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ENDONUCLEASE RESTRICTION ANALYSES AND TRANSDUCTION ANALYSES ON BACTERIOPHAGES OF THE PLANT PATHOGEN PSEUDOMONAS SYRINGAE

by

RUSSELL O NORDEEN

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Approved by:

Thomas C Currier

Major Professor

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PREFACE

This thesis is dedicated to the memory of Louis Pasteur whose genius and humanistic soul have been a constant source of inspiration to me during the course of this research.

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SECTION I

INTRODUCTION

INTRODUCTION

Pseudomonas syringae, first isolated by Van Hall in 1902, was originally considered a lilac pathogen but other isolates of this species infect a wide range of plants of at least 40 genera. This organism is distributed throughout the world and causes a variety of plant diseases including bacterial canker of deciduous fruit trees, blossom blight of pear, holcus spot on maize, bacterial brown spot on beans, and systemic necrosis of cowpeas. Plants affected include many of economical importance such as peach, plum, cherry, apricot, bean, sugarbeets, and various citrus fruits.

The taxonomy of the organism has presented problems due to the traditional method of assigning specific names to *Pseudomonas* spp. found attacking 'new' plant hosts. Thus, the nomenclature of Dye, et al. will be followed (13). Isolates originally referred to as *P. syringae* (Van Hall 1902) are now referred to as *P. syringae* pathovar *syringae* Van Hall 1902. I will refer to this organism as *P. syringae* throughout.

Attempts to correlate closely related plant pathogenic *Pseudomonas* spp. have been made. These methods have included biochemical, serological, nucleic acid homology, phage typing and bacteriophage typing tests, (11, 15, 24, 26, 33). These methods do not allow definitive separation of such closely related species as *P. syringae* and *P. mors-prunorum*. Certain biochemical properties have been useful in distinguishing phytopathogenic *Pseudomonas* spp. from saprophytic species, e.g. the oxidase test, the arginine dihydrolase test and tobacco hypersensitivity. Other tests which appear to be more useful have included pathogenicity against various plants including peach seedlings (26) and cowpea seedlings (23). The specifity of these methods of identification relies on the fact that the production of a wide spectrum biocidal agent, syringomycin (SR), appears to be limited to *P. syringae*. There seems to be a specific relationship

between the infecting strain of P. syringae and bean hosts (30). A lack of specifity with regard to the infecting P. syringae strain and the host, (cowpea), has also been shown (23).

Two different toxins [syringomycin, or SR, (12) and syringotoxin, or ST, (20)] have been associated with P. syringae. Most strains of P. syringae produce SR regardless of host origin (12). Production of SR by P. syringae appears to be optimal in still cultures of potato dextrose broth supplemented with 0.4% casamino acids (18). SR has been implicated in disease development in various hosts including maize, cowpea and peach seedlings (12, 17). SR isolated from peach seedlings is able to produce necrotic symptoms similar to infection with P. syringae (32). Other factors appear to be related to pathogenesis since strains which do not produce SR have been shown to be pathogenic (26), and certain strains which do produce SR are not pathogenic (2). Several explanations for these observations are possible: these include possible suboptimal culture conditions (18), the fact that some plants are more sensitive to SR than the standard bioassay fungus Geotrichium candidum (32), and the need for the infecting organism to grow in the host. The latter apears to be related to the ability of P. syringae to proliferate in the host (17) or possibly to nutritional requirements that the host provides the infecting organism.

SR and ST are low molecular weight peptides. Various compositions have been reported for SR. These include D-glutamic acid, serine, glycine, α-alanine, valine, D-phenylalanine and D-lysine (32) as well as a 2:1:2:1 mole ratio of serine, phenylalanine, an unidentified basic amino acid and arginine. ST has also been purified and is very similar to SR in its mode of action although it is easily distinguished from SR on the basis of electrophoretic mobility. The structure of ST was found to be threonine, serine, glycine, ornithine and the same unidentified basic

amino acid as is found in SR in a 1:1:1:1:1 mole ratio (20). Both toxins have a broad spectrum of activity including antibiotic action against animal, plant, fungal, and bacterial cells. SR appears to act on cellular membranes to cause detergent like lysis of cells (2). SR also is capable of binding to salmon and calf thymus DNA and inhibits Flemming RNA polymerase when calf thymus DNA is the template. Both toxins are extremely acid stable. They are stable at pH 1-2 but not above pH 7 and are heat stable for 20 minutes at 121°C (12). It has been suggested that SR is a product of cell wall metabolism (2).

Other P. syringae characteristics may be important for pathogenesis. Sands reported a spontaneous change in colony type from smooth to rough in isolates from wheat and safflower (31). He also reported an association between this change and reduced ability to produce toxin. Earlier, Otta (27) reported that cells isolated from rough colonies were filamentous and had lengths of up to 176 m compared to a maximum length of 4 m for cells from smooth colonies. Otta reported no reversion of rough to smooth types. Other reports have attributed the ability of P. syringae to initiate ice crystal development on plants to be a predisposing factor in disease of maize (1). This ice nucleation activity, INA, has proven to be a useful taxonomic aid since phytopathogenic Pseudomonas spp. closely related to P. syringae are INA inactive or show differential INA activity at -5.5°C and -10°C (22, 28).

Recently Gonzalez and Vidaver implicated a 35 x 10^6 dalton plasmid, pCG131, in the holcus spot disease in maize caused by P. syringae (16). Plasmids of similar size to pCG131 were isolated from SR producing isolates of apricot, millet and almond. Also, some restriction endonuclease generated fragments of plasmids from these hosts were found to be of the same size as pCG131 fragments, suggesting some molecular relatedness between these plasmids.

The involvement of pCG131 in holcus spot disease is doubtful since curing and retransfer experiments have been unsuccessful. Currier and Morgan in a survey of both SR producing and ST producing isolates failed to find a correlation between toxin production and plasmid content (submitted). Hybridization studies using labelled plasmid probes with cured host DNA or wild type host DNA in excess, failed to reveal integration of plasmids into chromosomal DNA for selected strains. This data along with the finding that cured isolates retained the ability to produce SR suggests that genes for SR production are chromosomal in nature for these isolates.

To study the various loci involved in pathogenesis and toxin production, various genetic systems will need to be developed. These include transpositional analysis, conjugational mapping, transformation, transduction and the development of cloning vectors for various complementation analyses.

Recently Chatterjee was able to show that R68.45, a plasmid with ability to mobilize chromosomal markers from various sites could be used for conjugational analyses of Erwinia chrysanthemi (6). R68.45 was mated into P. syringae but failed to show chromosome mobilization activity (CMA). This apparently was due to the deletion of a 2.05 Kb fragment which is lost following unfavorable culturing conditions of the host (10). Proper maintenance of strains with R68.45 has allowed success in subsequent CMA experiments and it appears this plasmid will be of some use in mapping various loci on the P. syringae chromosome.

Tn5, Tn7 and Tn10 have been transposed into P. syringae strains in our lab. It is hoped that these elements can be transposed into chromosomal DNA and used for specific mutation of various loci, as well as to aid in the polarized transfer of chromosomal DNA using suitable plasmid vectors.

Transformation has also recently been accomplished in P. syringae (21, 25). The finding in this thesis, i.e. that certain phages are capable of generalized transduction, along with the transformation activity discovered, offers the prospect of fine structure mappping in the very near future. Fine structure mapping in Erwinia chrysanthemi has already been accomplished with a UV induced phage (7).

Viruses active against plant pathogens are usually isolated from soil or culture supernatants. Other sources include diseased plant material or seeds (3, 8, 9, 29). In the following chapter the isolation of P. syringae phages from sewage is described.

Viruses from P. syringae have been described with respect to plaque formation, lysogenicity, host range, heat stability, adsorption properties and morphology (3, 9, 14, 29). Baigent reported that turbid plaque forming phages isolated from culture supernatants were separable into two or more component phages, one a clear plaque forming derivative. Rigorous tests for lysogeny besides subculturing were not performed. It remains to be seen whether antisera analysis or hybridization studies will reveal lysogenic properties of various phage isolates of P. syringae. EM analysis indicated various morphologies among P. syringae phage isolates, including some phage with T3 morphology and others resembling the smaller coliphages (3, 4, 5, 29). Baigent reported no correlation between the presence or absence of the turbid plaque forming isolates and virulence of their respective host strains to peach seedlings, or the ability to produce an antibiotic active against G. candidum.

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SECTION II

ENDONUCLEASE RESTRICTION ANALYSES ON BACTERIOPHAGES OF THE PLANT PATHOGEN *PSEUDOMONAS SYRINGAE*

ABSTRACT

Bacteriophages isolated from culture supernatants of Pseudomonas syringae and from sewage were identified based on distinct restriction patterns with the restriction endonuclease EcoRl. Eight isolates were determined to be unique, with two isolates from sewage having restriction patterns identical to two phages from culture supernatants. Molecular weights of the phage DNA ranged from 24 to 49 kilobases for isolates from sewage and from 39 to 52.5 Kb for the isolates from culture supernatants. Buoyant densities of phage particles in CsCl varied from 1.498 g/cm^3 to 1.507 g/cm^3 for isolates from sewage and 1.506 g/cm^3 to 1.516 g/cm^3 for isolates from culture supernatants. Electron microscopy revealed four distinct morphological groups. A total of 31 out of 47 strains of P. syringae indicated they contained temperate phages based on plaque forming ability of culture supernatants.

