Two Different Classes of *avr*D Alleles Occur in Pathovars of *Pseudomonas syringae*

Irem Yucel, Carol Boyd, Qadriyyah Debnam, and Noel T. Keen

Department of Plant Pathology, University of California, Riverside 92521 U.S.A.
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Considerable variation was observed in the occurrence of avirulence gene D (*avr*D) in different isolates and pathovars of *Pseudomonas syringae*. Three functional alleles of *avr*D were cloned and characterized from *P. s.* pv. *phaseolicola* and *P. s.* pv. *lachrymans*. These *avr*D genes occurred on indigenous plasmids in both pathovars, like the allele originally cloned from *P. s.* pv. *tomato*. *P. s.* pv. *lachrymans* was unique in that it carried two different alleles on plasmids of different sizes. These alleles were cloned on 5.6- or 3.8-kb *Hin*dIII fragments that are conserved in several other *P. syringae* pathovars. Surprisingly, the two *avr*D alleles from *P. s.* pv. *lachrymans* were the most divergent of those compared, with only 85% amino acid identity. Allele 1 from *P. s.* pv. *lachrymans* was 95% identical to *avr*D from *P. s.* pv. *tomato* but less similar to the other three *avr*D genes. These two alleles were accordingly called homology class I. The *avr*D gene from *P. s.* pv. *phaseolicola* and allele 2 from *P. s.* pv. *lachrymans* were 97 and 98% identical, respectively, at the amino acid level with the nonfunctional *P. s.* pv. *glycinea* allele. These three alleles were therefore grouped into homology class II. Comparison of all the *avr*D alleles permitted the identification of four amino acid substitutions unique to the *P. s.* pv. *glycinea* allele at positions 19, 245, 280, and 304.

Additional keywords: hypersensitive response.

Avirulence gene D (*avr*D) cloned from *Pseudomonas syringae* pv. *tomato* (Kobayashi *et al.* 1990a,b) causes *P. s.* pv. *glycinea* race 4 (R4) to elicit the hypersensitive response (HR) on soybean cultivars containing the resistance gene, *Rpg*4 (Keen and Buzzell 1991). *Escherichia coli* and other gram-negative bacteria expressing *avr*D produce a cultivar-specific elicitor of the soybean HR, identified as two novel acyl glycosides called syringolides (Keen *et al.* 1990, Midland *et al.* 1993; Smith *et al.* 1993). The 311 amino acid protein encoded by *avr*D is thought to have an enzymatic activity that converts normal bacterial metabolites into the HR-inducing syringolides.

Corresponding author: N. T. Keen.
Current address of I. Yucel: Bldg. 011A, Rm. 254, USDA, Beltsville, MD 20705 U.S.A..
Current address of Q. Debnam: Department of Biology, Fisk University, Nashville, TN 37203 U.S.A.

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Some other pathovars, including *P. s.* pv. *glycinea*, harbor DNA sequences with considerable similarity to *avr*D (Kobayashi *et al.* 1990a). The *avr*D allele from *P. s.* pv. *glycinea* encodes a protein that has 86% amino acid identity with the *avr*D protein from *P. s.* pv. *tomato* yet does not confer the avirulence phenotype. Several amino acid substitutions throughout the length of the *P. s.* pv. *glycinea* protein distinguished it from that of *P. s.* pv. *tomato* (Kobayashi *et al.* 1990b), yet it directed the production of extremely small quantities of elicitor when overexpressed in *E. coli* (Keen *et al.* 1990). The amino acid substitutions in the *P. s.* pv. *glycinea* protein may therefore impair enzymatic activity required to produce the elicitor and/or reduce protein stability. Studies of recombinant genes (Kobayashi *et al.* 1990b) indicated that the carboxyl terminus of the *avr*D protein influences protein stability, but the specific amino acids required for the avirulence phenotype were not identified. In order to define the amino acids and regions of *Avr*D necessary for the avirulence phenotype, we surveyed several *P. syringae* pathovars for the occurrence of *avr*D genes and cloned and characterized three new *avr*D alleles from *P. s.* pvs. *phaseolicola* and *lachrymans*.

RESULTS

Occurrence of avirulence gene D in various pathovars of *P. syringae*.

