Generalized Transduction in the Phytopathogen *Pseudomonas syringae*†

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Received 22 December 1982/Accepted 5 April 1983

Bacteriophages isolated from culture supernatants of *Pseudomonas syringae* pv. *syringae* and from sewage transferred various chromosomal genes to *P. syringae* PS224. Linkage between arginine and tryptophan loci was demonstrated. The number of transductants recovered per milliliter was not altered appreciably by UV irradiation of selected phage isolates. In addition, the presence of the IncP2 plasmid R38 in a *P. syringae* PS224 arginine auxotroph did not increase the transduction frequency as it does in *Pseudomonas aeruginosa*. Increasing the multiplicity of infection of transducing phage Pssy15 from 1 to 10 resulted in up to a 10-fold increase in the number of transductants recovered, although the actual transductional frequency remained about the same. Treatment of transduction mixtures with DNase did not affect transductional frequency.

*Pseudomonas syringae* pv. *syringae* (7), hereafter referred to as *P. syringae*, 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 (13). Another toxin, called syringotoxin, which has properties similar to SR (9), has also been isolated from *P. syringae*. Certain strains appear to show a relationship between SR production and pathogenicity (8); however, SR-producing strains isolated from certain hosts may not be pathogenic on certain other plants (1). Also, strains which do not produce SR and which are still pathogenic have also been isolated (12). Thus, factors other than just toxin production appear to be involved in disease development.

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 and Brown reported the isolation of a generalized transducing phage of the plant pathogen *Erwinia chrysanthemi* (4). 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 partially characterized (11), were assayed for the ability to transduce various chromosomal markers. Four isolates from culture supernatants and one isolate from sewage possessed transductional ability, and one of the isolates from culture supernatants was shown to be capable of generalized transduction.

**MATERIALS AND METHODS**

**Organisms and culture conditions.** Two *P. syringae* prototrophs (PS179 and PS224) were used in this study and have been previously described (11). These strains were originally isolated from pear and apricot, respectively. Mutant strains were obtained in this laboratory by standard procedures, using N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis followed by penicillin enrichment. *P. syringae* KS35 is an arginine auxotroph of PS224 that was used extensively in preliminary studies. When other auxotrophic markers were introduced into this strain for linkage analyses, ethyl methane sulfonate was used as the mutagen. *Pseudomonas aeruginosa* PAO1670 (10) was obtained from Bruce Holloway, Monash University, Victoria, Australia. Stock cultures were maintained on NBY agar (14). Cells for experimental use were cultured in NBY broth at 29°C. Transductants were plated on Ng agar consisting of glucose and minimal salts (5). Medium for the *P. aeruginosa* PAO1670 × *P. syringae* matings consisted of Ng agar plus 50 μg of arginine, 60 μg of streptomycin, and 5 μg of tetracycline per ml. Media components, including agar, were obtained from Difco Laboratories (Detroit, Mich.).

**UV light 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 MgSO₄, pH 7.0). The samples for irradiation consisted of a 1.5-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 4 × 10^{-6} J/mm² per s. 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 PS224 diluted to 10⁵ cells per ml, using the double agar overlay method.

Bacteriophage adsorption. Equal volumes of bacteriophage lysate, about 10⁵ PFU per ml, and an overnight culture of P. syringae PS224 cells diluted to 10⁹ 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 PUF remaining in the supernatant were sequentially diluted in phage buffer and plated with 0.1 ml of an overnight culture of P. syringae PS224 cells diluted to 10⁹ cells per ml, using the double agar overlay method.

Transduction. Equal volumes (1.0 ml) of UV-irradiated or non-irradiated phage in phage buffer and an overnight culture of P. syringae PS224 auxotrophic bacterial cells diluted to 10⁹ cells per ml in NBY broth were mixed at various multiplicities of infection (MOIs), usually 1.0. Auxotrophic cells plus sterile phage buffer served as the control. To test for bacterial contamination, a 20-μl portion of the original non-irradiated phage stock (about 10^{10} PFU per ml) was plated in duplicate on Ng agar. To ensure maximal phage adsorption, all samples were incubated for 2 h at room temperature (25°C). The cells were then pelleted by centrifugation and suspended 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 were incubated 4 to 5 days before counting transductants.