INTRODUCTION

A number of bacteriophage isolates of the plant pathogen Pseudomonas syringae pv. syringae (12) have been isolated from various sources, including soil samples and culture supernatants of P. syringae strains (9, 14). Several strains of P. syringae have been shown to be lysogenic (3). Phage isolates have been characterized with respect to host range and by electron microscopy (3, 8, 9, 11, 15, 18). Many of these phage isolates have a wide host range and are able to plaque on saprophytic as well as phytopathogenic Pseudomonas spp. Electron microscopy has shown two major morphological groups of phages capable of infecting P. syringae. The first group of phages resembles the smaller coliphages ϕ2 and E1. The second group resembles the coliphage T3.

To date no attempts have been made to characterize P. syringae phage isolates at the molecular level. Recently a group of closely related Pseudomonas pseudoflava phages were identified as being homogeneous by cleavage with restriction endonucleases EcoR1 and HindIII (2). In this study we screened culture supernatants of 47 P. syringae isolates from various hosts to determine which isolates liberate temperate phages. Some of the strains tested are known to produce either syringomycin or syringotoxin. Raw sewage was also used as a source of phages active against two selected strains of P. syringae. From these sources we have identified 8 unique P. syringae phage isolates by restriction analysis with EcoR1. All isolates were characterized with respect to morphology, buoyant density, and genome size.

MATERIALS AND METHODS

Organisms and culture conditions. The strains used in this study are listed in Table 1. Stock cultures were maintained on NBY medium (17) solidified with 1.5% agar. Cells for experimental use were cultured in NBY broth at 29°C. Media components including agar were obtained from

Difco, Detroit, MI. The solidifying agent, Gelrite (Kelco, San Diego, CA) was used at 0.75% for plates and 0.15% for overlays. When agar was used in overlays, its concentration was 0.75%.

Phage isolation. Sewage isolates were obtained by centrifuging municipal raw sewage for 10 min at 10,000 rpm and then filtering the supernatant through a 0.22 μ Millipore filter. The filtrate was mixed with an equal volume of NBY agar (1.5%) at 50°C and overlaid on NBY agar using the double agar overlay technique of Adams (1). Temperate phage isolates were obtained by centrifuging an overnight culture of the test organism for 10 min at 10,000 rpm and then spotting 20 μl of successive 100X dilutions of filtered supernatants on P. syringae lawns using NBY agar overlay. Growing the strains well into stationary phase, i.e. Klett 400 (#66 filter) or higher, aided in the recovery of phages. Plaques with apparently different phenotypes were stabbed with a sterile needle or toothpick and resuspended in phage buffer (0.01 M Tris 0.01 M $MgSO_4$ pH 7.0). Several dilutions were plated by the double agar overlay method to obtain well isolated plaques, and the process was repeated two more times to obtain unique phage isolates. Phage plaque assays and enrichments were carried out at 29°C.

Phage enrichment and storage. Initial attempts were made to broth enrich phage stocks in NBY broth. Overnight cultures of P. syringae hosts were diluted to about 10^8 cells per ml and then allowed to double once prior to infection with phage. All phage isolates were tested with inputs ranging from MOI 0.001 to MOI 10; titers above 10^{10} were rarely achieved. A higher titer may have been obtained using a bubbler apparatus, but because of the number of phage stocks that we wished to enrich, a plate enrichment technique was chosen for convenience (5).

For plate enrichment, dilutions of phage stocks were made in phage buffer and 0.1 ml of the appropriate dilution plated with 0.1 ml of an

overnight culture of P. syringae 224 cells diluted to Klett 100. Plates showing optimal confluent lysis were cooled 30 minutes at 4°C and overlaid with 5 ml of phage buffer and then placed at 4°C overnight. The phage buffer was drawn off, centrifuged at 10,000 rpm for 20 min and then filtered or mixed with chloroform, and stored at 4°C. Titers of 10^{10} to $9X10^{11}$ plaque forming units (PFU) per ml were obtained by this technique. Use of NBY Gelrite for the underlay and overlay in place of NBY agar did not decrease phage titers during enrichment. Plate enrichment routinely gave titers of 10^{11} PFU per ml which were further concentrated to about 10^{12} PFU per ml by PEG 6000 precipitation (4).

The majority of isolates were unaffected by storage over chloroform; however, one isolate, 92-3, was both sensitive to chloroform and storage at 4°C. Since all isolates were to be used in later transduction studies, all stocks were routinely filter sterilized. Filtering phage isolates did not affect plaque formation. Some phage isolates, in particular the one from B15, were unstable upon prolonged storage. This has been previously reported for phage from this organism (3). During plate enrichment, certain clear plaque phage were noted for isolates ϕ421, ϕ422, 40-4 and B15. These clear plaque phage bred true.

Phage particle purification. Phage stocks were purified following enrichment by either adding them directly to CsCl or by precipitating the phage with 10% (w/v) polyethylene glycol 6,000 to increase phage titers (4). The phage pellets were resuspended in 0.1 of the original volume of phage buffer and then added to CsCl gradients. The CsCl gradients used contained 45 to 48% CsCl, usually 46%. Centrifugation was performed in a Sorvall TV865 rotor at 40,000 rpm to equilibrium. All phage isolates appeared stable for 40 hours in 45% CsCl. Phage bands were not always observed for all isolates even though titers of up to 10^{12} PFU per ml were achieved. The sewage isolates 40-1 and 92-2C were particularly difficult

to visualize and required fractionation and a bioassay to identify the location of the phage. Phage bands were removed either directly through the side of the tube with a 1 ml syringe and a 25 guage needle, or fractionated from the bottom of the tube with a Hoeffer FS-101 fractionator (Hoeffer Scientific Instruments, San Francisco, CA). In the latter case, the refractive index of selected fractions was determined. Gradients which did not form visible bands were fractionated and assayed for phage activity by spotting 10 µl of gradient fractions, appropriately diluted in phage buffer, onto P. syringae 224 lawns. Phages were dialyzed overnight against two changes of phage buffer at 4°C.

Electron microscopy. Phages purified in CsCl gradients were spread onto grids with carbon-coated Formvar support films, negatively stained with 1% uranyl acetate and examined using a Phillips EM201 electron microscope.

Phage DNA extraction. Phage DNA was extracted by adding 0.25% SDS to CsCl purified phage in 3% NaCl, and then heating the mixture at 60°C for 5 min (2). Phages which remained opalescent after this procedure were treated with 50 µl per ml of pronase E (type XIV protease, Sigma) for 5 min to complete phage head disruption. Phage DNA was then twice extacted with an equal volume of phenol (previously equilibrated with a 3% NaCl solution), and the aqueous phase was dialyzed overnight against two changes of TS buffer (0.01 M Tris 0.005 M NaCl pH 8.0). The DNA was then added to 57% CsCl gradients containing 0.2 µg per ml of ethidium bromide and centrifuged to equilibrium at 50,000 rpm in a TV865 rotor. DNA bands were visualized by UV fluorescence and collected by fractionation. Yields of DNA were about 1-10 µg per ml per 10^{11} phage, depending on the phage stock. The sewage isolate 40-1 and 92-2C consistently gave smaller yields of DNA. The DNA was then dialyzed overnight against two changes of TS buffer. DNA concentrations were determined using a modification of the

'plastic wrap and ring method' of determining DNA concentrations (6). 10 µl aliquots of DNA were diluted into 10 µl volumes of TES (0.05 M Tris, 0.005 M EDTA, 0.05 M NaCl, pH 8.0) containing 2 µg per ml of ethidium bromide. 5 µl of these dilutions were successively diluted 1:2 into 5 µl of TES containing 1 µg per ml ethidium bromide and 5 µl aliquots of each dilution were spotted on the back of a plastic petri dish and photographed on a transilluminator (Ultraviolet Products, Model 63B) emitting 320 nm light with Polaroid Type 107C film through an ultraviolet filter and a Wratten 23A filter. Salmon DNA, 11 µg per ml, served as a standard. A dilution of the unknown giving the same relative fluorescence as a dilution of the standard was used to calculate the DNA concentrations.

Restriction endonuclease digests and agarose gel electrophoresis. EcoRl and HindIII were purchased from Bethesda Research Laboratories. New England Biolabs was the source of HaeIII restriction endonuclease fragments of bacteriophage ϕX 174 RF DNA, and Sigma Chemical Co. was the source of the bacteriophage lambda ($CI_{857}S7$) DNA used to generate HindIII fragment standards. Reaction mixtures contained from 5 to 54 µl of phage DNA in either 2X or 10X buffer mix recommended by the supplier. Varying amounts of the restriction enzyme were added, usually 1 µl (1-3 units). When used in the restriction digestion ribonuclease A (Sigma) and spermidine trihydrochloride (Sigma) were at a final concentration of 10 µg per ml and 0.005 M, respectively. Reactions were incubated at 37°C for two to three hours and then terminated by the addition of 1/8 volume of stop solution; 60% sucrose, 75 mM EDTA, 0.6% sodium sarcosinate and 0.01% bromocresol green (6). A volume of 20 to 60 µl was then subjected to electrophoresis at 0.35 V per cm in 3 mm thick vertical slab gels consisting of 0.7% or 1.5% agarose, 0.089 M boric acid, 0.089 M Tris and 0.0025 M EDTA. The running buffer was the same, but without agarose. Gels were stained with 0.2 µg per ml of ethidium bromide for 30 min and

destained for 30 min in water. Gels were photographed as described above.

RESULTS

Isolation, host range, and enrichment of bacteriophages. Table 2 lists the culture supernatants which exhibited plaque forming activity and the host range of the activity. Strains PS179 and PS224 were of particular interest as hosts because they have been extensively studied in this laboratory and a variety of auxotrophs have been previously isolated. Because our major goal was to develop a transductional system (see following paper) we concentrated mainly on the phages active in a single organism, i.e., PS224.

