Isolation and Partial Characterization of Three Classes of Mutant in *Pseudomonas syringae* Pathovar *pisi* with Altered Behaviour towards Their Host, *Pisum sativum*

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Mating with *Escherichia coli* strain SM10 carrying the Tn5 vector pSUP2011 was used to mutagenize *Pseudomonas syringae* pv. *pisi* strain 299A. The resulting transconjugants were each tested by stem-inoculation into several pea (*Pisum sativum*) cultivars. Three classes of mutant, which probably resulted from insertion of part or all of RP4-2-Tc::Mu into the genome of strain 299A, showed reduced virulence towards one or more pea cultivars. The single class I mutant was avirulent on all pea cultivars tested and had lost the ability to induce a hypersensitive response in tobacco (*Nicotiana tabacum*) cv. White Burley; the single class II mutant induced a hypersensitive response on all pea cultivars and tobacco; class III mutants showed reduced virulence towards pea cv. Early Onward, while remaining fully virulent towards other normally susceptible pea cultivars, and inducing a hypersensitive response in tobacco.

INTRODUCTION

Pseudomonas syringae pv. *pisi* causes bacterial blight of peas (*Pisum sativum*) (Lawyer, 1984). In common with some other plant-pathogenic pseudomonads, this organism exhibits a well-defined race structure and strains can be distinguished on the basis of differential interaction with a range of host cultivars (Taylor, 1986). The interactions of host and pathogen are essentially of two kinds: compatible and incompatible. A compatible interaction results in the establishment of the pathogen and development of disease symptoms in the host. An incompatible interaction results in the induction of host defences leading to the development of a hypersensitive response (HR) in the host. HR limits the development of the pathogen and results in a localized necrotic lesion close to the site of infection (Klement, 1963). HR also develops when plant-pathogenic bacteria are introduced into the leaves of a non-host plant such as tobacco (*Nicotiana tabacum*).

In an attempt to investigate genes involved in pathogenicity we have used the transposon vector pSUP2011 (Simon *et al.*, 1983) to mutagenize the race 1 strain, 299A, of *P. syringae* pv. *pisi*. A rapid stem-inoculation technique permits screening of mutagenized transconjugants against a range of pea cultivars. This paper describes the isolation and initial characterization of three classes of mutant affected in their ability to cause disease in one or more pea cultivars.

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Abbreviation: HR, hypersensitive response.

Table 1. *Bacterial strains and plasmids*

Strain no.	Relevant properties	Reference/source
<i>P. syringae</i> pv. <i>pisi</i>		
299A	Race 1, wild type; derivatives prefixed PF	Taylor (1972 <i>b</i>)
PF21	Path ⁻ class I (Km ^r)	299A
PF22-25	Path ⁻ class III (Km ^s)	299A
PF26-27	Path ⁻ class III (Km ^r)	299A
PF28	Path ⁻ class I (Km ^s)	PF21
PF32	Path ⁻ class II (Km ^s)	PF35
PF35	Path ⁻ class II (Km ^r)	299A
PF49-91	Path ⁻ class III (Km ^r); HR on cv. Early Onward	299A
PF92-108, 110-123	Path ⁻ class III (Km ^r); reduced virulence on cv. Early Onward	299A
PF109, 124	Path ⁻ class III (Km ^r Sm ^r Tn5); reduced virulence on cv. Early Onward	299A
PF201	Wild type, RP4-4	299A × J53(RP4-4)
<i>Escherichia coli</i>		
J-53	Pro ⁻ Met ⁻	Clowes & Hayes (1968)
SM10	Thr ⁻ Leu ⁻ Thi ⁻ RP4-2-Tc::Mu in chromosome	Simon <i>et al.</i> (1983)
Plasmids		
RP4	Ap ^r Km ^r Tc ^r	Datta <i>et al.</i> (1971)
RP4-2-Tc::Mu	Km ^r Tra ⁺ (Ap ^s due to ISR1 in TnI)	Simon <i>et al.</i> (1983)
RP4-4	Km ^s derivative of RP4	Hedges & Jacob (1974)
pSUP2011	Ap ^r Km ^r Cm ^r Tn5 (pBR325 replicon)	Simon <i>et al.</i> (1983)
pKAN2	Internal HindIII fragment of Tn5 cloned in pBR322 (used as probe for Tn5)	M. J. Daniels, John Innes Institute

METHODS

Bacterial strains and plasmids. Strains of *P. syringae* pv. *pisi* and *Escherichia coli*, and plasmids, are listed in Table 1.