Toxin assay. Wild-type strains, auxotrophic derivatives, and transductants were screened for SR production by picking colonies to potato dextrose agar plates (Difco) supplemented with 0.4% Casamino Acids and adjusted to pH 7.0 before autoclaving. The plates were incubated for 6 days at 29°C, and toxin activity was then determined by spraying a mycelial suspension of the fungus Geotrichum 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 transferring transductants onto NBY agar plates; after 24 h of growth, the plates were flooded with 1% p-aminodimethylaniline 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 × 10⁸ to 5 × 10⁹ 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 removed and suspended in 1 ml of 0.01 M sodium phosphate buffer (about 10^{10} cells). Sequential dilutions of these cells were plated on Ng medium containing arginine, streptomycin, and tetracycline. Resultant colonies were subcultured twice on selective media 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 suspended 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 sodium dodecyl sulfate solution according to the procedure of Cassé et al. (3). After 30 min at 34°C, the lysates were neutralized, and plasmid DNA was purified by the method of Currier and Nester (6).

DNaese treatment. MgCl₂ and bovine pancreas DNase (grade 1; Boehringer Mannheim Corp., Indianapolis, Ind.) were added to 1 ml of bacteriophage lysate (10⁹ PFU) to yield final concentrations of about 20 μg of DNase per ml (approximately 50 Kunitz U) and 0.005 M MgCl₂. The mixture was incubated for 2 h at 37°C and assayed directly for PFU and transducing activity. As a control, 100 ng of EcoRI digestion fragments of bacteriophage lambda were added to the phage and digested as above.

RESULTS

Screening phage for transducing activity. Eleven phages which were previously shown to have plaque-forming activity on P. syringae PS224 (11) are listed in Table 1. All phage isolates were screened for their ability to transduce an arginine marker in P. syringae KS35. This strain was chosen to screen phage for their transducing activity.

### Table 1. Phage isolates and frequency of transduction in P. syringae KS35

<table>
<thead>
<tr>
<th>Phage stock</th>
<th>Origin</th>
<th>Transduction frequency of arginine locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pssy15</td>
<td>Culture supernatant of P. syringae B15</td>
<td>3.0 × 10⁻⁷</td>
</tr>
<tr>
<td>Pssy4210</td>
<td>Unknown</td>
<td>1.3 × 10⁻⁸</td>
</tr>
<tr>
<td>Pssy42</td>
<td>Culture supernatant of P. syringae PS179</td>
<td>5.0 × 10⁻⁸</td>
</tr>
<tr>
<td>Pssy420</td>
<td>Clear plaque variant of Pssy42</td>
<td>4.3 × 10⁻⁸</td>
</tr>
<tr>
<td>Pssy4220</td>
<td>Unknown</td>
<td>1.4 × 10⁻⁸</td>
</tr>
<tr>
<td>Pssy923</td>
<td>Sewage</td>
<td>1.2 × 10⁻⁷</td>
</tr>
<tr>
<td>Pssy41</td>
<td>Culture supernatant of P. syringae PS179</td>
<td>ND²</td>
</tr>
<tr>
<td>Pssy9220</td>
<td>Sewage</td>
<td>ND³</td>
</tr>
<tr>
<td>Pssy401</td>
<td>Sewage</td>
<td>ND³</td>
</tr>
<tr>
<td>Pssy403</td>
<td>Sewage</td>
<td>ND³</td>
</tr>
<tr>
<td>Pssy404</td>
<td>Sewage</td>
<td>ND³</td>
</tr>
</tbody>
</table>

¹ Pssy4210 and Pssy4220 are very similar; they differ only in the mobility of the fourth largest EcoRI digestion product. Pssy42, Pssy420, and Pssy923 are indistinguishable by restriction analysis with EcoRI (11).

² See reference 11.
³ Transduction frequencies are expressed per PFU.
⁴ ND, Not detected.
ability because it has a low reversion frequency and it produces prototrophic exconjugants in matings with strain PS224 carrying the plasmid R68.45 (unpublished data). Four phages from sewage (Pssy401, Pssy403, Pssy404, and Pssy9220) and one from the culture supernatant of P. syringae PS179 (Pssy41) did not demonstrate transductional activity. Two of these isolates (Pssy41 and Pssy403) were indistinguishable by restriction analysis with EcoRI (11).

Of the remaining six phage isolates that exhibited transductional activity, phage Pssy15 showed the highest frequency and was therefore the most intensively analyzed isolate. DNase did not affect the transduction frequency of phage Pssy15 as compared to an untreated control and it did not diminish phage titer (Table 2), but it did totally eliminate the presence of added EcoRI fragments of bacteriophage lambda as determined by running a sample on an agarose gel before and after DNase treatment. Also, all arginine transductants (approximately 300) that were tested were oxidase negative and produced a UV fluorescent pigment, indicating that they were in fact P. syringae.