Culture supernatants of strains B3A, B15, PS148 and three independently lyophilized samples of strain PS179 (designated PS179-59, PS179-421 and PS179-422) all formed plaques on PS224. The phage titer in these supernatants was approximately 10^6 PFU per ml for B3A, B15 and PS148 and approximately 10^2 PFU per ml for the three PS179 cultures. Once isolated by three successive plaque purifications, the phage from the three PS179 cultures were readily plate enriched to titers of 10^{10} to $9X10^{11}$ PFU per ml. The phage from strain B15 could be enriched to 10^{10} PFU per ml after two cycles of plate enrichment but the phage from B3A could not be enriched above 10^6 PFU per ml even after several more cycles. Enrichment of the phage from PS148 was not attempted because this isolate is presumably identical to B3A but was obtained from a different source.

Phage from sewage were isolated by plating filter sterilized sewage on PS179 and PS224. Approximately 15 plaques of varying morphology were observed. The differences in plaque morphology were in some cases subtle and somewhat subjective. All isolates were readily enriched to titers of phage approximately 10^{11} PFU per ml. Phage isolate 40-1 formed plaques only on PS224, while all other isolates formed plaques on both PS179 and PS224.

Phage plaque morphology of selected isolates. The plaque

morphologies of the phage having activity on strain PS224 and which could be enriched to high titers are given in Table 3. Two of the isolates from sewage (40-3 and 40-4) produced small turbid plaques while the remaining three from sewage produced distinctly different clear plaques.

The phage from PS179 and B15 were small and turbid. Clear plaques occurred frequently for phage stocks ϕ421 and ϕ422. These clear plaque phage were extremely difficult to eliminate and seemed to be preferentially enriched following subsequent plant enrichment.

Virion morphology. Of the phages described in this study, four distinct morphological groups were observed (Figure 1). These morphologies resemble those previously reported for P. syringae phage isolates (3,7,8,16). Morphology group IV (panel 5), containing only the B15 phage, grossly resembles the type I (panel 1 and 2) and the type II (panel 3) morphologies in that all have a long flexible tail and a hexagonal head. However, there are obvious differences. For example, the head of the B15 isolate is more narrow and three of the six head angles are not distinct. This gives the head a rounded appearance where it connects to the tail structure. Also at the resolution in Figure 1 the tail appendages for the five type I phages resembles a tail plate; however, those appendages may be an amorphous bundle of tail fibers as reported by Baigent (3). Better resolution of tail appendages is required to positively identify isolates with tail plate structures. Two type III phage (panel 4) were identified. These are similar to the Escherichia coli phage T3.

Restriction endonuclease analyses. Phage DNA was prepared from phage particles purified in CsCl density gradients. Early attempts to obtain complete digests resulted in failure, presumably because of agar inhibition of EcoRl (6). Cleaner digests were obtained by adding spermidine trihydrochloride at 0.005 M and ribonuclease A at 10 µg per ml;

however, better digests were obtained by using overlays and plates containing Gelrite. Addition of spermidine and ribonuclease A was not required to obtain complete digests of phage DNA obtained from plate enrichment on Gelrite.

To obtain separation of both large and small fragments, agarose gels of 1.5 (Fig. 2, left) and 0.7% (Fig. 2, right), respectively, were used. The molecular weights of the phage genomes were calculated by summing the molecular weights of the individual fragments as determined by their mobilities relative to standards. Some of the results of the restriction digests of the phage from PS179 were unexpected. Both PS179-421 and PS 179-422 gave rise to cloudy plaques containing phage of a Type 1 morphology and clear plaque phage frequently arose from phage purified from both culture supernatants when PS224 was used as host. It was not expected that the two cloudy plaque phage from PS179-421 and PS179-422 would be so distinctly different. (Compare lane f and e.) However, as expected, the cloudy plaque phage from PS179-422 was indistinguishable in restriction digests from a clear plaque variant, ϕ422-1. Both digests are not shown, however, the pattern representing both is given in lane e. It was also surprising that a second phage ϕ422-2 (lane d), initially thought to be a clear plaque variant of ϕ422, was quite distinct from the assumed parental phage (lane e). Likewise, distinctly different restriction digests were obtained for the clear plaque phage, ϕ421-1 (lane c) and the cloudy plaque phage, ϕ421 (lane f). The former was isolated from a lawn of PS224 which was confluently lysed with the latter. However, the clear plaque phage, ϕ421-1 (lane c) and ϕ422-2 (lane d) are identical except for a difference in mobility of the fourth largest band.

Inspection of the restriction digests of DNA from the phage isolated from sewage reveals that the two phages, 40-3 and 92-3, are indistinguishable from ϕ421 and ϕ422-1, respectively. (Compare lane e to h and lane f

to g.) Three additional unique phage isolates from sewage were identified and their restriction digests are shown in lanes i-k.

DISCUSSION

Our screening experiments are interesting in comparison to the earlier findings by Crosse (13) and Baigent (3). Crosse reported about 40% of Pseudomonas mors-prunorum isolates liberated phages but that none of the nine P. syringae pear isolates did. Our studies show that phage can be found in two of six isolates from pear (PS179 and B301). In fact, two distinct phage (ϕ421 and ϕ422) were obtained from the pear isolate PS179. Baigent indicated in her work that temperate phages were spontaneously released by several P. syringae isolates (3). Similarly, we have shown the liberation of phages by a number of P. syringae isolates from different hosts. However, lysogeny has not been proven for any of our isolates.

The host range of these isolates varies quite extensively. Our data indicate that several phages from strains known to produce SR (B15, PS146, PS270, PS359, PS362) can infect some strains known to produce ST (B455#1, B460). Likewise some strains known to produce ST (B427, B452, B454, B455#2, B456, and B457) produce phage which can infect B362, a strain known to produce SR. However, in general, phage have a broader host range in strains producing the same toxin as the strain from which they were isolated.

The origin of some of the clear plaque phage obtained from ϕ421 and ϕ422 on lawns of PS224 is at present not clear. Baigent speculated that the clear plaque forming isolates which appeared on confluently lysed plates inoculated with turbid plaque forming phage, were mutants. It is likely that the clear plaque phage ϕ422-1, which was isolated from plates confluently lysed with the turbid phage ϕ422, may be the result of a mutation (e.g. point mutation or a small deletion) since the digests of

these two phage isolates are identical. On the other hand, the pattern of ϕ421-1 and ϕ421 are distinct enough to eliminate the possibility that ϕ421-1 arose from ϕ421 by mutation or a simple genomic rearrangement. The same can be said for the relationship between ϕ422 and ϕ422-2. However, it is likely that ϕ421-1 and ϕ422-2 are of similar origin because their restriction digests differ only by the mobility of the fourth largest *Eco*R1 restriction fragment. Since ϕ421-1 and ϕ422-2 probably did not arise from PS179, then they must have arisen from PS224. If this is true, then during the induction and or replication of these prophages (ϕ421-1 and ϕ422-2) they must have become altered in their immunity properties or they would not be able to plaque on PS224. This alteration might have occurred by mutation or by recombination with the immunity region of the infecting phage from PS179 to give a new recombinant phage. In either case distinctly different restriction patterns would be expected for the clear plaque phage and the original infecting phage, and this is what was observed. The experiments needed to test this or other hypotheses have not been performed.

Finally, our data indicate that temperate phage can be found in sewage since two different isolates from culture supernatants were indistinguishable from phage isolated from sewage. Rigorous tests will need to be performed on the isolates from sewage before one can conclusively state that these phage are capable of lysogenizing *P. syringae* 224.

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Table 1. *P. syringae* strains tested for phage production.

Strain	Source[a]	Host	Toxin[b]
PS2	ICPB	Unknown	+?
B3A	DeVay	peach	+SR
PS9	ICPB	lilac	+?
PS9 rough	PS9	lilac	+?
PS9 mucoid	PS9	lilac	+?
B15	DeVay	almond	+SR
PS21	ICPB	pear	+SR
PS135	ICPB	pear	wSR
PS146	ICPB	plum	+SR
PS148	ICPB	peach	+SR
PS178	ICPB	cherry	wSR
PS179	ICPB	pear	+SR
PS179 rough	PS179	pear	+SR
PS224	ICPB	apricot	+SR
PS229	ICPB	raspberry	w?
PS230	ICPB	apple	+?
PS251	ICPB	plum	-
PS259	ICPB	*Cyamopsis*	w?
PS269	ICPB	pear	+SR
PS270	ICPB	apple	+SR
PS295	ICPB	foxtail	wSR
B301	DeVay	pear	+SR
B326	DeVay	cherry	+SR
B359	DeVay	millet	+SR
B359 white	B359	millet	+SR
B359 rough	B359	millet	+SR
B362	DeVay	bean	+SR

Strain	Source	Host	Toxin
B366	DeVay	sugarbeet	+SR
B368	DeVay	tomato	+SR
B382	DeVay	corn	+SR
B407	DeVay	foxtail	+SR
B427	DeVay	foxtail	-
PS955	Chatterjee	pear	+SR
PS134A	ICPB	grapefruit	w?
PS265	ICPB	citrus	w?
B475	DeVay	lemon	+ST
B452	DeVay	valencia orange	+ST
B453	DeVay	navel orange	+ST
B454	DeVay	navel orange	+ST
B455#1	DeVay	grapefruit	+ST
B455#2	DeVay	grapefruit	+ST
B456	DeVay	tangelo	+ST
B457	DeVay	navel orange	+ST
B458	DeVay	navel orange	wST
B459	DeVay	navel orange	+ST
B460	DeVay	lemon	+ST
Nesmyth #10	Nesmyth	?	+?