Confirmation of the identity of P. syringae pv. *pisi* isolates. Tests to confirm the identity of mutants which had lost pathogenicity involved the detection of a heat-stable antigen specific for *P. syringae* pv. *pisi* and were done as described previously (Taylor, 1972*a*; Taylor & Dye, 1972).

Growth conditions. Strains of *P. syringae* pv. *pisi* were grown at 25 °C in nutrient broth (NB, Oxoid no. 1) or on nutrient glycerol agar (NGA, Garrett *et al.*, 1966) containing, g l⁻¹: NB, 8; glycerol, 20; Difco Bacto agar, 20; L-tryptophan, 0.05; L-methionine, 0.05. Strains of *E. coli* were grown at 37 °C in Luria broth (LB) containing, g l⁻¹: Difco Bacto tryptone, 15; Difco Bacto yeast extract, 5; NaCl, 5. Minimal medium (MSM) for both organisms was the minimal salts medium of Clowes & Hayes (1968). Glucose was added to MSM as a sterile solution (50%, w/v) to give a final concentration of 0.5% (w/v) immediately prior to use. Stock solutions of amino acids (10 g l⁻¹) and vitamins (0.2 g l⁻¹) were separately sterilized and added to MSM as required to give final concentrations of 50 and 1 µg ml⁻¹, respectively. Antibiotics, freshly prepared as solutions in sterile water, were added to MSM at the following final concentrations (µg ml⁻¹): kanamycin sulphate, 50; dihydrostreptomycin sulphate, 1000; chloramphenicol (dissolved in 0.1 vol. ethanol and diluted in water, 80 mg ml⁻¹), 40; ampicillin, 50.

Use of phage PRD1. The pilus-specific phage PRD1 (Olsen *et al.*, 1974) was used to detect cells harbouring Inc P group plasmids. A lysate (10 µl) of PRD1 (10⁹ p.f.u. ml⁻¹) was spotted on a freshly inoculated NB culture (16 h) streaked on an NGA plate. After 24 h incubation, sensitivity was detected by interruption of the line of growth.

Filter-matings. A procedure similar to that of Towner & Vivian (1976) was used. Equal volumes of NB or LB cultures (16 h) were mixed and passed through a Millipore filter (25 mm diam.; 0.45 µm pore size), which was incubated (24 h, 25 °C) on the surface of an NGA plate. Growth on the filter was resuspended in quarter-strength Ringer's solution (3 ml) and suitable dilutions were plated on selective media. For matings involving *E. coli* strain SM10(pSUP2011) and *P. syringae* pv. *pisi* strain 299A, the plasmid transconjugants were selected on MSM plus kanamycin.

Southern transfer and hybridization. Colony hybridization was done as described by Grunstein & Hogness (1975). Filters were hybridized to nick-translated probes (Rigby *et al.*, 1977), labelled to 10⁶ c.p.m. per ml of hybridization solution containing 50% (v/v) formamide, 2 × SSC (24 h, 42 °C) (1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7). Filters were washed once (30 min, 60 °C) in 0.1% (w/v) SDS in 2 × SSC prior to autoradiography using Kodak X-OMAT X-ray film at -70 °C with intensifying screens (Cronex Lighting Plus).

Pathogenicity testing. A stem-inoculation procedure was used to test strains of *P. syringae* pv. *pisi* with pea cultivars grown (10-14 d) in 3.2 cm peat blocks. Bacteria cultured (16 h) as patches on an NGA plate were scraped

Fig. 1. Inoculation of pea seedlings. *P. syringae* pv. *pisi* stem-inoculated (see Methods) into seedlings (10 d) of pea and recorded after 7 d further growth. (a) Strain 299A on cv. Kelvedon Wonder: compatible interaction; a typical water-soaked lesion spreading from the site of inoculation can be seen. (b) Strain PF22 on cv. Early Onward: incompatible interaction; a typical localized, necrotic lesion can be seen at the site of inoculation. (c) Strain PF21 on cv. Kelvedon Wonder: a null interaction; only the wound made by the inoculation is visible.

from the surface with a sterile entomologist's mounting pin and stabbed into the main stem at its junction with the stipules at the youngest two nodes (two inoculations per plant). The plants were maintained for 5–10 d before recording the results. A compatible (pathogenic) response typically showed an area of water-soaking spreading from the site of inoculation (Fig. 1a), whereas an incompatible response resulted in necrosis localized at the point of inoculation (Fig. 1b). Some mutants and *E. coli* showed a null response (Fig. 1c) in which no symptoms other than the inoculation wound were visible. Mutants that were reduced in their pathogenicity were identified by reduction of the area of water-soaking, together with the presence of visible necrosis.