To test for the presence of *avr*D in a collection of *P. syringae* pathovars, bacteria were assayed for elicitor production in two different culture media, and Southern blots were performed using total DNA restricted with *Hin*dIII and an *avr*D-specific probe. The different isolates varied in elicitor production when grown on two different media (Table 1). Certain isolates did not produce elicitor activity on either medium, whereas other isolates were positive on at least one medium. Generally, when bacteria were grown on M9 medium, elicitor activities were weaker than in bacteria grown on induction medium or were not detected. This was assumed to result from the higher activity of the *avr*D promoter on induction medium, as shown by Shen and Keen (1993). Although *P. s.* pvs. *aptata* 2042, *dendropanacis*, *garcae,* and *hibisci* produced weak elicitor activity on M9 medium that appeared specific to soybean cultivars Harosoy and Flambeau, this activity was not detected when the bacteria were grown on induction medium. It is accordingly possible that the weak activity on M9 medium may be artifactual.

Southern blots of total bacterial DNA restricted with *Hin*dIII generally showed agreement between the production of elicitor activity in one or both culture media and the pres-

Table 1. *avr*D elicitor production and the presence of *avr*D-hybridizing DNA in several different *Pseudomonas syringae* pathovars

Pathovar and isolate number	Elicitor activity[a]		Southern blot[b]	
	M9	IM	Band	kb[c]
aesculi 2894	−	+	+	3.8
apii 1089-5	w+	+	+	1.8
apii 1089-6	w+	+	+	1.8
apii 0790-4	w+	+	+	1.8
apii 0890-9	+	+	+	1.8
aptata 1617	−	−	−	
aptata 2042	w+	−	−	
aptata 2134	−	−	−	
atrofaciens 2213	−	−	−	
atropurpurea 2340	−	+	+	3.8
avellanae 10963	−	−	−	
ciccaronei 2342	ND	−	−	
cilantro 0788-9	+	+	+	5.6
cilantro 0790-2	+	+	+	5.6
coronafaciens 2216	+	+	+	5.6
dendropanacis 3226	w+	−	−	
dysoxyli 2356	−	−	−	
eriobotryae 2343	−	−	+	5.6
glycinea 2214	−	−	+	5.6
helianthi 2043	−	−	−	
helianthi 2067	+	+	+	3.8
helianthi 2149	−	−	−	
hibisci 2895	w+	−	−	
impatiens 0789-1	−	−	−	
japonica 2896	−	−	+	
lachrymans	+	+	+	3.8/5.6
maculicola 4326	−	−	−	
mellea 2344	−	−	−	
mori 1642	−	+	+	3.8
morsprunorum 1565	−	+	+	3.8
morsprunorum 2351	−	−	−	
morsprunorum 2115	−	−	−	
myricae 2897	−	−	−	
oryzae 3228	−	−	−	
panici 2345	−	−	−	
papulans 1754	−	−	+	3.8
persicae 1573	−	−	−	
phaseolicola G50	ND	+	+	5.6
phaseolicola 3121	+	+	+	3.8
philadelphi 2898	+	+	+	5.6
photiniae 2899	−	−	−	
pisi 2105	−	−	−	
porri 1908	+	+	+	5.6
porri 1912	+	+	+	5.6
porri 2360	−	+	+	3.8
savastanoi 1670	−	−	−	
sesami 1671	−	−	+	3.8
tabaci 2106	−	−	−	
theae 2353	−	−	−	
tomato PT23	w+	+	+	5.6
tomato 10862	−	−	−	
viburni 1702	ND	−	−	
zizaniae 11040	−	w+	+	3.8

[a] All cultures except those noted in Table 4 were supplied by R. Samson and bear the collection number of the French Collection of Phytopathogenic Bacteria (CFBP). Cultures were grown for about 24 hr in 50 ml of the respective media, and the culture fluids were processed through a Waters Sep-Pak C18 cartridge as described in Materials and Methods. IM = induction medium. The final preparations were taken to dryness and dissolved in 1 ml of water prior to bioassay in leaves of various soybean cultivars. Preparations classed as positive produced necrotic reactions in cultivars Harosoy and Flambeau, which were not observed in cultivars Acme and Merit. w+ = Weakly positive; + = positive; − = negative; ND = not done.