[a] ICPB, International Collection of Phytopathogenic Bacteria, Davis, CA; J. DeVay, Davis, CA; A. K. Chatterjee, Manhattan, KS; B. Nesmyth, Lexington, KY.

[b] SR, syringomycin; ST, syringotoxin; ?, toxic activity not identified. Toxin production criteria:

(-) colony completely overgrown with fungus

(w) colony not overgrown but no zone of inhibition

(+) at least 1 mm zone of inhibition

Identification of the toxic activity as SR or ST was determined for PS179, PS224 and PS955 by Currier and Morgan (submitted). All other determinations were previously reported (DeVay et al. 1978; Gross 1976; Vidaver and Buckner 1978).

Table 2. Plaque forming activity of culture supernatants.

Active supernatant	Host range[a]
B3A	B15, PS179, PS179 rough, PS224, PS229
PS9	PS135, B445#1
PS9 rough	B3A, PS135, PS179, B359, B455#1
PS9 slimy	PS135, PS362, B455#1
B15	B3A, PS148, PS224, PS229, PS955, B458
PS146	B455#1
PS148	PS179 rough, PS224, PS229
PS178	PS9
PS179	PS224
PS179 rough	PS224
PS230	PS9, B455#1, B460
PS251	B3A, B326
PS134A	PS9 slimy, B455#1, B455#2, B456, B457
PS265	B455#1, B460
PS270	B455#1
PS301	B475
PS359	B455#1
PS362	B455#1, B458
PS368	PS955
B427	B362, B454, B455#1, B455#2, B456, B457, B459
B452	B362, B455#1, B455#2, B460, Nesmyth#10
B454	B455#1, B455#2, B460, Nesmyth#10
B455#2	B362, B460, Nesmyth#10
B456	B362, B455#1, B455#2
B457	B455#1
B459	B455#2
B460	B454, B455#1, B456

Active supernatant	Host range[a]
B475	PS9
Nesmyth #10	B454, B455#1, B455#2, B456, B457, B459

[a]See Table 1 for strain description.

Table 3. Description of Phage Isolates having activity on stains 179 and 224.

Isolate	Source	Plaque morphology	Genome size-Kb	Particle size-nm (ρ-g/cc^3)	EM
92-2C	Sewage	Large, clear 3-5mm with halo	24	Head 54x48 (1.507) Tail 20x10	III
40-1	Sewage	Large, clear 3-5mm	25	Head 54x48 (1.498) Tail 20x10	III
40-3	Sewage	Small, turbid 1-2mm	39	Head 54x48 (1.507) Tail 162x 12	I
40-4	Sewage	Small, turbid 1-2mm	32	Head 54x48 (1.507) Tail 172x12	I
92-3	Sewage	Small, clear 1-2mm	49	Head 54x48 (1.505) Tail 175x12	I
ϕ421	PS179(421)	Small, turbid 1-2mm	39	Head 54x48 (1.508) Tail 162x12	I
ϕ421-1	PS179(421)	Small, clear 1-2mm	51	Head 70x48 (1.513) Tail 195x12	II
ϕ422-1	PS179(422)	Small, clear 1-2mm	49	Head 54x48 (1.510) Tail 184x12	I
ϕ422-2	PS179(422)	Small, clear 1-2mm	50	Head 70x54 (1.507) Tail 185x12	II
B15	*P. syringae* B15	Pinpoint, 0.5-1mm turbid	52.5	Head 54x42 (1.516) Tail 172x12	IV

Fig. 1 Electron micrographs of the representative phage morphologies (bar = 100nm). (1) Type I - (ϕ421) (2) Type I - (92-3) (3) Type II (ϕ421-1) (4) Type III (40-1) (5) Type IV (B15).

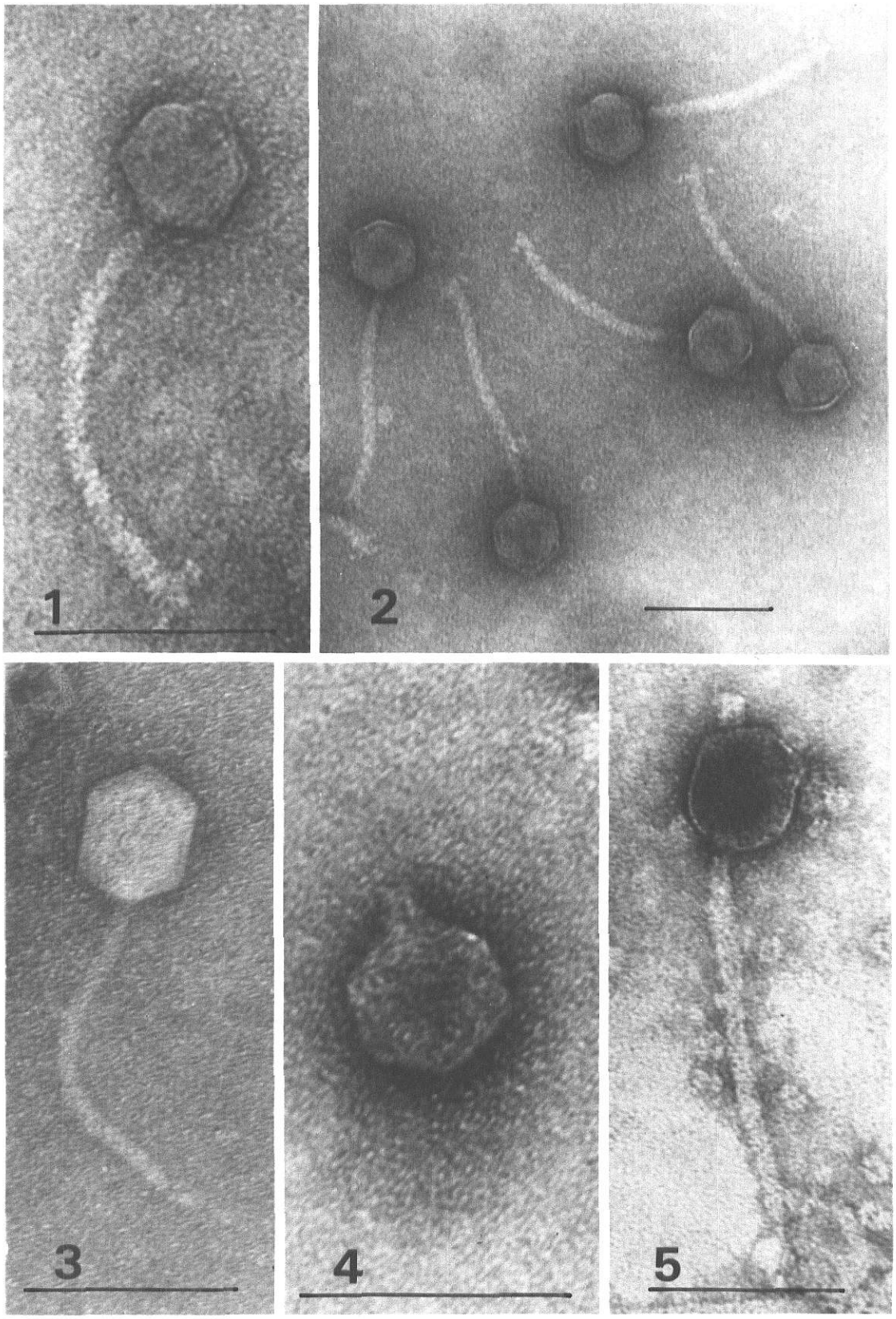
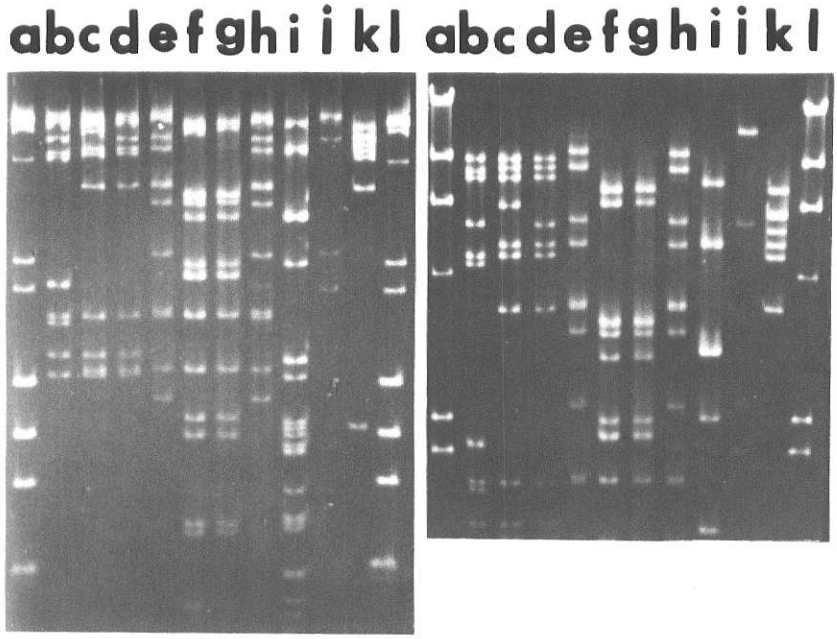

1
2
3
4
5

Fig. 2 Electrophoretic patterns (1.5% gel left/0.75% gel right) of EcoR1 digests of bacteriophage DNA. Lanes a and l, markers consisting of a mixture of bacteriophage lambda DNA digested with HindIII and a HaeIII digestion of the bacteriophage ϕX174. The phage DNA samples in each lane are: (b) B15 (c) ϕ421-1 (d) ϕ422-2 (e) ϕ422 or ϕ422-1 (f) ϕ421 (g) 40-3 (h) 92-3 (i) 40-4 (j) 40-1 (k) 92-2C. Size of lambda standards in Kb: 23.7, 9.46, 6.67, 4.26, 2.25, and 1.96. Size of ϕX174 standards in Kb: 1.35, 1.08, 0.87, and 0.6.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SECTION III

GENERALIZED TRANSDUCTION IN THE PLANT PATHOGEN *PSEUDOMONAS SYRINGAE*

ABSTRACT

Bacteriophages isolated from culture supernatants of Pseudomonas syringae and from sewage, transferred various chromosomal genes to P. syringae 224. The number of transductants recovered per ml was not altered appreciably by UV irradiation of selected phage isolates or by mating the Inc P2 plasmid R38 into a P. syringae 224 arginine auxotroph. Increasing the MOI of one transducing phage, B15, from MOI 1 to MOI 10, resulted in up to a ten fold increase in the number of transductants recovered, although the actual transductional frequency remained about the same. Treatment of transduction mixtures with deoxyribonuclease did not affect transductional frequency.