Testing for hypersensitivity. Bacterial cultures were tested for HR by inoculation of *Nicotiana tabacum* cv. White Burley (Klement, 1963).

Plasmid detection. The single-colony lysate technique (Eckhardt, 1978) was used to examine the plasmid content of *P. syringae* pv. *pisi* strains.

RESULTS

Use of the transposon vector pSUP2011

Transposon Tn5 was chosen for attempted mutagenesis of *P. syringae* pv. *pisi*. Preliminary experiments indicated that pSUP2011, one of a series of Tn5 vectors developed by Simon *et al.* (1983), gave high frequencies (up to 10^{-1} per recipient) of inheritance of kanamycin resistance (Km^r) with efficient 'suicide' of the delivery vector as shown by the failure to inherit chloramphenicol resistance or detect a plasmid of similar size to pSUP2011 in the recipients. The *E. coli* host carrying pSUP2011 has a derivative of the IncP group plasmid RP4 integrated into its chromosome. The RP4 derivative has a deletion in Tn1 and an insertion of phage Mu in the tetracycline-resistance gene, so that only Km^r is expressed. The plasmid pSUP2011 has narrow-host-range replication functions and chloramphenicol resistance derived from pBR325, but can be mobilized by the chromosomal RP4 derivative because it carries the mobilization (Mob) region from RP4. Once transferred to hosts such as *P. syringae* pv. *pisi*, pSUP2011 is unable to replicate and is not maintained, although transposition of Tn5 may occur.

Selection of mutants showing altered pathogenicity

The *P. syringae* pv. *pisi* race 1 strain 299A was filter-mated with *E. coli* strain SM10(pSUP2011) and approximately 500 Km^r transconjugants were isolated. Each was stem-

Table 2. *Interaction of P. syringae* pv. *pisi* mutants with plants

Plant inoculation tests were performed as described in Methods. All strains were confirmed as *P. syringae* pv. *pisi* on the basis of serological tests (see Methods).

Class of mutant	Strain no.	Interaction* with:			Approx. frequency of isolation
		Pea cv. Kelvedon Wonder	Pea cv. Early Onward	Tobacco cv. White Burley	
I	PF21	n	n	n	1/500
II	PF35	—	—	—	1/500
III	PF22-27, PF49-91	+	—	—	49/500
	PF92-124	+	+/-	—	33/500
Wild-type	299A	+	+	—	500/500

* n, no (null) reaction; +, susceptible (compatible); —, resistant with hypersensitivity (incompatible); +/-, weakly susceptible with hypersensitivity (reduced compatibility).

inoculated on pea cvs. Kelvedon Wonder and Early Onward, both of which are susceptible to the wild-type, 299A. All potential mutants showing reduction or loss of virulence towards either cultivar were tested on two further occasions. Three classes of mutant were identified and were also used to infect tobacco (Table 2).

A single class I mutant, designated PF21, showed a null response (Fig. 1c) on both the cultivars tested and also failed to elicit HR on tobacco. A spontaneous Km^s derivative of PF21, designated PF28, retained the non-pathogenic phenotype of PF21. Both PF21 and PF28 were confirmed as *P. syringae* pv. *pisi* on the basis of serological tests. In addition, both strains harboured the plasmids pAV213 (50 kb) and pAV212 (54 kb) which were present in the wild-type strain 299A (Malik, 1985).

A single class II mutant, designated PF35, produced a typical incompatible response on both cv. Kelvedon Wonder and cv. Early Onward, and retained its ability to induce HR in tobacco. A spontaneous Km^s derivative of PF35, designated PF25, maintained the class II phenotype on both pea cultivars, and also induced HR in tobacco. Both PF35 and PF25 were shown to be *P. syringae* pv. *pisi* on the basis of serological tests and harboured pAV213 and pAV212.