[b] DNA was extracted from bacteria grown on King's medium B. Following restriction with *Hin*dIII and electrophoresis on a 0.7% agarose gel, the DNAs were blotted to a nylon membrane and hybridized with an *avr*D-specific probe.

[c] Approximate size of the hybridizing band(s) in kilobases.

ence of DNA bands hybridizing to an *avr*D-specific probe (Table 1). In the cases of *P. s.* pvs. *eriobotryae*, *glycinea* 2214, *papulans*, and *sesami*, elicitor activity was not detected in either culture medium tested, but hybridizing DNA bands were observed. While it is possible that these isolates did not produce elicitor activity despite carrying a functional *avr*D allele, they may contain nonfunctional *avr*D genes, as in the case of the *P. s.* pv. *glycinea* isolates thus far examined (Kobayashi *et al.* 1990b). All of the tested bacteria except *P. s.* pv. *lachrymans* (with two bands) yielded single *avr*D-hybridizing bands that were generally either about 5.6 or 3.8 kb (Table 1). Sizes of the hybridizing *Hin*dIII fragments appeared to vary by as much as 100–200 bp in some cases, but we currently have no explanation for this behavior. Also, all of four *P. s.* pv. *apii* isolates gave single 1.8-kb hybridizing *Hin*dIII fragments (Table 1). Since these bacteria yielded *avr*D elicitor activity on both culture media, they must harbor functional *avr*D genes and carry a new *Hin*dIII polymorphism in the *avr*D region. Variation was also observed when different isolates of the same bacterium were tested for *avr*D elicitor activity and *avr*D hybridization (Table 1). Similar to the

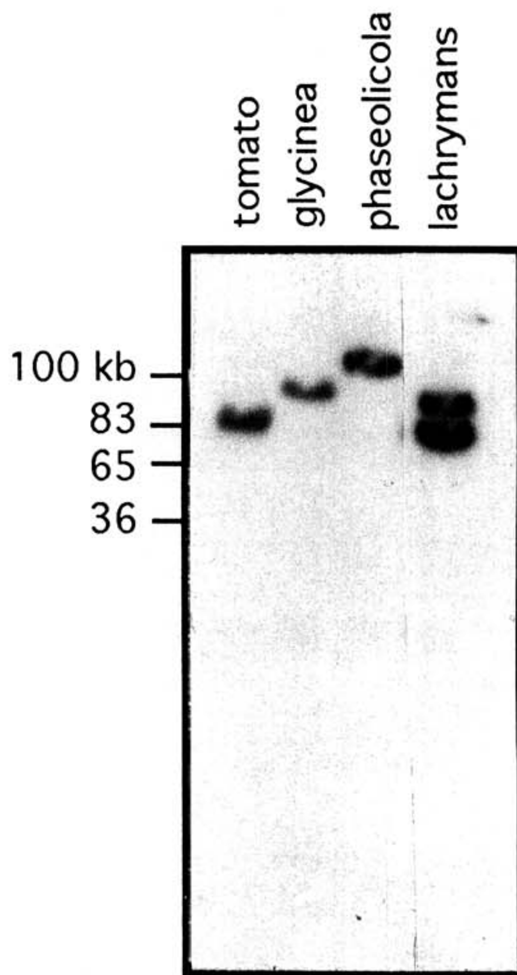

Fig. 1. Southern blot of undigested indigenous plasmids from four *Pseudomonas syringae* pathovars. Following electrophoresis of the plasmids and blotting to nylon membranes, the DNA was hybridized to a ^{32}P-labeled *avr*D-specific probe from *P. s.* pv. *tomato*. Lane 1, *P. s.* pv. *tomato*; lane 2, *P. s.* pv. *glycinea* R4; lane 3, *P. s.* pv. *phaseolicola*; lane 4, *P. s.* pv. *lachrymans*.

previously investigated *P. s.* pv. *morsprunorum* isolate (Kobayashi *et al.* 1990a), isolates 2351 and 2115 were negative, but *P. s.* pv. *morsprunorum* isolate 1565 was elicitor-positive on induction medium and yielded a 3.8-kb *avr*D-hybridizing band (Table 1). Similarly, only one of the three tested *P. s.* pv. *helianthi* isolates produced elicitor and contained hybridizing DNA. *P. s.* pv. *tomato* isolate 10862 lacks detectable plasmids (Denny 1989) and, unlike isolate PT23, neither produces elicitor nor contains *avr*D-hybridizing DNA. This is probably explained by the plasmid location of *avr*D in strain PT23 and other *P. syringae* pathovars (Fig. 1).