INTRODUCTION

Pseudomonas syringae pv. syringae (7) is an important plant pathogen which is capable of infecting at least 40 genera of plants. Most strains of P. syringae regardless of host origin produce syringomycin (SR) a low molecular weight toxin with a broad spectrum of activity (12). Other toxins have also been isolated from P. syringae including syringotoxin (ST) which has properties similar to SR (9). Certain strains appear to show a relationship between SR production and pathogenicity (8); however, strains which produce SR but which are not pathogenic have also been isolated (1). Also, strains which do not produce SR or ST and which are still pathogenic have also been isolated (11). Thus, other factors appear to be involved in disease development. These may include the ability to grow epiphytically on the host, the ability to grow within the host, and the ability to produce toxin.

To determine the nature of toxin production and other prerequisites for disease development, genetic exchange systems will need to be developed including the ability for fine structure mapping of genes. Recently, Chatterjee reported the isolation of a generalized transducing phage of the plant pathogen Erwinia chrysanthemi (5). Temperate phages have been reported for P. syringae (2), but no indication of transductional activity with any of these isolates has been reported. In this study phages obtained from culture supernatants of P. syringae and sewage, which have been characterized earlier (see previous chapter) were assayed for the ability to transduce various chromosomal markers. Four isolates from culture supernatants and one isolate from sewage possessed transductional ability and at least one of the isolates from culture supernatants was capable of generalized transduction.

MATERIALS AND METHODS

Organisms and culture conditions. The P. syringae prototrophs used

in this study have been previously described (see previous chapter). Mutant strains were obtained by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis, followed by penicillin enrichment. Streptomycin resistant P. syringae 224 strains were isolated as spontaneous variants. An overnight culture of cells grown in NBY broth was concentrated by centrifugation to about 10^{10} cells per ml and then 0.1 ml was plated onto NBY agar containing 100 µg per ml of streptomycin (Sigma). Resistant clones were subcultured onto NBY agar containing 100 µg per ml of streptomycin and then stored on NBY agar. Pseudomonas aeruginosa PAO 1670 (10) was obtained from Dr. Bruce Holloway, Monash University, Australia. Stock cultures were maintained on NBY agar (13). Cells for experimental use were cultured in NBY broth at 29°C. Transductants were plated on Ng agar consisting of glucose and minimal salts (4). Media for the P. aeruginosa PAO 1670 X P. syringae matings consisted of Ng agar plus 50 µg per ml of arginine, 60 µg per ml of streptomycin and 5 µg per ml of tetracycline. The arginine and drug solutions were filtered through a 0.22 µ Millipore filter prior to being added to autoclaved media. Media components including agar were obtained from Difco (Detroit, MI).

UV irradiation. For bacteriophage killing curves and transduction experiments, stock phage lysates with a titer of about 10^{10} PFU per ml in NBY broth, were diluted 10 fold in phage buffer (0.01 M Tris, 0.01 M $MgSO_4$, pH 7.0). The samples for irradiation consisted of a 15 ml volume of the above mixture in a glass petri dish (100 mm diameter). The samples were stirred while being exposed to UV rays (254 nm) at a rate of 40 ergs per mm^2 per sec. For killing curves a 0.1 ml sample was withdrawn at regular time intervals and sequentially diluted in phage buffer and then plated with 0.1 ml of an overnight culture of P. syringae 224 diluted to 10^9 cells per ml, using the double agar overlay method.

Bacteriophage adsorption. Equal volumes of bacteriophage lysate,

about 10^9 PFU per ml, and an overnight culture of P. syringae 224 cells diluted to 10^9 cells per ml, were mixed and incubated at room temperature (25°C). At various times 50 µl samples were withdrawn and diluted in 5.0 ml of NBY broth to stop further adsorption. Cells with adsorbed bacteriophage were removed by centrifugation. The PFU remaining in the supernatant were sequentially diluted in phage buffer and plated with 0.1 ml of an overnight culture of P. syringae 224 cells diluted to 10^9 cells per ml using the double agar overlay method.

Transduction. Equal volumes (1.0 ml) of UV irradiated or nonirradiated phage in phage buffer and an overnight culture of P. syringae 224 auxotrophic bacterial cells diluted to 10^9 cells per ml in NBY broth were mixed at various multiplicities of infection, usually 1.0. Auxotrophic cells plus sterile phage buffer served as the control. To control for bacterial contamination, a 20 µl aliquot of the original unirradiated phage stock (about 10^{10} PFU per ml) was plated in duplicate on Ng agar. All samples were incubated for two hours at room temperature (25°C). The cells were then pelleted by centrifugation and resuspended in 1.0 ml of 0.01 M sodium phosphate buffer (pH 7.0). A 0.1 ml sample was plated in duplicate on Ng agar and the plates incubated 4 to 5 days prior to counting transductants.

Toxin assay. Wild type strains, auxotrophic derivatives, and transductants were screened for syringomycin production by picking colonies to potato dextrose agar plates (Difco) supplemented with 0.4% casamino acids and adjusted to pH 7.0 prior to autoclaving. The plates were incubated 6 days at 29°C and toxin activity was then determined by spraying a conidial suspension of the fungus Geotrichium candidum onto the plates. Zones of inhibition were observed after overnight incubation at 29°C.

Oxidase and fluorescence tests. P. syringae isolates are oxidase negative but do produce a fluorescent pigment. Oxidase activity was

determined by plating transductants onto NBY agar plates; after 24 hours of growth, the plates were flooded with 1% p-amino-dimethylanaline oxalate (Difco). Fluorescent pigment production was monitored by subjecting transductants on Ng plates to UV light at 360 nm.

Mating experiments. Cells for mating experiments were grown overnight in NBY broth at 29°C to stationary phase (about 2 X 10^9 to 5 X 10^9 cells per ml). Cell concentrations of the donor and recipient were adjusted to achieve a 1:1 ratio. Nonselective mating was performed by plating 20 µl of each culture onto NBY agar and incubating the plates overnight at 29°C. Growth in the area of the mating was scraped off and resuspended in 1 ml of 0.01 M sodium phosphate buffer (about 10^{10} cells). A 0.1 ml sample of this mixture was plated onto Ng media containing arginine, streptomycin, and tetracycline (NgAST). Sequential dilutions of the resuspension, diluted into 0.01 M sodium phosphate were also plated. Resultant colonies were subcultured twice on NgAST and assayed for the R38 plasmid by the mini-lysate technique.

Mini lysate plasmid isolation. Overnight cultures were grown to stationary phase and then centrifuged at low speed and resuspended in TES buffer (0.05 M Tris, 0.005 M EDTA, 0.05 M NaCl, pH 8.0). Cells were then lysed in an alkaline SDS solution according to the procedure of Casse' et al. (3). After 30 min at 34°C the lysates were neutralized and plasmid DNA purified by the method of Currier and Nester (6).

DNAase treatment. $MgCl_2$ and Bovine Pancreas DNAase (grade 1 Boehringer, Indianapolis, IN) was added to 1 ml of bacteriophage lysate (10^9 PFU) to yield a final concentration of about 20 µg per ml (approximately 50 Kunitz units) of DNAase and 0.005 M $MgCl_2$. The mixture was incubated for 2 hrs at 37°C and assayed directly for PFU and transducing activity. As a control 100 ng of *Eco*R1 digestion fragments of bacteriophage lambda were added to the phage and digested as above.

RESULTS

Phage Isolation. The isolation of the phages used in this study was previously reported (see previous chapter). Initially, a turbid plaque forming phage, ϕ422, was used. This isolate came from a lyophilized culture of P. syringae 179 designated PS179-422. This phage was dropped in favor of a clear plaque variant ϕ422-1 which had an identical restriction endonuclease pattern with EcoR1 and appeared to transduce at about the same frequency and was easier to enrich. When the phage isolate B15 was finally brought to high titer, it was used in all subsequent work since it had the highest transduction rate of any of the isolates tested (Table 1).

Phage properties. Adsorption studies were performed with selected phage stocks used in transduction studies. After two hours, the phage isolates tested (ϕ421, ϕ422, ϕ422-1 and B15) all showed greater than 99% adsorption to the P. syringae 224 arg A auxotroph (Fig. 1). The adsorption kinetics of ϕ422-1 were essentially identical to those shown in Figure 1 for ϕ422. Adsorption studies with a number of PS224 auxotrophs and phages ϕ421 and ϕ422 revealed that greater than 99% of the phage were adsorbed within two hours. The data indicate that B15 adsorbs much more rapidly than either ϕ421 or ϕ422 and it is possible that transduction experiments occurring over a one hour interval are feasible. The biphasic kinetics of adsorption shown has also been reported by Chatterjee for an Erwinia chrysanthemi transducer, Erch 12 (5).

