm. *Eco*R1 and *Hin*dIII endonucleases were purchased from Promega (Milwaukee, WI). Size standards for gel electrophoresis consisted of bacteriophage lambda DNA digested with *Hin*dIII. Following staining of gels with ethidium bromide (4 mg/ml), gels were destained in distilled water and documented with Polaroid film or an Alpha Innotech imaging system. Sizes of DNA fragments were determined by plotting the log of the molecular weight of DNA fragments observed against the distance traveled in mm.

Results

Three strains of *P. tomato* (B19, Pst6, and PDDCC3647) were used to enrich bacteriophages. These strains were selected on the basis of a preliminary survey for bacteriophage production by 44 *P. tomato* strains (Minor et al. 1996). Plate enrichments using these 3 hosts produced titers of >10^{10} pfu (plaque forming units) per ml after concentration with PEG6000. (Table 1).

Initial attempts to purify phage using CsCl block gradients containing three 2.4-ml layers of 1.7 g/ml, 1.5 g/ml, and 1.45 g/ml caused the phage to band in the upper third of the 12-ml tubes used. Centrifugations were performed in a SW41 rotor at 23,000 rpm for 2 hours at room temperature. Subsequent purifications used five 2-ml layers of 1.7 g/ml, 1.5 g/ml, 1.45 g/ml, 1.3 g/ml, and 1.2 g/ml and 1.0 ml of enriched phage in phage buffer. Using this protocol, phage banded near the middle of the centrifugation tubes; although both upper diffuse and

Table 1. Source of *P. tomato* bacteriophages.

Phage Isolate	Source Strain[a]	Geographical Origin	Propagation Strain[a]
*Pto*7	AV80	Nebraska	PST6
*Pto*9	PT30	California	PST6
*Pto*17	PT17	Oklahoma	PST6
*Pto*18	DC92-10	Canada	PST6
*Pto*3	CNBP1323	France	B19
*Pto*21	PST95	South Africa	B19
*Pto*27	T4B1	Canada	B19
*Pto*31	30555	Australia	B19
*Pto*33	B121	New Jersey	B19
*Pto*36	B125	Canada	B19
*Pto*40	10862	Canada	B19
*Pto*43	SM78-1	Georgia	B19
*Pto*5	JL1060	California	PDDCC3647
*Pto*10	JL1053	California	PDDCC3647
*Pto*14	PT23.2	Oklahoma	PDDCC3647
*Pto*15	PT14	California	PDDCC3647
*Pto*16	DC84-1	Canada	PDDCC3647
*Pto*22	JL1075	California	PDDCC3647
*Pto*23	PT21	California	PDDCC3647
*Pto*24	JL1031	California	PDDCC3647

[a]Minor et al. 1996

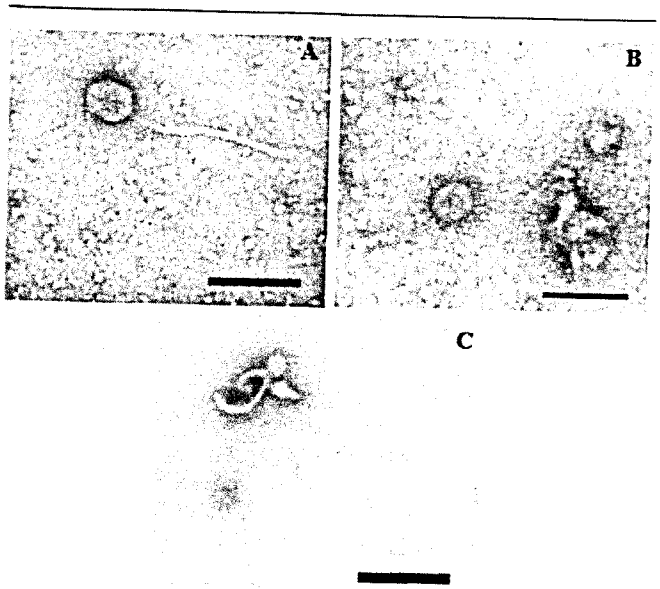

Fig. 1. Electron micrographs of negatively-stained *Pseudomonas tomato* phages **A**, *Pto*16, **B**, *Pto*21,**C**, *Pto*33. Bar in **A** and **B** is 100 nm. Bar in **C** is 200nm.

