Bacteriophages from Sewage Specific for Fluorescent Phytopathogenic Pseudomonads

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ABSTRACT

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Fifty-six bacteriophages were isolated on *Pseudomonas syringae* pv. *glycinea* from raw sewage obtained from four sources in Riverside and San Bernardino counties in California. Seven selected phages characterized further were specific for the fluorescent phytopathogenic pseudomonads, particularly for the *P. syringae* group. Not all pathovars of *P. syringae* were susceptible to these phages. One of these phages was specific to pv. *glycinea* race 4. Electron microscopic characterization of six of the seven selected phages revealed four distinct morphologies. All of the phages examined contained double-stranded deoxyribonucleic acid. The phages were tested for generalized transducing ability of several different chromosomal genes. No stable recombinants were recovered regardless of the conditions used in transduction experiments.

Genetic analysis of the fluorescent phytopathogenic bacterium *Pseudomonas syringae* pv. *glycinea* has produced a partial linkage map of a segment of the chromosome (11). This was accomplished by conjugational transfer of chromosomal material mediated by antibiotic resistance plasmids. This method is useful for producing such linkage fragments of the chromosome. To produce a fine-structure map of the pv. *glycinea* chromosome, including the genes for pathogenicity, generalized and specialized transducing phages are desirable.

The existence of bacteriophages infecting phytopathogenic pseudomonads has long been observed. Fulton (12) isolated and compared phages attacking pv. *tabaci*. Lysogeny was first reported in pv. *mors-prunorum* and pv. *syringae* by Garrett and Crosse (13). Other early work has been reviewed by Okabe and Goto (16). Phages of phytopathogenic bacteria have been isolated from soil (8) and diseased plant tissue (3,18), but isolation from sewage has not been reported. Several uses for these phages have been proposed, including taxonomic differentiation of the host, strain identification, and biocontrol of plant disease (7). Application of these uses has had only limited success for various reasons. Transduction of chromosomal markers has been reported in *Erwinia chrysanthemi* (6). The potential for phage-mediated genetic studies of phytopathogenic bacteria appears great.

The present study was inititated to determine whether bacteriophages can be isolated from raw sewage in areas where the host is not known to exist, to partially characterize these phages, and to evaluate their usefulness in the identification of pv. *glycinea* strains and, in particular, their capacity to transduce genetic material.

MATERIALS AND METHODS

Strains. The *P. syringae* pv. *glycinea* strains used have been described earlier (19). Other bacterial strains used in these studies are listed in Table 1.

Media. A complete medium (1% casein hydrolysate, 0.5% yeast extract, and 0.4% K_2HPO_4) and a minimal medium (0.7% K_2HPO_4, 0.15% KH_2PO_4, 0.1% NH_3NO_3, and 0.01% $MgSO_4$) were autoclaved separately from Difco bacto agar (1.5%). Glucose, autoclaved separately, was added to the minimal medium after autoclaving at a final concentration of 1%.

Phage isolation and purification. Raw sewage was collected from water treatment plants in Riverside and San Bernardino, CA. Phages were isolated by the method of Adams (2). Increase of phage titer, plaque assay, single-step growth curves, rabbit antisera production, and serological neutralization studies were performed by the methods of Adams (2). Phages were collected from filter-sterilized lysates by centrifugation at 54,000 *g* for 2 hr. Phage preparations were purified by centrifugation (at 77,000 *g* for 35 min) in a linear, 10–40% sucrose density gradient. Fractions absorbing light at 260 nm were collected and dialyzed to remove sucrose. Phages were stained with 2% aqueous uranyl acetate for 1 min and examined in a Hitachi model HU12 electron microscope. The actual magnification of the microscope was calibrated periodically by photographing a calibration grid (Ernst F. Fullam, Inc., Schenectady, NY 12301) and measuring the distance between the lines with a calibrator supplied by the manufacturer.

Designation of phage isolates. Bacteriophages isolated from sewage from different locations were given tentative designations to provide isolation information. Permanent names complying with established nomenclature (1) were deferred until comparisons with previously reported *P. syringae* phages could be made. A letter and number indicate the bacterial strain on which the phage was isolated; this is separated by a dash from a number that indicates the location from which the sewage was obtained. Phages from the same source that gave distinctly different plaque morphologies on the same host were given a terminal letter designation. For example, phage R4-0B was isolated on pv. *glycinea* strain PgR4 (race 4) from sewage collected in Riverside (-1, -2, and -3 indicate San Bernardino locations), and this phage gave small plaques on race 4.