Transduction studies. In order to increase the transduction frequency observed (Table 1), various approaches were tried. The first involved UV irradiation of the phage. Survival data of selected phage isolates are indicated by Figure 2.

The number of transductants per ml increased about 2-5 times following UV irradiation of transducing phage for the ϕ422-1 isolate and

about 2 times for the B15 isolate (data not shown). These studies also indicated that the sewage isolates 40-1 and 92-2C were much more sensitive to UV than the other isolates. Most phage isolates showed about 99% killing at 90 seconds but 40-1 and 92-2C showed greater than 99.9% killing at this time point (data not shown). UV irradiation did not appear to affect adsorption of ϕ422 or ϕ422-1 phages except after extremely long periods of irradiation, i.e. greater than 150 seconds. Although UV irradiation did not increase the number of transductants per ml, the actual transduction frequency rose as exposure to UV was increased. This rise was presumably due to an increase in the relative number of transducing praticles as viable phage particles were killed by UV.

The use of stationary phase cells as recipients in transduction experiments increased the transduction frequency about two-fold (data not shown). Broth cultures of P. syringae 224 arg A were allowed to grow well into stationary phase, i.e. 48 to 72 hours, and cultures from both 48 and 72 hours both showed a two-fold increase in the number of transductants recovered.

Mating the Inc P2 plasmid R38 into PS224 arg A did not increase the transduction frequency observed as has been reported for phage E79 in Pseudomonas aeruginosa (10). R38 transfer was verified by the expression of plasmid markers by the recipient and agarose gel electrophoresis (data not shown). The PS224 arg A strain does not contain any resident plasmids, and the presence of a 140 X 10^6 dalton band after mating indicated R38 had been transferred.

Increasing the MOI dramatically increased the number of transductants recovered per ml for phage B15, although the actual transductional frequency remained about the same (Figure 3). This same effect was not observed in preliminary work with phage ϕ422-1 where an MOI of 1 appeared to give optimal transduction frequency. Increasing the MOI actually

decreased the number of transductants.

All transductants that were tested were oxidase negative and produced both syringomycin and a UV fluorescent pigment. All these tests are indicators that the transductants were P. syringae.

Transduction of auxotrophic markers. Table 2 shows the transductional frequency of a number of markers with phage isolate B15. This data indicates that B15 is a generalized transducing phage. DNAase did not affect transduction frequency compared to a nontreated control, and it did not diminish phage titer (data not shown), but it did totally eliminate the presence of added EcoR1 fragments of bacteriophage DNA as determined by running a sample on an agrose gel before and after DNAase treatment. Transduction frequency appeared to vary depending on the marker transduced. Arginine transductants were tested for toxin production and while 100% of Arg A auxotrophs were SR^-, 100% of transductants were SR^+. Cells transduced with the phage isolate ϕ422 were found to be sensitive to phage ϕ422. On the other hand, all B15 transductants tested were resistant to phage B15. These clones have not yet been tested for the ability to liberate B15. About 70% of strains isolated from turbid plaques (formed by phage ϕ422-1 on PS244 arg A) and purified by repeated subculture were resistant to phage ϕ422-1. These strains adsorbed ϕ422-1 equally as well as the PS224 arg A controls, so it is presumed they have been lysogenized. When used as recipients in transduction experiments, these cultures did not yield an increase in transduction frequency. Supernatants of these cultures did not form plaques on P. syringae 224 arg A. This same isolation procedure was used in attempts to find cultures resistant to B15, however, no such isolates have yet been obtained.

DISCUSSION

The transducing phages described in this work are the first phages

reported to mediate transduction in P. syringae. The large increase in transductants recovered per ml by increasing the MOI of phage B15, indicates that this phage readily lysogenizes upon infecting the recipient cell. On the other hand the phage sensitivity of the transductants obtained with ϕ422-1 indicate that these transductants are not lysogenized. This may explain why increasing the MOI decreased the number of transductants. However, it is difficult to perceive how the transductants could escape infection at an initial MOI of 1.

Although it had no effect in transduction, the ability of the PS224 arg A auxotroph to accept the R38 plasmid at a frequency of about 10^{-3} per donor indicates the possibility of other Inc P2 plasmids being used in genetic analysis of P. syringae. Some of these may be of importance in increasing transductant survival in transduction experiments with the various clear plaque forming transducers.

The phage isolate 92-3 showed the ability to plaque on P. syringae 224 and 179. It is hoped that 92-3 will show transducing activity in PS 179 since no turbid plaque forming isolates have been discovered for this strain.

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Table 1. Transducing Phage Isolates and Frequency of Transduction.

Phage Stock[a]	Transduction Frequency of arg A[b]
B15	$3.0X10^{-7}$
ϕ421-1	$1.3X10^{-8}$
ϕ422	$5.0X10^{-8}$
ϕ422-1	$4.3X10^{-8}$
ϕ422-2	$1.4X10^{-8}$
92-3	$1.2X10^{-7}$

[a]For description of phage isolates see previous chapter.

[b]Transduction frequencies are expressed per plaque forming unit.

Table 2. Generalized Transduction by Phage Isolate B15.

Marker	ArgA	TrpA	TrpB	TrpC	HisA	SerglyB	MetA	AroA
Transduction frequency[a]	$3x10^{-7}$	$7.5x10^{-7}$	$1.8x10^{-7}$	$1.7x10^{-7}$	$8.2x10^{-8}$	$2.8x10^{-7}$	$2.4x10^{-8}$	$1.3x10^{-7}$
Reversion frequency	$<10^{-9}$	$2x10^{-9}$	$2x10^{-9}$	$6x10^{-8}$	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$	$<10^{-9}$
DNAase Transduction frequency	$3.2x10^{-7}$	ND	ND	ND	ND	ND	ND	ND

[a]Transduction frequencies based on plaque forming unit input.

ND-Not done.

Fig 1. Adsorption of selected Pseudomonas syringae bacteriophage isolates to P. syringae 224 wild-type. Adsorption experiments were performed as described in the text.

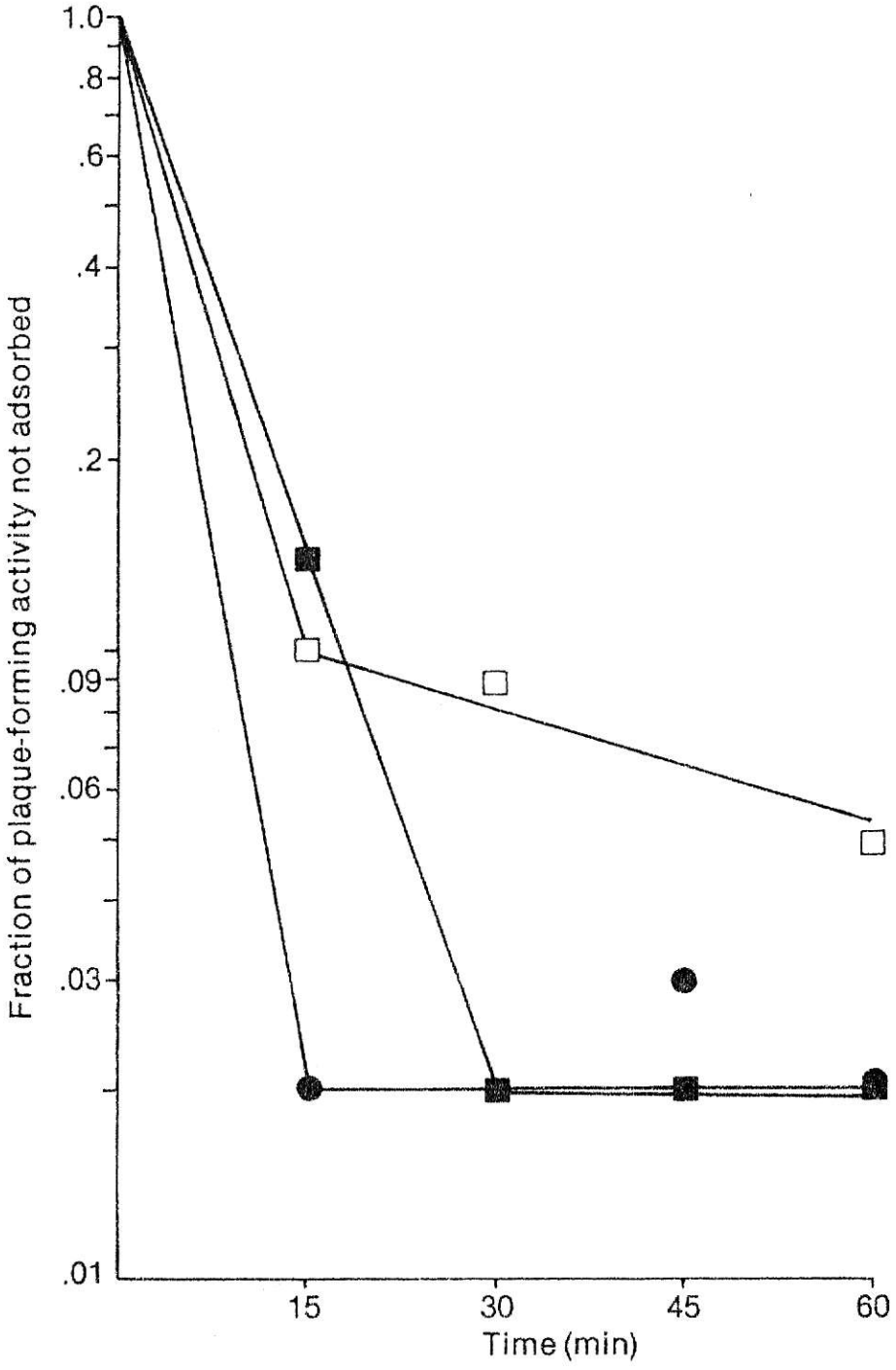

1.0
.8
.6
.4
.2
.09
.06
.03
.01
Fraction of plaque-forming activity not adsorbed
15
30
45
60
Time (min)

Fig. 2. UV survival curves of selected Pseudomonas syringae bacteriophage isolates. Phage were irradiated at 40 ergs/mm^2 per sec as described in the text.