Hybridization intensities varied with the different *P. syringae* isolates despite attempts to load equal amounts of DNA on the gels (data not shown). This suggested that sequence variation occurs in the *avr*D genes harbored by different *P. syringae* isolates. However, because such differences could also be accounted for by gene copy number, it was impossible to make firm conclusions based only on the Southern blot hybridization intensities.

*avr*D homologues found on indigenous plasmids of *P. s.* pvs. *tomato, glycinea, phaseolicola,* and *lachrymans*.

Previous studies indicated that the *avr*D gene found in *P. s.* pv. *tomato* PT23 resided on an approximately 80-kb indigenous plasmid (Kobayashi *et al.* 1990a). *P. s.* pv. *glycinea* race 4, pv. *phaseolicola,* and pv. *lachrymans* also contained several indigenous plasmids, which were separated on an agarose gel. Southern analysis showed that the *avr*D alleles occurred on the largest plasmids in *P. s.* pvs. *glycinea* and *phaseolicola*, estimated to be 95 and 120 kb, respectively (Fig. 1). In *P. s.* pv. *lachrymans*, the largest two plasmids of about 90 and 75 kb both harbored DNA homologous to *avr*D from *P. s.* pv. *tomato*. This confirmed the results of Kobayashi *et al.* (1990a) and our own blots (Table 1).

P. s. pv. *lachrymans* has two different *avr*D alleles.

P. s. pv. *lachrymans* contained two different *Hin*dIII fragments that have homology with *avr*D (Table 1) (Kobayashi *et al.* 1990a). To determine the location of these *Hin*dIII fragments, plasmids were isolated from *P. s.* pv. *lachrymans* and digested separately with *Hin*dIII. A total plasmid preparation was also digested with *Hin*dIII as a control. The digested fragments were resolved on an agarose gel, blotted, and hybridized with an *avr*D-specific probe. The largest plasmid contained only the 5.6-kb *Hin*dIII fragment, whereas the second plasmid carried only the smaller *Hin*dIII fragment (data not shown). The smaller DNA fragment was determined to be about 3.8 kb and not 4.3 kb as previously reported (Kobayashi *et al.* 1990a). The 3.8-kb DNA fragment hybridized less intensely to the *avr*D probe from *P. s.* pv. *tomato* than did the 5.6-kb fragment, suggesting that their *avr*D genes differed in sequence.

Occurrence of an *avr*D allele on cosmid clone pPsp01.

Cosmid clone pPsp01 from *P. s.* pv. *phaseolicola* 3121 conferred the HR on a spectrum of soybean cultivars when introduced into *P. s.* pv. *glycinea* R4 (D. Dahlbeck and B. Staskawicz, unpublished data). We observed that clone pPsp01 contains an insert of about 26 kb and, when introduced into *P. s.* pv. *glycinea* R4, directed the HR on all soybean cultivars carrying *Rpg*4. However, pPsp01 also directed an avirulence phenotype on the cultivar Acme. Consequently, this clone was initially not suspected to contain an *avr*D allele until it was shown to direct production of the *avr*D elicitor in culture by *P. s.* pv. *glycinea* R4 (data not shown). It was probable, based on the results in Table 1, that *P. s.* pv. *phaseolicola* 3121 contained a functional *avr*D allele, and Southern analysis also revealed the presence of a DNA region in pPsp01 with considerable homology to *avr*D from *P. s.* pv. *tomato* (data not shown).

Cloning and characterization of the *avr*D alleles from *P. s.* pvs. *phaseolicola* and *lachrymans*.

A 3.8-kb *Hin*dIII fragment was cloned from pPsp01 (pMTLK44) and found to confer the *avr*D phenotype to *P. s.* pv. *glycinea* race 4. In addition, *E. coli* cells carrying this fragment (pMTLK4) produced *avr*D elicitor activity (Table 2). The 3.8-kb DNA fragment was mapped (Fig. 2), and a 1.8-kb *Hin*dIII-*Xba*I subclone in pRK415 was isolated (pPAD5) that retained the *avr*D phenotype when introduced into *P. s.* pv. *glycinea* R4. *E. coli* cells carrying the same DNA fragment in pUC129 (pPAD1) produced *avr*D elicitor activity (Table 2).