Class III mutants showed the normal compatible response with cv. Kelvedon Wonder but showed loss or reduction of virulence on cv. Early Onward. This class of mutants arose at high frequency, being represented by some 82/500 transconjugants tested. The interaction with cv. Early Onward varied but was reproducible for a given isolate. Several class III mutants gave rise to spontaneous Km^s variants, but this did not affect their phenotype towards pea.

About 40% of the class III mutants had undergone detectable changes in their plasmid content. These included apparent loss of either pAV213 or pAV212 or the acquisition of a larger plasmid accompanied by loss of one of the two plasmids normally present in strain 299A. No strains which had lost both pAV213 and pAV212 were detected and all remaining class III mutants showed no apparent change of plasmid profile.

The genetic basis of the mutants

The results described in the previous section cast doubt upon the role of Tn5 in the generation of the mutants, since frequently the loss of Km^r was not accompanied by reversion of the mutant. It was therefore of some importance to determine whether Tn5 were present in the mutants, and also, since RP4 could also confer Km^r, whether some or all of this plasmid were present. Colonies lysed on filters were probed with radiolabelled pKAN2 (Table 1) to detect Tn5 and RP4 to detect RP4-2-Tc::Mu from strain SM10 (Table 1). Only two mutants, PF109 and PF124, showed strong hybridization with pKAN2: both also expressed high-level (1000 µg ml⁻¹) resistance to streptomycin (see below), which is specified by Tn5. The remainder showed only weak hybridization to pKAN2 and were sensitive to streptomycin.

All of the mutants, including the Km^s variants described above, hybridized strongly with RP4. There was weak hybridization between pKAN2 and RP4. Further, all of the mutants and

Table 3. Inheritance of RP4 and RP4-4 in strain 299A and pathogenicity mutants

Filter matings were performed as described in Methods. Strains are listed in Table 1.

Donor	Recipient	Selected resistance*	Frequency of plasmid inheritance per recipient
J5-3(RP4)	299A	Tc	7.5×10^{-1}
		Km	1.0×10^{-1}
J5-3(RP4)	PF201	Km	6.9×10^{-4}
J5-3(RP4)	PF21	Tc	1.2×10^{-1}
J5-3(RP4)	PF35	Tc	6.9×10^{-1}

* Km, kanamycin resistance; Tc, tetracycline resistance.

the Km^s variants were sensitive to lysis by the IncP group pilus-specific phage, PRD1. The wild-type 299A was resistant to PRD1. These results suggest that the mutants harbour at least part of RP4-2-Tc::Mu, inherited from strain SM10, and that loss of Km^r was not accompanied by loss of the entire RP4-derivative sequences. Transconjugants of *P. syringae* pv. *pisi* strain 299A carrying RP4 were fully virulent on cv. Early Onward, indicating that the presence of this replicon was not responsible for the class III phenotype.

Examination of the plasmid content of the mutants failed to show the presence of additional plasmid bands, suggesting that RP4-2-Tc::Mu or part of it had integrated into the *P. syringae* pv. *pisi* chromosome. Using a Km^s derivative of RP4, designated RP4-4 (Hedges & Jacob, 1974), it was possible to demonstrate a 100- to 1000-fold reduction in inheritance of RP4 in a strain (PF201) harbouring an autonomous RP4-4 (Table 3). However, the mutants PF21 and PF35 did not show any reduction in the frequency of inheritance of RP4 (Table 3), indicating that an autonomous RP4 replicon was not present.

Tn5 expresses high-level resistance to streptomycin in P. syringae pv. *pisi*

The demonstration that mutants PF109 and PF124 harboured Tn5 and exhibited high-level resistance to streptomycin was used to determine the relative frequencies of Tn5 and RP4 inheritance in *P. syringae* pv. *pisi* from *E. coli* SM10(pSUP2011). In matings with strain 299A, Tn5 (streptomycin resistance) was inherited at 10^{-4} , while RP4 (Km^r) was inherited at 10^{-1} . This further supports the conclusion that part or all of the integrated RP4 present in SM10 is efficiently transferred to recipient strains in this case.