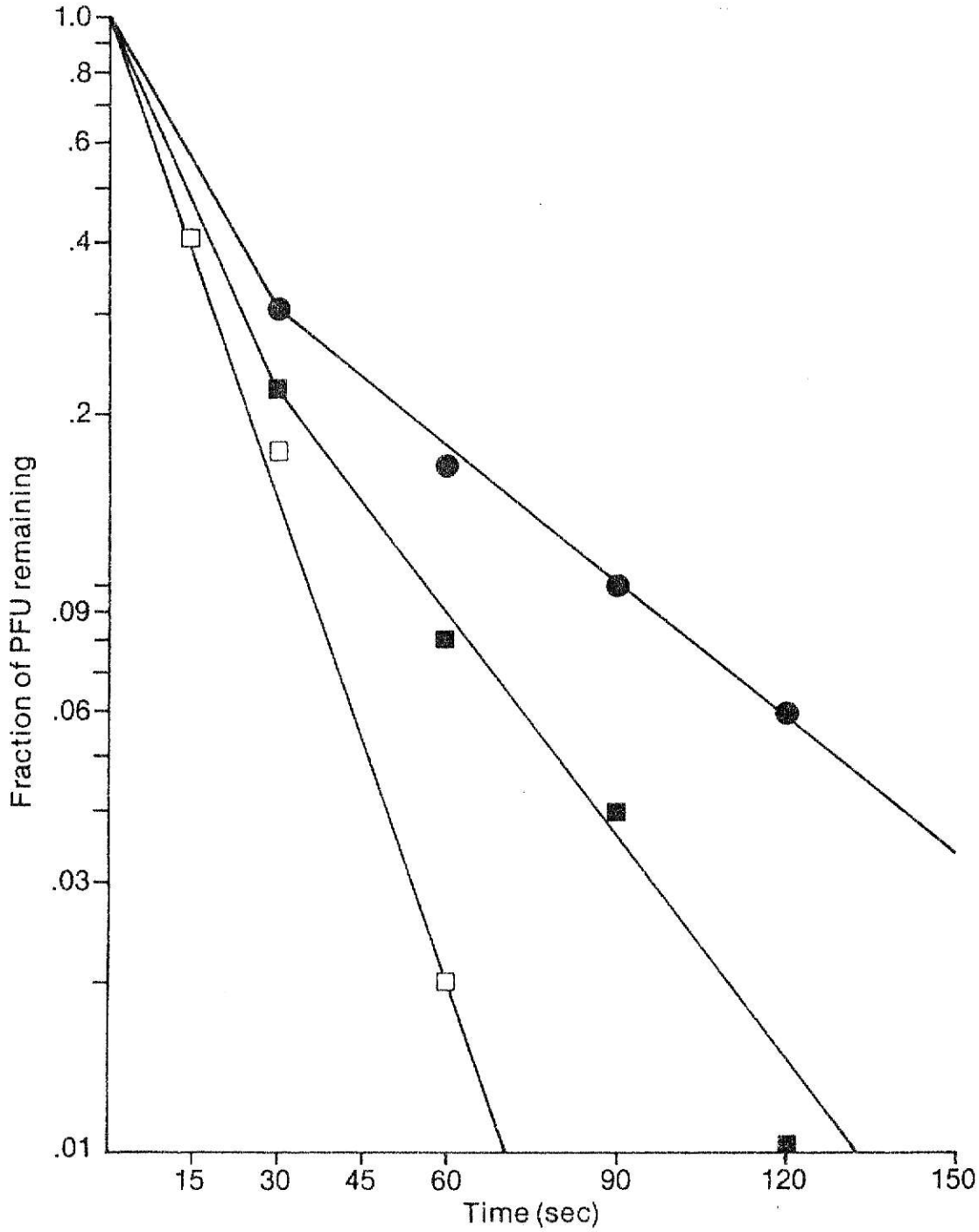
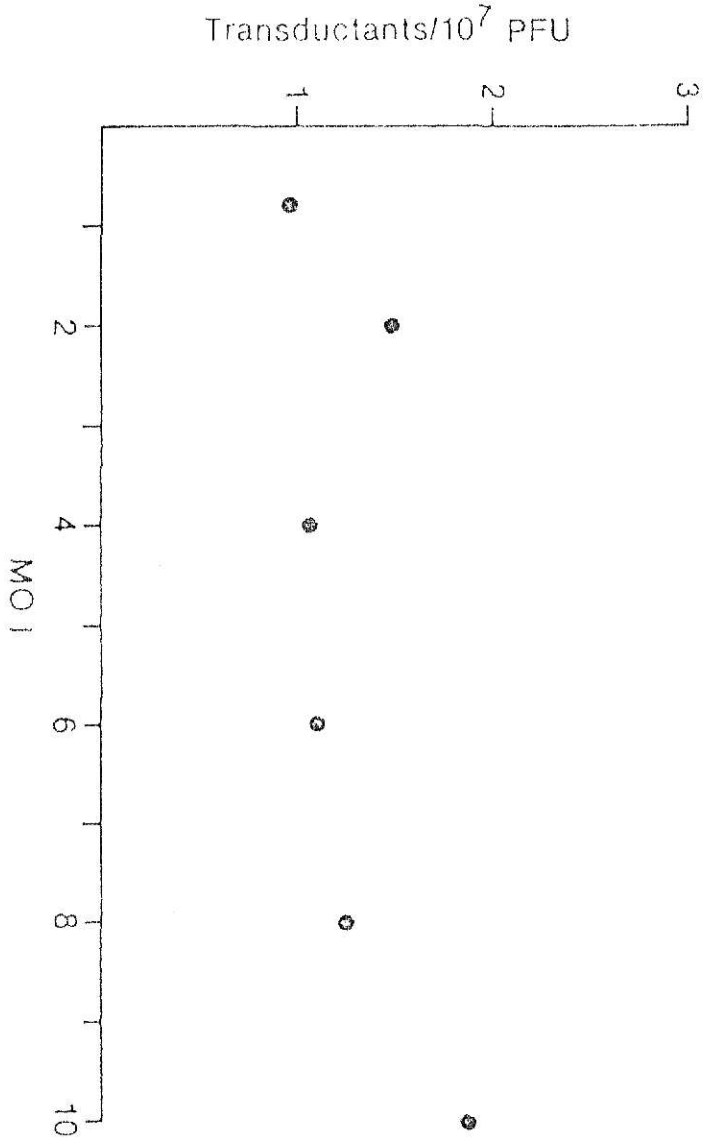

1.0
.8
.6
.4
.2
.09
.06
.03
.01
Fraction of PFU remaining
15
30
45
60
90
120
150
Time (sec)

Fig. 3 Effect of varying the multiplicity of infection of bacteriophage isolate B15.


Transductants/10^7 PFU
1
2
3
MOI
2
4
6
8
10

SECTION IV

APPENDIX

APPENDIX

Culture growth characteristics. To isolate viruses from culture supernatants of PS179 lyophils, B3A, KS444, and 1797 (the latter two strains are derivatives of 179 wt), these strains were grown in Luria broth, NBY broth and a simple salts medium (Ng). Growth as measured by turbidity was maximal in Luria broth. The PS179 lyophils were in stationary phase after 18 hours growth in NBY, i.e. Klett 300 (#66 filter) or above. All incubation was at 30°C unless stated otherwise. Strains PS148, PS224 and B3A were also above Klett 300 after 18 hours growth in NBY. PS229 on the other hand grew very slowly and was late log or about Klett 180 at 18 hours in NBY.

The doubling time of PS179-422 was about 1 hour in NBY and the doubling time of PS224 was about 50 minutes in NBY. After about 8 hours and 10 hours in NBY (10 ml shake cultures at 150 RPM) strains PS224 and PS179 (422), respectively, were in the stationary phase of growth. The doubling time of most PS224 auxotrophs in NBY was about 2 hours. Stationary cultures (above Klett 300) were achieved after 24 hours of incubation. Isolates with slower growth rates included the ser/gly B, met A and pro A auxotrophs. The ser/gly B and met A auxotrophs had doubling times of about 3 hours and the pro A auxotroph had a doubling time of about 4 hours. Approximately 36 hours of incubation time was needed to achieve stationary phase with the pro A auxotroph.

Overnight cultures of PS179-422 and PS224 diluted to Klett 100 formed uniform lawns of desirable density using NBY media, but lawns were poor using Luria media and extremely poor using Nutrient Agar media. Cultures of strains PS179-422 and PS224 were tested for lawn formation at 0.1 ml Klett 100, 50 and 10 inputs using NBY media. Klett 100 and Klett 50 cultures formed uniform lawns of desirable density, however, the density of Klett 10 lawns was not satisfactory. Plating lawns with stationary or

log cultures of PS224 did not affect plaque formation of phage B3A. Optimal lawn densities were achieved with 0.1 ml of Klett 100 and 0.2 ml of Klett 100 cultures for PS224 and PS179-422, respectively.