The two *avr*D homologues from *P. s.* pv. *lachrymans* were identified by screening 300 colonies of a plasmid-specific *Hin*dIII library using colony hybridization. Twenty-three colonies hybridized with the 1.8-kb *Hin*dIII-*Xba*I fragment from pPAD1. These colonies were confirmed to contain *avr*D sequences by hybridization of isolated plasmid DNA with *avr*D. Sixteen of the colonies contained the cloned 3.8-kb

Table 2. Avirulence phenotypes and elicitor production directed by *avr*D alleles from *Pseudomonas syringae* pvs. *phaseolicola* and *lachrymans*

Construct	Pathovar source of *avr*D	*P. s.* pv. *glycinea*[a] Soybean hypersensitive response	*P. s.* pv. *glycinea*[a] Active elicitor	*Escherichia coli* active elicitor[a]
pMTLK4	*phaseolicola*	NA	NA	+
pMTLK44	*phaseolicola*	+	+	NA
pPAD1	*phaseolicola*	NA	NA	+
pPAD5	*phaseolicola*	+	+	NA
pLADA	*lachrymans*	NA	NA	+
pLAD3	*lachrymans*	NA	NA	+
pLAD5	*lachrymans*	+	ND	NA
pLADB	*lachrymans*	NA	NA	+
pLADB4	*lachrymans*	+	ND	NA
pLLAD1	*lachrymans*	NA	NA	+

[a] + = Present; ND = not done; NA = experiment not applicable.

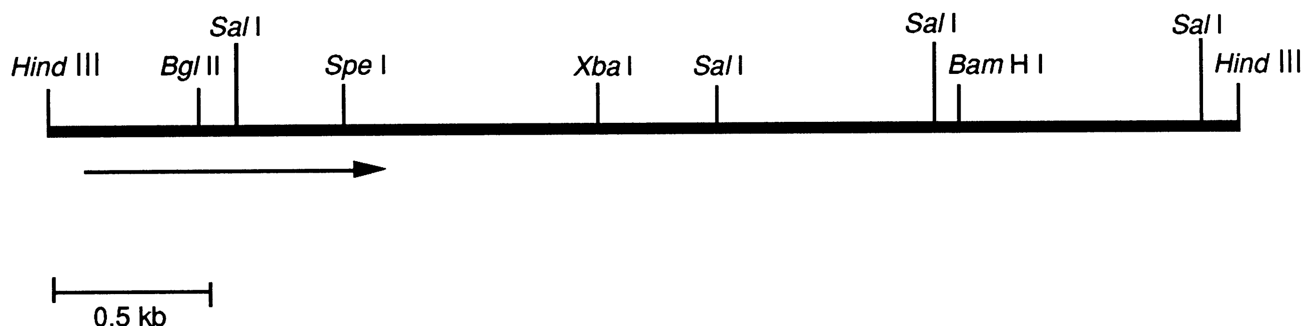

Fig. 2. Restriction map of the 3.8-kb *Hin*dIII fragment found in both *Pseudomonas syringae* pv. *phaseolicola* and *P. s.* pv. *lachrymans*. The arrow indicates location of the *avr*D open reading frame and direction of transcription.

```
AvrD from
P. syringae          harp box
tomato          TTTGCATGGAACCAAATCCGTCCCAAAGGCCACACAGAG
lachrymans 1    TTTGCATGGAACCAAATCCGTCCCAAAGGCCACACAGAG
lachrymans 2    TTTGCATGGAACCAAATCCGTCCCAAAGGCCACACATGG
phaseolicola    TTTGCATGGAACCAAATCCGTCCCAAAGGCCACACAGAG
glycinea        TTTGCATGGAACCAAATCCGTCCCAAAGGCCACACA---
                                σ 54 promoter
tomato          CCACAATTTTTATAAAAATTGGTAGCTGTATAGCTTCCG
lachrymans 1    CCACCATTTTTATAAAAATTGGTAGCTGTATAGCTTCCG
lachrymans 2    CACAAA-TTTTCTAAAACTTGGTGGCTGTATAGCTTCAG
phaseolicola    CCACAATTTTCTAAAACTTTGGTGGCTGTATAGCTTCAG
glycinea        ------TTTTCTAAAAC-TTGGTGGCTGTATAGCTTCAG
```