Determination of nucleic acid content. Twenty of the 56 bacteriophages were selected at random, and their nucleic acids were isolated and purified according to the methods of Richardson (17). Whether the nucleic acid was RNA or DNA and whether it was single or double stranded were determined by acrylamide gel electrophoresis and enzyme digestion with bovine pancreas RNase A (Sigma Chemical Co., St. Louis, MO 63178), DNase I (Millipore Corp., Freehold, NJ 07728), and DNase S1 ([*Aspergillus oryzae*] Calbiochem, La Jolla, CA 92037) according to the methods of Morris and Dodds (15) and Dodds (9,10). Tobacco mosaic virus double-stranded RNA and citrus tristeza virus double-stranded RNA were used as molecular weight standards and RNA controls. Calf thymus DNA was used as a DNA control.

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TABLE 1. Bacterial strains used to determine the host ranges of selected bacteriophages from raw sewage

Strain number	Species	Source[z]	Response[y] to phage						
			B3-0	B3-2	B3-3	B5-1	R1-1	R3-1	R4-0B
11775	*Escherichia coli*	a	−	−	−	−	−	−	−
14468	*Mycobacterium smegmatis*	a	−	−	−	−	−	−	−
PA02700	*Pseudomonas aeruginosa*	e	−	−	−	−	−	−	−
L161	*P. fluorescens*	d	−	−	−	−	−	−	−
13312	*Salmonella choleraesius*	a	−	−	−	−	−	−	−
TA98	*S. typhimurium*	a	−	−	−	−	−	−	−
12600	*Staphylococcus aureus*	a	−	−	−	−	−	−	−
PM137	*P. marginalis*	b	−	−	−	−	−	−	−
PS297	*P. solanacearum*	b	−	−	−	−	−	−	−
PS148	*P. syringae* pv. *syringae*	b	+	+	+	+	+	+	−
CC227	*P. syringae* pv. *syringae*	c	−	−	+	+	+	+	−
RYS21	*P. syringae* pv. *syringae*	c	+	−	+	+	+	+	−
PC104	*P. syringae* pv. *coronafaciens*	b	−	−	−	−	−	−	−
PH101	*P. syringae* pv. *helianthi*	b	−	−	−	−	−	−	−
PL115	*P. syringae* pv. *lachrymans*	b	+	+	+	+	+	−	−
PM166	*P. syringae* pv. *mors-prunorum*	b	+	+	+	+	+	+	−
CC230	*P. syringae* pv. *phaseolicola*	c	+	+	+	+	+	−	−
BB5,BB31, BB58,CC218	*P. syringae* pv. *phaseolicola*	c	+	+	+	+	+	+	−
CC217	*P. syringae* pv. *phaseolicola*	c	−	−	−	−	−	−	−
PP133	*P. syringae* pv. *pisi*	b	+	−	−	−	−	+	−
RYE5	*P. syringae* pv. *pisi*	c	+	+	+	+	+	−	−
RYE9	*P. syringae* pv. *pisi*	c	+	+	+	+	+	+	−
PS194	*P. syringae* pv. *savastanoi*	b	+	+	−	+	+	+	−
PT5	*P. syringae* pv. *tabaci*	b	−	+	−	+	+	−	−
11582	*P. syringae* pv. *tabaci*	a	+	+	+	−	−	+	−
PT116	*P. syringae* pv. *tomato*	b	+	+	+	+	+	+	−

[y] + = Plaques formed, − = plaques not formed.
[z] Sources: a = American Type Culture Collection; b = International Collection of Phytopathogenic Bacteria, M. P. Starr, University of California, Davis; c = C. Hayward, University of Queensland, Brisbane, Australia; d = R. H. Olsen, University of Michigan, Ann Arbor; e = Bruce Holloway, Monash University, Melbourne, Australia.