**Adsorption studies.** Adsorption studies were performed with selected phage stocks (Pssy41, Pssy42, Pssy420, and Pssy15). All of these except Pssy41 were capable of transduction. The adsorption kinetics (data not shown) of Pssy420 were indistinguishable from those of Pssy42 but were different from those observed for Pssy41 and Pssy15 (Fig. 1). However, the adsorption kinetics for all isolates were similar in that an initial period of rapid adsorption was followed by a period of greatly decreased adsorption. For all isolates tested, rapid adsorption had ceased after 30 min, with Pssy15 adsorbing more rapidly than either Pssy41 or Pssy42. At 30 min, at least 90% of the phage were adsorbed to P. syringae KS35, and after 2 h greater than 99% of the phage were adsorbed (data not shown). Seven other auxotrophs were tested for their ability to adsorb Pssy41 and Pssy42, and after 2 h greater than 99% of the phage were adsorbed in all cases. With all transducing phages tested, it is possible that transduction experiments occurring over a 30-min interval are feasible, but a 2-h period was normally used to ensure maximal adsorption.

**Optimization of transduction.** To increase the transduction frequency, various approaches were tried. These included UV irradiation of the phage, the use of stationary-phase cells, the introduction of a plasmid known to inhibit phage replication in P. aeruginosa, varying the MOI, and using presumptive lysogens as recipients.

Survival data of selected phage isolates irradiated with UV light are shown in Fig. 2. The survival curve for Pssy404, a nontransducing isolate, is included for comparison. The UV killing kinetics of Pssy42 were indistinguishable from those shown in Fig. 2 for its clear plaque derivative Pssy420. These studies also indicated that the sewage isolates Pssy401 and Pssy9220 were much more sensitive to UV light than the other isolates. Most phage isolates showed about 99% killing at 90 s, but these phages showed greater than 99.9% killing at this time point (data not shown).

Three phage stocks (Pssy42, Pssy420, and Pssy15) were irradiated for 0, 30, 60, 90, and 120 s and were tested for transduction of an arginine marker, using P. syringae KS35 as a recipient. At 90 s, these three isolates showed maximal increases in transduction frequencies which were two- to fivefold for Pssy42 and Pssy420 and twofold for Pssy15. All other phage isolates were tested for an increased transduction frequency after 90 s of UV irradiation. Only in those cases where activity was present before irradiation was any effect of the irradiation observed. The observed increases were again about twofold. UV irradiation did not appear to...
affect adsorption of Pssy15, Pssy42, or Pssy420 phages, except after extremely long periods of irradiation, i.e., greater than 150 s. Although UV irradiation did not increase the number of transductants per milliliter, the actual transduction frequency (i.e., transductants per viable phage) rose approximately two- to fivefold as exposure to UV light was increased. This rise may be partially due to an increase in the relative number of transducing particles as viable phage particles were killed by UV light.

The use of stationary-phase cells as recipients in transduction experiments also did not produce dramatic effects. For most phage isolates, the transduction frequency increased about twofold. Broth cultures of KS35 were allowed to grow well into stationary phase, i.e., 48 to 72 h, and cultures from both 48 and 72 h showed a twofold increase in the number of transductants recovered as compared with log-phase cells.

Mating the IncP2 plasmid R38 into KS35 did not increase the transduction frequency for Pssy15 and Pssy420 as has been reported for phage E79 in P. aeruginosa (10). R38 transfer was verified by the expression of plasmid mark-

ers by the recipient and by agarose gel electrophoresis. Strain KS35 does not contain any resident plasmids, and the presence of a 140 × 10^6-dalton band after mating indicated R38 had been transferred.

Increasing the MOI dramatically increased the number of transductants recovered per milliliter for phage Pssy15, although the actual transductional frequency remained about the same. This same effect was not observed with phage Pssy420, where an MOI of 1 appeared to give optimal transduction frequency. Increasing the MOI actually decreased the number of transductants recovered per milliliter.