Fig. 3. Comparison of the promoter regions of *avr*D alleles from *Pseudomonas syringae* pvs. *tomato, lachrymans, phaseolicola,* and *glycinea* R4. Locations of the "harp box," *cis*-acting element (GCCACACA), and the σ^{54} promoter sequences are indicated by bracketed lines over the regions.

fragment, and one of them was retained and called pLADA. The other seven colonies harbored plasmids with the 5.6-kb *Hin*dIII fragment, and one was retained and called pLADB. The restriction maps of pLADA and pLADB were similar to the previously mapped pMTLK4 (Fig. 2) and pPTP120 (Kobayashi *et al.* 1990a), respectively. They both yielded 1.8-kb *Hin*dIII-*Xba*I subclones that directed *avr*D elicitor production in *E. coli* as well as the avirulence phenotype in *P. s.* pv. *glycinea* R4 (Table 2).

pLAD3 and pLLAD1 from *P. s.* pv. *lachrymans* were digested to completion with *Sau*3A, and the DNA fragments resolved on a 5% polyacrylamide gel (data not shown). Since only a few bands varied, pLAD3 and pLLAD1 contained similar but not identical *avr*D alleles.

DNA sequence analyses.

The *avr*D allele from *P. s.* pv. *phaseolicola* and the two alleles from *P. s.* pv. *lachrymans* were sequenced. The promoter regions of all five *avr*D alleles thus far sequenced are shown in Figure 3. A sequence similar to the consensus "harp box" found in *P. syringae hrp* genes (Fellay *et al.* 1991) occurred in all five alleles. Another sequence motif consisting of GGCCACACA was also present in all the *avr*D alleles as well as in avirulence genes from several other *P. syringae* pathovars (Innes *et al.* 1993; Tamaki *et al.* 1988; Shen and Keen 1993). This region is postulated to be a *cis*-acting element necessary for *avr*D transcription (Shen and Keen 1993). Finally, a putative σ^{54} RNA polymerase promoter element defined by GG-10 bp-GC was also present in all of the promoter regions (Kustu *et al.* 1989; Minton and Clark 1985; Shen and Keen 1993). The promoter regions of the *avr*D alleles from *P. s.* pv. *glycinea* and *P. s.* pv. *lachrymans* allele 2 had nine and one base deletions, respectively, relative to the other alleles (Fig. 3).

DNA sequences of the three newly characterized *avr*D alleles have been entered in GenBank. The predicted translation products are shown in Fig. 4. Previous sequence comparison of the *avr*D alleles of *P. s.* pv. *tomato* PT23 and *P. s.* pv. *glycinea* R4 (Kobayashi *et al.* 1990b), revealed that both genes encoded entirely colinear 311 amino acid proteins with 41 amino acid substitutions dispersed throughout the length of the proteins. The three new alleles also encoded proteins with 311 amino acids that were entirely colinear with those from the earlier alleles. Comparison of sequence data from all five alleles showed that only four amino acid substitutions were unique to the nonfunctional *P. s.* pv. *glycinea* R4 allele. The first substitution occurred at position 19, in which an arginine residue in the functional alleles was replaced by a cysteine in the nonfunctional protein. The last three changes, all clustered at the carboxyl end of the nonfunctional *P. s.* pv. *glycinea* R4 protein, were a serine to leucine substitution at position 245, valine to alanine at position 280, and a second serine to leucine change at position 304 (Fig. 4).

Table 3 summarizes the percent identity at the nucleotide and amino acid levels among the five *avr*D alleles as determined with the best fit and gap limit programs in the Genetics Computer Group, University of Wisconsin package. The proteins from the *P. s.* pv. *phaseolicola* allele and *P. s.* pv. *lachrymans* allele 2 (pLLAD1) had the highest percentages of amino acid identity at 99%. The lowest levels of identity with 92 and 85% at the nucleotide and amino acid levels, respectively, were shared between the *P. s.* pv. *glycinea* R4 allele and allele 1 from *P. s.* pv. *lachrymans* (pLAD1), and between the two *P. s.* pv. *lachrymans* alleles.