TABLE 2. Host range and single-step growth characteristics[a] of selected bacteriophages isolated from raw sewage and producing plaques on *Pseudomonas syringae* pv. *glycinea*

Phage	pv. *glycinea* strains				Burst size (pfu)[c]	Latent period (min)
	PgR2	PgR4	PgR7	All others[b]		
B3-0	+	−	+	+	90	45
B3-2	+	−	−	+	100	45
B3-3	+	−	+	+	nd	nd
B5-1	−	−	+	+	120	150
R1-1	+	−	+	+	nd	nd
R3-1	+	−	+	+	75	60
R4-0B	−	+	−	−	nd	nd

[a] − = No plaques formed, + = plaques formed, nd = not determined.
[b] PgR1, PgR3, PgR5, PgR6, PgB1, PgB2, PgB3, PgB4, PgB5, and PgB6.
[c] pfu = Plaque-forming units.

Screening for transducing ability. Bacteriophages specific to *P. syringae* were screened for the ability to transduce chromosomal genes by the methods of Miller (14), Watson and Holloway (20), and Buchanan-Wollaston (5). All 56 of the bacteriophage isolates were tested at a multiplicity of infection varying from 0.1 to 10. Transductions were attempted using all 56 phages grown on wild-type pv. *glycinea* strain PgB3 as the donor and with strains PgB3 21.15 *met*-25 *ura*-9 *rif*-15 *nal*A2 and PgB3 35.19 *met*-38 *his*-62 *ura*-15 *ile*-36 *ade*-132 *ilv*-90 *xyl*-19 *nal*A6 as recipients. Selection was made for recombination resulting in prototrophy at each of the auxotropic loci. Controls included 1) plating the filter-sterilized-phage preparations on complete medium, 2) plating recipient strains alone on selection media, and 3) using phages grown on the recipient strain instead of the wild-type PgB3 for transduction. Selected phages were tested against additional recipient strains. The recipient bacteria contained a total of 11 different auxotrophic and several antibiotic resistance markers. Colonies appearing on the original selection plates were subsequently transferred to fresh plates of the same medium and scored for growth after 48 hr at 25 C.

RESULTS AND DISCUSSION

All sewage samples tested contained bacteriophages virulent against at least one of the 14 pv. *glycinea* strains. Variations in the efficiency of plating existed among some strains. A host strain was scored as sensitive if discrete plaques containing viable phages were formed by at least one of three phage dilutions. Preliminary screening for differences in bacteriophage host range among the pv. *glycinea* isolates permitted us to select seven of the 56 phage isolates for further characterization (Table 2). In general, the phage isolates were virulent on most of the pv. *glycinea* strains. The exception was phage R4-0B, which, in several tests, was virulent only on pv. *glycinea* strain PgR4.

The seven selected phages were tested on several phytopathogenic pseudomonads and other genera of bacteria, including animal pathogens and saprophytes (Table 1). These bacteriophage isolates were absolutely specific for the fluorescent phytopathogenic pseudomonads, particularly those pathovars assigned to *P. syringae*. However, none of the seven phages were virulent on pv. *coronafaciens* or on pv. *helianthi*. Also, none of the seven phages were virulent on the pv. *phaseolicola* strain CC217, although all but phage R4-0B produced normal plaques on four other pv. *phaseolicola* strains. Additional phage isolates (data not presented) were tested on these pathovars with similar results. The lack of virulence of the seven phages on *P. marginalis* was not surprising. Even though it is a fluorescent phytopathogenic pseudomonad, it can be classified as *P. fluorescens* Biotype II (4). Phages purified by sucrose density gradient centrifugation were examined by electron microscopy (Fig. 1). The dimensions of the individual phages are presented in Table 3. When phages representative of the four different morphologies were centrifuged together on sucrose density gradients, each migrated to a different point in the gradient.