The difference between Pssy15 and Pssy420 in the effect of MOI on transduction frequency may relate to the observation that all of the transductants obtained with Pssy15 were phage resistant and apparently lysogenic for Pssy15, whereas all transductants obtained with Pssy420 were sensitive to the transducing phage. This observation suggested that the transduction frequency might be increased by using a recipient strain already lysogenic for the transducing phage. Bacterial strains resistant to both Pssy15 and Pssy420 could be obtained by streaking surviving cells from confluently lysed cells of KS35. Some of the resulting isolates were still capable of adsorbing the phage to which they were resistant, and of these isolates lytic activity
was associated with cells resistant to Pssy15 but not with cells resistant to Pssy420. When strains resistant to either phage were used as recipients with their respective transducing phage, an increase in the transduction frequency was not observed. However, it was apparent from these studies that resistance to Pssy15 and the associated lytic activity were rapidly lost, indicating that the cells presumed to be lysogenic for Pssy15 were unstable.

**Generalized transduction and linkage analyses.** Because the highest transduction frequency of an arginine marker was observed with Pssy15, this phage was tested for a generalized transducing ability. Table 2 shows that a number of auxotrophic markers were converted to prototrophy with Pssy15 and that the transduction frequency varied with the marker. Because Pssy15 had generalized transducing ability, it should be useful in fine-structure linkage studies. Therefore, we have isolated a number of strains auxotrophic for arginine and another compound. So far, linkage (10% frequency of coinheritance) between arginine and tryptophan loci has been identified. In these preliminary tests for marker linkage, experiments were performed at an MOI of 10 so that a large number of transductants could be easily isolated. Because most of the transductants were at least doubly infected, the observed frequencies of cotransduction may not be a precise estimate of linkage. However, we feel that linkage does exist between the tryptophan and arginine loci because coinheritance was not observed between the arginine locus and 15 other auxotrophic markers. In addition, the arginine locus appears to be linked to one or more loci which are involved with toxin biosynthesis or excretion. This is suggested because the arginine auxotroph (KS35) does not produce toxin in our standard bioassay, but approximately 90% of the prototrophic transductants do. Furthermore, of those that do produce toxin, about 5% produce a noticeably larger zone of inhibition than does the wild type. Further analysis of this region should establish the number of genes involved and their map order relative to the arginine and tryptophan loci.

**DISCUSSION**

The transducing phages described in this work are the first reported to mediate transduction in *P. syringae*. Of 11 phage isolates tested, 6 were capable of transduction and 1 was shown to mediate generalized transduction. This indicates that transduction is a fairly common property of *P. syringae* phages. The data also show that transduction is not limited to phage isolated from culture supernatants or to phage giving rise to turbid plaques. Of the six transducing isolates, one (Pssy923) was from sewage. This isolate produced clear plaques and had the same EcoRI restriction pattern as Pssy42 and Pssy420, both of which are also transducing phages. Pssy420 arose as a clear plaque variant from Pssy42, which was isolated from the culture supernatant of *P. syringae* PS179. Even though Pssy923 is indistinguishable from Pssy42 and Pssy420 by EcoRI restriction analysis, we are confident that it is a different isolate because its host range is distinctly different than those observed for Pssy42 (11) and Pssy420.

Phages Pssy15 and Pssy420 were initially judged to be most useful in transduction studies because they could routinely be obtained in high titer and exhibited among the highest transduction frequencies. Phage Pssy15 appears to readily lysogenize the recipient cells, since all transductants tested were phage resistant and spontaneously released phage. This is in contrast to Pssy420 in which all transductants tested were phage sensitive. This difference may explain why the number of transductants per milliliter increases with increased MOI in the case of Pssy15 and why it decreases in the case of Pssy420.

Various attempts were made to increase the frequency of transduction. However, all approaches failed to give more than two- to five-fold increases. These effects were not considered great enough to make modification of our standard protocol worthwhile. In our preliminary studies, only $10^8$ cells were plated, and this routinely gave 20 to 30 transductants at an MOI of 1. However, with many auxotrophic recipients as many as $2 \times 10^9$ cells can be plated before the efficiency of selection for transductants decreases. Therefore, 200 to 300 transductants per plate can usually be obtained, and this is sufficient to obtain accurate linkage data. Therefore, we feel that Pssy15 will be very useful in fine-structure mapping in *P. syringae* PS224 and possibly in several other strains since it can infect at least 31 other natural isolates of *P. syringae* (11).

**ACKNOWLEDGMENTS**

This work was supported by the Kansas Agricultural Experiment Station and by grant PCM-8021628 from the National Science Foundation.

The assistance and helpful advice of Arun K. Chatterjee is gratefully acknowledged. We thank Michael K. Morgan for providing the *P. syringae* auxotrophs used in this study and for many helpful suggestions and Michael Fugate for assisting in certain phases of this study.

**LITERATURE CITED**