DISCUSSION

A survey of several *P. syringae* pathovars showed considerable variation in the occurrence of *avr*D-hybridizing DNA and the production of *avr*D elicitor activity (Table 1). The results therefore confirm and extend the pattern previously observed by Kobayashi *et al.* (1990a). Significantly, variation also occurs within certain isolates grouped as a single

```
tomato         MQDLSFSNIE NHLGPAKDRF FGDGFKHVEY SARHVNLTES
lachrymans 1   ---------- ---------- ---------- ----------
lachrymans 2   -------T-- ---------- ---------- ----------
phaseolicola   -------T-- ---------- ---------- ----------
glycinea       -------T-- --------C- ---------- ----------

               41                                       80
tomato         AVDANITLSY PANWSKKNGS SELVPHLSTI DALTISTNLS
lachrymans 1   ---------- ---------- ---------- ----------
lachrymans 2   EANRS-S--- --------D- G--I----S- ------I---
phaseolicola   EANRS-S--- --------D- G--I----S- ------I---
glycinea       -AN-S-S--- --------D- G--I----S- ------I---

               81                BglII                 120
tomato         QDILLNSFKS IDHCWVKGIS IKAGNKPEED LRNINAKITK
lachrymans 1   -S-------- -----M-R-- ---------- ----------
lachrymans 2   ------R--- ------RR-- -R--K----- ----------
phaseolicola   ------R--- ------RR-- -R--K----- ----------
glycinea       ------R--- ------RR-- -R--K----- ----------

               121                                     160
tomato         ESQVLDSQGD TNLFFVGNVG AMTVQLELIM PAAHEIETVK
lachrymans 1   --RG------ -Y-IL-V--- -------F-- -------S--
lachrymans 2   ---G------ ---I-G---- T------F-I -----VD-I-
phaseolicola   ---G---K-- ---I-G---- T------F-I -----VD-I-
glycinea       ---G------ ---I-G---- T------F-I -----VD-I-

               161                                     200
tomato         DSAEKSCYSL HFKNRTQFID DIIFYSPLNA ISTLFVAYDK
lachrymans 1   ---------- ---------- N--------- ----------
lachrymans 2   --T--N---- ---------- ---------- --K----N-N
phaseolicola   --T------- ---------- ---------- --K----N-N
glycinea       --T--N---- ---------- ---------- --K----N-N

               201                                     240
tomato         EPHFLPGGIE AGYPNIMNPV DSLVSHAQIA QALLYKLDGL
lachrymans 1   ----S-S--- ---------- ---------- ----------
lachrymans 2   ---------- -N----I--- ---------- ----------
phaseolicola   ---------- -N----I--- ---------- ----------
glycinea       ---------- -N----I--- ---------- ----------

               241                           SpeI      280
tomato         TRDESNTLWM RSLNIIAENP AKRIAATRLL VTELKRANIV
lachrymans 1   --G------- ---------- ---------- ----------
lachrymans 2   --G------- -N-------- ---R------ ----------
phaseolicola   ---------- -N-------- ---R------ ----------
glycinea       --G-L----- -N-------- ---R------ ---------A

               281                            311
tomato         SVKGKNWRIA EVAGHMNGIT LSSSVAHLLP L
lachrymans 1   ---------- ---------- ---------- -
lachrymans 2   -L--E---V- ---------- F--------- -
phaseolicola   -L--E---V- ---------- F--------- -
glycinea       -L--E---V- ---------- ---L------ -
```

Fig. 4. Amino acid sequence comparison of *avr*D alleles from *Pseudomonas syringae* pvs. *tomato, lachrymans, phaseolicola,* and *glycinea* R4. The full sequence of the *P. s.* pv. *tomato* allele is shown, and differences occurring in the other alleles are shown below. Four amino acid substitutions unique to the *P. s.* pv. *glycinea* allele are in bold and underscored. The positions of *Bgl*II and *Spe*I restriction sites that are conserved in all five sequences are also indicated.