lower intense bands were noted for most phage. Multiple bands, especially of lower density, may contain phage ghosts devoid of DNA. Phage titers of lower-density bands had 2-10 times fewer pfu/ml than higher-density bands (data not shown). Phage were removed from the sides of the centrifuge tubes using a sterile 18-gauge needle. They were dialyzed twice overnight against 1.0 liter of phage buffer at 4° C in dialysis tubing with an 8,000 dalton molecular weight cutoff.

Phage densities were determined using a refractometer. Phage densities ranged from 1.36 to 1.51 g/ml as shown in Table 2. Purified phage with titers of $>10^{10}$ pfu/ml were used for EM analysis and DNA extraction. Only one morphological group was observed in EM studies of the enriched and purified phage (Fig. 1). This morphology has been previously reported for phage from *P. tomato* strains (Cuppels 1983). The phage examined by EM in this study each had a long flexible tail and a polyhedral head. Dimensions of phage heads and tails are given in Table 2.

*Eco*R1 and *Hin*dIII restriction endonuclease patterns indicated 3 unique phage isolates as shown in Fig. 2. Phage from group I has a genome size of 52 kB, phage from group II has a genome size of 40 kB, and phage from group III has a genome size of 48 kB (Table 2). All phage propagated on *P. tomato* strain 3647 (group I) gave similar restriction fragment patterns with *Eco*R1 and *Hin*dIII (data not shown). All phage except *Pto*21 (group II) propagated on *P. tomato* strain B19 and strain Pst6 also gave similar *Eco*R1 and *Hin*dIII DNA fingerprints (group III) with the exception of *Pto*18. *Pto*18 has an extra DNA fragment of approximately 5 kB for a total genome size of 53 kB. We suspect that this additional DNA fragment may be an insertion

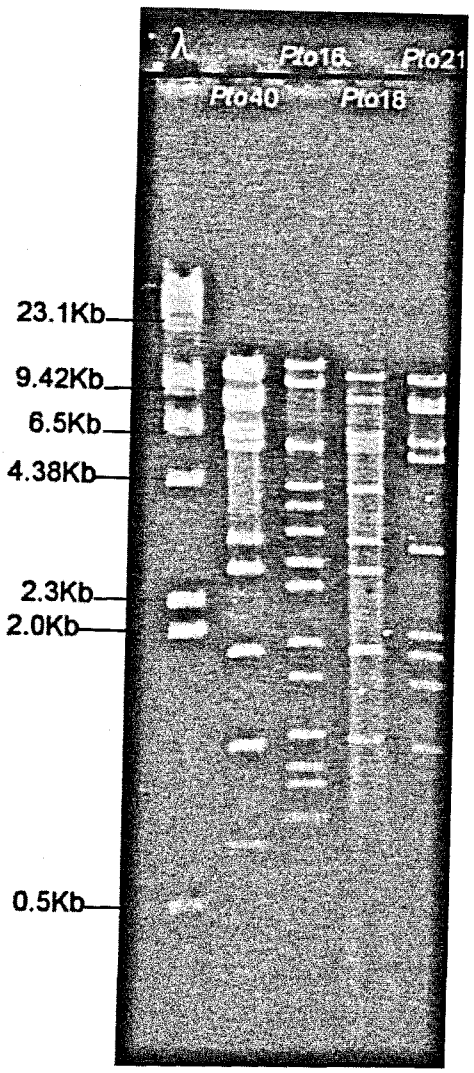

Fig. 2. Electrophoretic patterns of *Eco*R1 digests of bacteriophage DNA (0.7% gel). λ, bacteriophage lambda digested with *Hin*dIII. Size of lambda standards are indicated to the left in kB.

sequence since these are known to be common in the *P. tomato* genome (Buell et al. 2003). *Pto*21 appears to be unique based on restriction fragment patterns with *Eco*R1 and *Hin*dIII.