DISCUSSION

The observation that Tn5 expresses high-level streptomycin resistance in *P. syringae* pv. *pisi* permits selection with that antibiotic in preference to kanamycin. We have noted that it is extremely difficult to purify *P. syringae* pv. *pisi* transconjugants from *E. coli* in matings. However, use of streptomycin-resistant recipients or selection of Tn5 with streptomycin completely prevented growth of the *E. coli* donor. The expression of the streptomycin-resistance determinant on Tn5 has been reported for several other bacteria, including *Rhizobium meliloti* (Putnoky *et al.*, 1983; Selveraj & Iyer, 1984), *Pseudomonas putida* and *Acinetobacter calcoaceticus* (O'Neill *et al.*, 1984).

Three classes of mutant were isolated which showed loss or reduction of virulence towards one or more cultivars of pea. The class I mutant, PF21, exhibited a null reaction with all pea cultivars tested and failed to produce HR in tobacco. The class II mutant, PF35, resembled the class I mutant but retained the ability to induce HR in pea and tobacco. Mutants of similar phenotype to classes I and II were isolated in *P. syringae* pv. *phaseolicola* by Panopoulos *et al.* (1984). Class III mutants were obtained at a much higher frequency than the other two classes and were unique in showing loss or reduction of virulence towards a single cultivar (Early Onward) of pea. Anderson & Mills (1985) reported the isolation of several mutants of *P. syringae* pvs. *syringae* and *phaseolicola* which showed reduced virulence towards *Phaseolus lunatus* cv. PI 199791 and

Phaseolus vulgaris cv. Red Mexican respectively. These were induced after conjugation with *E. coli* SM10(pSUP1011) and were apparently due to Tn5 insertion into the genes involved.

The precise cause of the mutations obtained with pSUP2011 remains in doubt. It has been observed in *E. coli* that when RP4 is integrated into the bacterial chromosome it no longer exhibits incompatibility towards autonomous RP4 derivatives present in the same cell (Watson & Scaife, 1980; Grinter, 1984). A clear demonstration of surface exclusion by *P. syringae* pv. *pisi* harbouring an autonomous RP4 towards the inheritance of RP4-4 here, but an absence of exclusion in the two mutants tested may further support the notion that RP4-2-Tc::Mu has integrated in these mutants. Turner *et al.* (1984) reported the anomalous behaviour of the closely related pSUP1011 in *Xanthomonas campestris* pv. *campestris*. These workers demonstrated that the RP4 derivative in the chromosome of *E. coli* SM10 had excised and transferred to the recipient *X. campestris* pv. *campestris*, thus introducing RP4 sequences and, potentially, phage Mu into the recipient genome. Clearly a similar situation pertains to the mutants isolated here. Integration of either RP4 or Mu into the recipient genome could cause mutations, in addition to the induction of mutants by Tn5 transposition. The balance of evidence presented here suggests that the phenotypes observed are probably due to integration of at least part of RP4 derived from strain SM10, although their precise basis must await further molecular analysis.

Whatever their basis, these mutants exhibited stable, (except for PF35), well-defined phenotypes in relation to the pea host. The class I mutant showed loss of both virulence towards pea and the ability to induce HR in pea and tobacco. Saprophytic bacteria are unable to induce HR, suggesting that this ability is in some way associated with the role of the pathogen in causing disease. However, Azad & Kado (1984) obtained mutants of *Erwinia rubrifaciens* which were unable to induce HR in tobacco, but remained pathogenic toward Harley walnut trees. The class II mutant, PF35, was unstable and spontaneous reversion to weak virulence was observed in some isolates.

The reason for the relatively frequent isolation of class III mutants is not known. Clearly the gene or genes responsible may be a preferred site for integration of RP4 or Mu. Approximately 40% of these mutants showed changes in their plasmid profile compared with that of strain 299A. It is not clear whether this represents plasmid involvement in the phenotype of the class III mutants. There is an indication that this class of mutants may be heterogeneous in nature, perhaps resulting from slight disturbances in a number of genes involved in pathogenicity, but to which cv. Early Onward is particularly sensitive.

This is the first report of a class of mutant showing loss of virulence towards a specific host cultivar. A commonly held interpretation of the gene-for-gene hypothesis (Flor, 1956) would predict that a mutation involving loss of function should be a mutation from avirulence to virulence, since specificity is considered to reside in the incompatible interaction between host and pathogen (Ellingboe, 1981; Crute & Norwood, 1986). It is difficult to see how insertion of DNA at random into a gene could activate its expression, but if there were a preferred site for integration of DNA in these mutants, it might result in the insertion of a strong promoter close to a gene or genes responsible for avirulence which are otherwise inoperative toward cv. Early Onward.

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