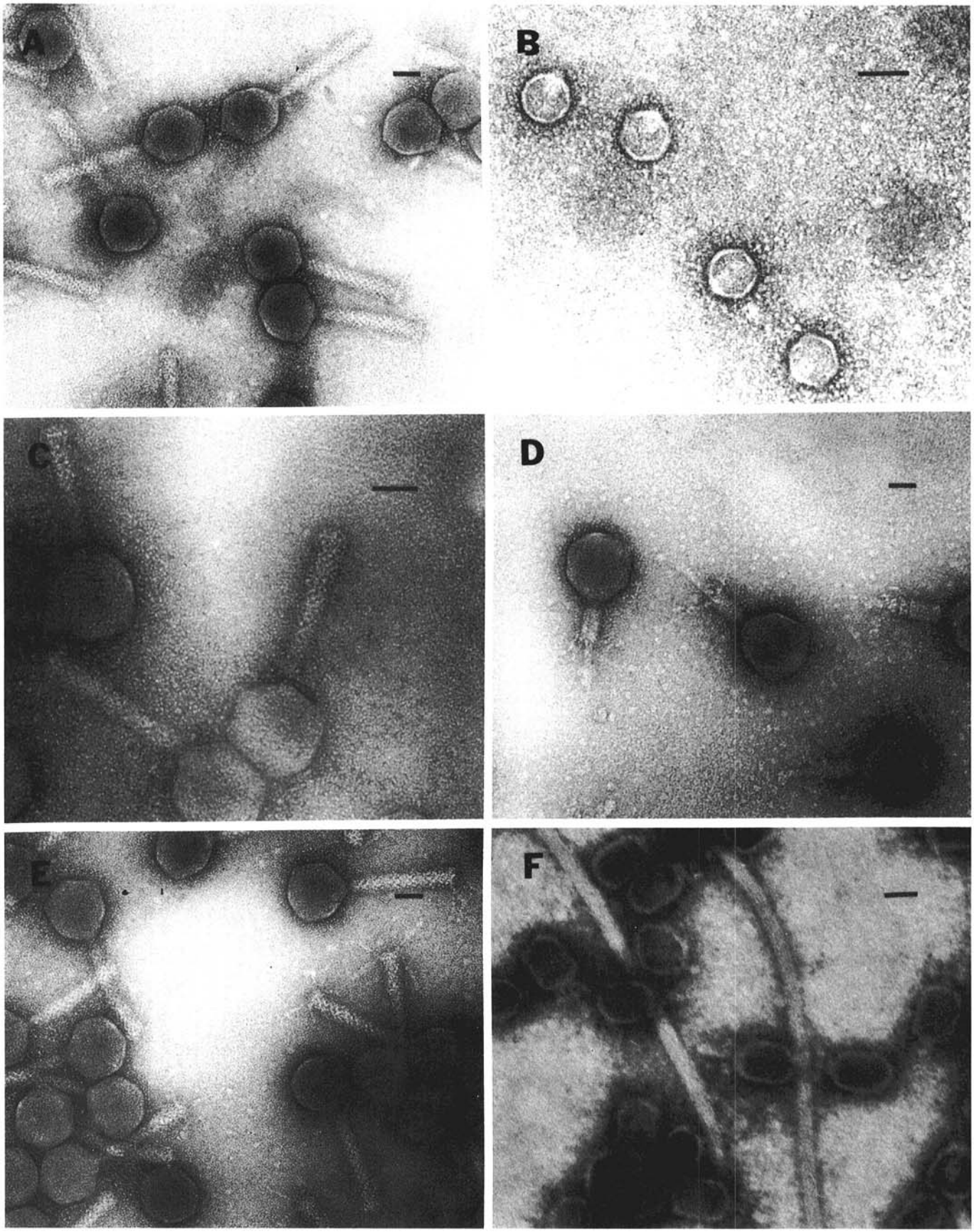

Fig. 1. Electron micrographs of particles of bacteriophages isolated from sewage on *Pseudomonas syringae* pv. *glycinea* and purified in sucrose density gradients. Tentative identifications of phage in **A,** B3-3; **B,** B5-1; **C,** B3-0; **D,** R3-1; **E,** B3-2; and **F,** R4-0B (B, ×330,000; C, ×247,500; others, ×165,000). Scale bar = 40 nm.