Discussion

Our results confirm earlier investigations of *P. tomato* phage that indicated that approximately 50% of *P. tomato* strains contain what appear to be temperate phage (Cuppels 1983). Induction studies using UV and mitomycin C failed to yield phage from the propagating strains (data not shown). Group I phage failed to plaque on *P. tomato* strains PST6 and B19. Group

Table 2. Phage characteristics.

Phage	Plaque Morphology*	Genome Size (kB)	Phage Size (nm) Head	Tail	Density (g/ml)	Type
*Pto*7	<1mm, T	48	N/D		1.36	III
*Pto*9	1-2mm, T	48	N/D		1.39	III
*Pto*17	1-2mm, T	48	N/D		1.42	III
*Pto*18	1-2mm, T	53	N/D		1.41	III
*Pto*3	2-3mm, C, H	48	N/D		1.41	III
*Pto*21	2-3mm, C	40	55 X 48	190 X 6	1.40	II
*Pto*27	2-3mm, C, H	48	N/D		1.43	III
*Pto*31	2-3mm, C, H	48	N/D		1.39	III
*Pto*33	2-3mm, C, H	48	34 X 34	151 X 14	1.39	III
*Pto*36	2-3mm, C, H	48	N/D		1.41	III
*Pto*40	2-3mm, C, H	48	N/D		1.42	III
*Pto*43	2-3mm, C, H	48	N/D		1.39	III
*Pto*5	1-2mm, T	52	N/D		1.49	I
*Pto*10	1-2mm, T	52	N/D		1.51	I
*Pto*14	<1mm, T	52	N/D		1.49	I
*Pto*15	1-2mm, T	52	N/D		1.49	I
*Pto*16	1-2mm, T	52	57 X 42	162 X 7	1.49	I
*Pto*22	1-2mm, T	52	N/D		1.51	I
*Pto*23	<1mm, T	52	N/D		1.37	I
*Pto*24	<1mm, T	52	N/D		1.49	I

* C = clear, H = halo, T = turbid N/D=not determined

II and III phage failed to plaque on *P. tomato* strain 3647. This may indicate a host range difference of these phage or possibly immunity due to prophage in the propagating strains that can not be induced. Phage *Pto*21 appears to produce plaques with clear edges indicating that this phage may be capable of rapid lysis. The phage in this study appear similar in morphology to those isolated by Cuppels (1983) and Nordeen (Nordeen et al. 1983); although the heads of group I and II phage were elongated and not isometric as those isolated by Cuppels.

The long, flexible, tail and polyhedral heads place these phage in the family Siphoviridae (Ackerman 1973). The phage propagated on *P. tomato* strain 3647 appear to be identical based on DNA fingerprinting with *Eco*R1 and *Hin*dIII. This result is not surprising since the *P. tomato* strains from which these phage were isolated all came from California initially (including strain PT23.2) except *Pto*16, which was isolated from the Canadian strain DC84-1. The history of these strains is unknown, but given their limited geographic origin, it was not unexpected that the phage isolated from them might be similar or identical. Surprisingly, the type III phage isolates came from bacterial host strains with a diverse geographical origin. The host strains that produced these phage were isolated in North America, Europe, and even Australia (strain 30555), and yet DNA fingerprinting indicates the phage isolates are identical. Phage *Pto*21 from a South African strain appears to be distinct from the other phage based on DNA fingerprinting. Although this is not surprising given the geographical origin of this strain, the significance of this finding is not clear at present. All of the phage isolated in this study appear to be suitable for transduction analyses based on their genome size. These studies would be useful in determining lateral transmission of virulence genes on the phylloplane surface (Kidambi et al. 1993). Sequencing of these phage should elucidate the role they play in disease development and may reveal additional novel genes of interest as demonstrated in the recent sequencing of 18 *P. aeruginosa* phage (Kwan et al. 2006) in which over 50% of open reading frames were novel.

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