Phage induction. Overnight cultures of PS179-422, KS444 and B3A grown in NBY were diluted to Klett 5 to 10 and then allowed to grow to Klett 20 to 30 prior to adding 0, 10, or 30 µg per ml of mitomycin C. Flasks were then placed in the dark and shaken at 150 RPM. The Klett was monitored at regular intervals to determine phage induction. At minimal Klett values, cultures were centrifuged at 10,000 RPM for 10 minutes, and the supernatant was filtered through a 0.22µ filter or treated with chloroform and then stored at 4°C. Supernatants (0.1 ml-1.0 ml) were plated with 0.1 ml of PS224 cells at Klett 100. Indications of plaque forming activity were noticed at 30 µg per ml of mitomycin C for PS179-422 and 30 µg per ml for B3A. These plaque like clearings could not be propagated. Some plates were completely void of lawn growth. A bacteriocin(s) may have been induced but since this was not followed up, this reasoning is only speculative.

Phage that were spontaneously liberated and enriched were fairly stable once titers above about 10^8 to 10^9 PFU per ml were obtained. The phage stocks were slightly more stable in phage buffer compared to NBY broth. Except for phage isolate 92-3, all phages appeared stable when stored over chloroform at 4°C. It must be noted that when phage were at low titer, i.e. below about 10^6 PFU per ml, they appeared to be somewhat sensitive to chloroform, so filtering following initial enrichment is suggested.

Phage adsorption studies. To determine optimal ion concentrations and mixtures for phage adsorption, the salt solutions listed in Table 1 were buffered with 0.001 M Tris pH 7.0 and used in an adsorption experiment with phage ϕ421 and host PS224 utilizing the procedure

mentioned in Section II. No differences in adsorption were noted using the various buffers, except for a slight increase in adsorption when the 0.01M KCl buffer was used. This was only about a two fold increase compared to adsorption with $MgSO_4$; therefore, it was decided to continue with the $MgSO_4$ buffer in subsequent experiments. All buffers allowed a one log adsorption of phage in ten minutes. Due to rapid phage adsorption it was not necessary to pre-adsorb phage prior to plating. Phage ϕ421 was used in a separate adsorption experiment with a PS224 culture with phage being added at various stages of the host growth cycle to determine the effect of host age on phage adsorption. No change in adsorption was noticed as the culture progressed in age from 14 to 42 hours.

Phage enrichment. Table 2 shows phage titers achieved by using PEG 6000 at 10% w/v and NaCl at 0.5 M. PEG 6000 concentrations lower than 10% did not appear to result in titers as high as those obtained using 10% PEG. The titers shown are the maximum titers obtained, however, all isolates were capable of being routinely enriched to 10^{11} PFU per ml by this technique. After precipitation supernatants were assayed for phage and found to contain only about 0.1% of the total phage titer. Also, during plate enrichment studies, phage trapped in the underlayer were titered and found to represent less than 0.1% of the total phage present in the overlay. Table 2 also indicates the approximate number of plaques per overlay to obtain maximum phage titers using either NBY agar or NBY Gelrite overlays.

Estimating phage DNA concentration and buoyant density. The Saran wrap and ring or 'spot' method outlined in Section I gave more accurate estimations of actual DNA concentrations than using a Gilford spectrophotometer and scanning samples from 230 nm to 320 nm. This was especially true for DNA samples prepared from plate enrichments that contained agar.

A260/A280 ratios of phage samples ranged from 1.02 to 2, with most isolates indicating a value of about 1.2. It was also noted that there was a strong absorbance at 230 nm for most samples. The nature of this absorbance was not determined. The highest concentration of DNA achieved was approximately 120 µg per ml for ϕ422-1 phage isolate. This estimation was based on a Gilford reading and is therefore not an accurate determination. Using the 'spot' method, concentrations of about 20 µg per ml were obtained, but most isolates yielded only 10-20 µg per ml. Following rebanding yields of 5-20 µg per ml of DNA were obtained. For a ϕ421-1 stock with a titer of 7×10^{11} prior to CSCl purification, 10 µg per ml of DNA was obtained following rebanding. Buoyant densities of phage DNA in 0.2 µg per ml of EtBr are listed in Table 2.

Phage DNA restriction analysis. Table 3 indicates other restriction enzymes tested on the phage preps listed. HindIII yielded a multitude of bands below 2 Kb in size for both ϕ421 and 40-4 phage isolates. The 92-2A isolate, which is the same as the 92-2C isolate based on restriction analysis, gave a usable digest with HindIII, i.e. one from which MW could be easily ascertained.

To restrict agar enriched phage DNA, spermidine trihydrochloride, STHC, or spermidine phosphate was added to digestion mixtures at concentrations ranging from 0.001 to 0.01 M and pre-incubated for 0 minutes to 120 minutes with the DNA prior to enzyme addition. STHC and spermidine phosphate gave optimal restriction at 0.005 M. No pre-incubation was required for STHC to exert its effect. Agar enriched phage DNA was also rebanded in saturated ethidium bromide CsCl gradients (20 µg per ml EtBr) and in 0.2 µg per ml EtBr CsCl gradients. Inhibition of restriction appeared to be less for the 0.2 µg per ml samples, but results were ambiguous and this experiment would have to be repeated for conclusive results. Attempts to find fractions containing maximal amounts of DNA

following CsCl phage purification by diluting 5 µl of each fraction sample in a solution of 5 µl of 2% sodium sarcosinate (Sarkosyl) and 2 µg per ml of EtBr failed to consistently indicate fractions with maximum concentrations of DNA. This method when modified may prove successful for selected isolates, however, it was particularly unsuitable for the lytic sewage isolates 40-1 and 92-2C. Bioassays of fractions proved more reliable than the Sarcosyl assays in determining fractions containing maximum amounts of DNA. The Sarcosyl assay revealed that the fractions with the most DNA did not alway correspond to the fraction that gave the best bioactivity, but the two fractions were always with 1 or 2 0.2 ml fractions of each other.

UV mediated transduction. A preliminary experiment with ϕ422 phage isolate indicated UV enhanced transduction at a transductional frequency of about 6×10^{-7} for phage irradiated 90 seconds. This high transductional frequency was not repeatable however. In a separate experiment old PS224 arg A cells (from a plate stored at 4°C for one month) were diluted to Klett 100 and gave a five fold increase in transductants using ϕ422-1 phage irradiated with UV for 90 seconds. The increase was presumably due to increased recombination efficiency in the recipient. This experiment was also not repeatable. UV irradiation of the PS224 arg A host did not increase the number of transductants recovered using the B15 phage (non-irradiated phage). Cells were irradiated for 15 seconds and 30 seconds at 16 ergs/mm^2 per sec. The viability of the culture irradiated for 15 seconds was 99% and that of the culture irradiated 30 seconds was 95%. This experiment should be repeated since proper controls were not run, i.e. plating irradiated cells plus sterile phage buffer on minimal media to check for the effects of UV on reversion frequency.

Table 1. Salt solutions used in phage adsorption studies.

A. .01 M KCl, .1 M NaCl, .001 M $CaCl_2$

B. .1 M NaCl, .01 M KCl

C. .01 M KCl, .1 M NaCl, .01 M $MnCl_2$, .001 M $CaCl_2$

D. .01 M $MgCl_2$, .1 M NaCl, .01 M $MnCl_2$

E. .01 M KCl

F. .001 M $CaCl_2$

Table 2. Phage enrichment and DNA buoyant density[a].

Isolate	PEG titer (PFU/ml)	Confluency[b]	DNA ρ (g/cc^3)
40-1	8×10^{11}	10^3-10^4	1.714
40-3	1×10^{12}	10^6	1.703
40-4	1×10^{12}	10^6	1.703
92-2C	2×10^{11}	10^4	1.716
92-3	1×10^{12}	10^6	1.699
ϕ421	3×10^{12}	10^5	1.705
ϕ421-1	2×10^{12}	10^4-10^5	1.700
ϕ422-1	1×10^{12}	10^5	1.703
ϕ422-1	1×10^{12}	10^4-10^5	1.713
B15	3×10^{11}	10^6-10^7	1.713

[a]Preps centrifuged in 57% CsCl containing 0.2 g per ml EtBr.

[b]Approximate number of plaques to achieve maximum phage titers.

Table 3. Restriction endonulease survey of selected phage preps.

Enzyme	Phage tested	Phage restricted
HindIII	ϕ42l, 40-4, 92-2A	ϕ42l, 40-4, 92-2A
SmaI	ϕ42l, 40-4, 92-2A	None
XhoI	ϕ42l, 40-4	None
KpnI	ϕ42l, 40-4	None
PvuII	ϕ42l, 40-4	None
AvaI	ϕ42l, 40-4	None
SalI	ϕ42l, 40-4	None

ENDONUCLEASE RESTRICTION ANALYSES AND TRANSDUCTION ANALYSES ON BACTERIOPHAGES OF THE PLANT PATHOGEN PSEUDOMONAS SYRINGAE

by

RUSSELL O NORDEEN

B.A., University of California at Los Angeles, 1975

AN ABSTRACT OF A MASTER'S THESIS

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Bacteriophages isolated from culture supernatants of the plant pathogen Pseudomonas syringae and from sewage, were assayed for cleavage by the restriction endonuclease Eco R1 and for the ability to transfer various chromosomal genes to P. syringae auxotrophs. Eight unique phages were identified based on restriction analysis. The buoyant density, electron microscope morphology and molecular weight of DNA was determined for these isolates. Selected phage isolates transferred various chromosomal genes to P. syringae 224 auxotrophs. The number of transductants recovered with one transducing phage, B15, was increased up to ten fold by using an MOI of 10, although actual transductional frequency compared to MOI 1 was not signficantly altered. UV irradiation of phage isolates did not appreciably increase transductional frequencies observed. The Inc P2 plasmid, R38, did not increase transductional frequency when mated into a P. syringae 224 arg derivative.