The seven phages were further characterized with respect to burst size and latent period (Table 2). Antisera produced against four of the phages were used to assay neutralization of infectivity of six phages (Table 4). Phage R1-1 was not included because of initial problems with density-gradient purification. Within 1 hr after centrifugation of phage R1-1 in a sucrose density gradient, a gel composed mainly of polymerized sugars formed in the gradient (a detailed study of this phenomenon will be reported later). Phage R1-1 was therefore purified on glycerol gradients. The differences in antigenic determinants of the selected phages cannot be generalized. Lack of a reciprocal cross-reaction involving phages B5-1 and R3-1 and the antiserum produced to these two phages is interesting: phage B5-1 is neutralized by R3-1 antisera, but phage R3-1 is not neutralized by B5-1 antiserum. This lack of reciprocity was observed repeatedly, but the reasons for it are not known. Perhaps the antigenic site on R3-1 that is recognized by B5-1 antisera does not, for some reason, induce rabbit antibody production. Fractionation of the antisera or, preferably, production of monoclonal antibodies may resolve this anomaly.

All of the phage genomes examined contained a single species of double-stranded DNA with molecular weights varying from 5.6 to 8.0×10^6 as determined by comparison with the standards. The phage DNA was completely digested within 8 hr by DNase 1, a nonspecific DNase that digests both single- and double-stranded DNA. Neither DNase S1 (a DNase specific to single-stranded DNA) or RNase A exhibited any activity against the bacteriophage DNA. The activity of the nucleases was monitored as the disappearance of the ultraviolet-fluorescing band from the acrylamide gels incubated in the presence of the enzyme. Each of the enzymes acted as expected on the control nucleic acids. Although the phages tested amounted to less than half of the 56 isolates, they represented all of the morphological groups, and therefore we feel that all of the remaining phages probably also contain double-stranded DNA.

Conditions known to affect transducing frequencies in other systems were varied to optimize the chances of observing transduction of chromosomal markers. The multiplicity of infection was varied from 0.1 to 10; different adsorption media were used; and the phage and/or host was occasionally preexposed to mild doses of ultraviolet irradiation. In no case, however, were stable recombinants for any of the chromosomal loci recovered.

TABLE 3. Dimensions of phages isolated from raw sewage and producing plaques on *Pseudomonas syringae* pv. *glycinea*

Phage	Dimensions[a] (nm)	
	Head	Tail
B3-0, B3-2, B3-3	80 ± 8	23 ± 3 × 140 ± 6
R4-0B	43 × 65 ± 5	6 ± 1 × 31 ± 9
R3-1	95 ± 4	20 ± 4 × 96 ± 9
R1-1, B5-1	35 ± 2	very short

[a] Based on measurements of 20 phage particles each.

TABLE 4. Phages screened against antisera and scored[a] for reduction in plaque forming units

Phage	Host[b]	Antisera			
		B5-1	R3-0B	R3-1	R4-0B
B3-0	PgB3	+	−	+	−
B3-2	PgB3	−	−	−	−
B5-1	PgB3	+	−	+	−
R3-0B	PgB3	+	+	+	−
R3-1	PgB3	−	+	+	−
R4-0B	PgR4	−	−	−	+

[a] + = Antisera neutralized phage, − = phage not affected by antisera. Phage concentrations were adjusted to give near-confluent lysis on host. Antisera titers were adjusted to one, two, and four times the concentration required to neutralize at least 99% of the homologous phage.
[b] Strains of *Pseudomonas syringae* pv. *glycinea*.

We feel that the most significant aspects of these results are 1) the ease and efficiency with which bacteriophages specific for the pathovars of *P. syringae* can be isolated from raw sewage, and 2) the isolation of a bacteriophage specific for a single strain of pv. *glycinea* race 4.

We do not know why phages capable of lysing strains of pv. *glycinea* are found in sewage from a geographical area in which no common diseases are caused by pv. *glycinea* and from which the occurrence of pv. *glycinea* has not been reported. In addition, the occurrence of disease problems caused by other pathovars of *P. syringae* is very limited in these southern California counties.

Initially, we thought that the phage host ranges among strains of pv. *glycinea* could be useful for race differentiation. However, when phages (other than R4-0B) were cloned by picking single plaques and propagated on pv. *glycinea* strains other than the strain originally used for isolation, the host range of the progeny varied. These phages may be useful for screening field isolates, but additional confirmation is necessary because of this observed variability in host range. The differential sensitivity of hosts to these bacteriophages has been useful in this laboratory to differentiate between phytopathogenic pseudomonads and nonphytopathogenic bacterial isolates.

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