Table 3. Comparisons of percent identity at the nucleotide and amino acid levels of *avr*D alleles from various *Pseudomonas syringae* pathovars

Pathovar source of *avr*D	Percent identity							
	lachrymans allele 1		*lachrymans* allele 2		*phaseolicola*		*glycinea*	
	NT[a]	AA	NT	AA	NT	AA	NT	AA
tomato	97	95	93	87	92	87	93	86
lachrymans allele 1	...	...	92	85	91	85	92	85
lachrymans allele 2	...	...	...	...	98	99	99	98
phaseolicola	...	...	...	...	...	...	97	97

[a] NT = nucleotide; AA = amino acid.

pathovar (e.g., *P. s.* pv. *maculicola* 4326 compared with the isolate used by Kobayashi *et al.* [1990a] and *P. s.* pvs. *helianthi, tomato,* and *morsprunorum* [Table 1]). In addition, some isolates within a single pathovar (e.g., *P. s.* pvs. *phaseolicola* and *porri*) exhibited polymorphism with respect to size of the hybridizing *Hin*dIII band. Most bacteria that yielded *avr*D-hybridizing *Hin*dIII bands also produced *avr*D elicitor activity in culture, indicating the presence of functional *avr*D genes. However, three other bacterial isolates, including *P. s.* pvs. *eriobotryae, papulans,* and *sesami* as well as the newly examined *P. s.* pv. *glycinea* isolate 2214 (Table 1), did not produce detectable elicitor activity. Further work will be required to determine if these bacteria in fact carry nonfunctional *avr*D alleles, as previously shown for other isolates of *P. s.* pv. *glycinea* (Kobayashi *et al.* 1990b), or fail to produce elicitor for other reasons.

Although the *avr*D region seems highly conserved in the studied *P. syringae* isolates (Kobayashi *et al.* 1990a), three different *Hin*dIII polymorphisms have now been observed. Thus *avr*D may occur on *Hin*dIII fragments of about 5.6, 3.8, or 1.8 kb (Table 1). Although not definitive, *avr*D hybridization to some of the bacterial DNAs was less intense despite apparent equal DNA loading (Table 1). This suggestion of heterogeneity in *avr*D sequences among the various isolates was subsequently fully confirmed by DNA sequence analysis of three new *avr*D alleles.

We cloned and sequenced three additional alleles of *avr*D, previously characterized from *P. s.* pvs. *tomato* and *glycinea* R4 (Kobayashi *et al.* 1990a,b). All of the newly sequenced alleles exhibited the *avr*D phenotype (Tables 1 and 2), including both of the *P. s.* pv. *lachrymans* alleles. This is consistent with previous indications (Kobayashi *et al.* 1990a,b) and the data in Table 1 showing that functional alleles of *avr*D occur in several *P. syringae* pathovars, with the notable exception of the soybean pathogen, *P. s.* pv. *glycinea.*

The promoter regions of the newly sequenced alleles were similar to those previously characterized from *P. s.* pvs. *tomato* and *glycinea* (Innes *et al.* 1993; Kobayashi *et al.* 1990b; Shen and Keen 1993). On the basis of the configuration of the harp box, the GCCA repeats, and the σ^{54} elements, we predict that the promoters of these genes should also be functional.

The newly sequenced *avr*D alleles all encoded proteins with 311 amino acids, precisely the same as the previously sequenced *avr*D genes. Based on the levels of amino acid identity of the five alleles studied, they fall into two homology classes (Table 3, Fig. 4). Class I contained the *avr*D gene from *P. s.* pv. *tomato* and *P. s.* pv. *lachrymans* allele 1, and the remaining three sequences comprised class II. Amino acid identity within class II was higher than in class I, ranging from 97 to 99%, indicating that the *avr*D alleles from *P. s.* pvs. *phaseolicola* and *glycinea* and *P. s.* pv. *lachrymans* allele 2 were more closely related to each other than those within class I (95% identity with each other). Although the two classes had identical amino acids in 275 of 311 positions, they contained divergent residues dispersed throughout the proteins (Fig. 4). Legitimacy of the two homology classes was confirmed following the discovery that each class of *avr*D allele directs different elicitors when expressed in gram-negative bacteria (Yucel *et al.* 1994).

Sequencing of the three new *avr*D alleles provided additional data permitting deduction of the amino acid substitutions accounting for the nonfunctional nature of the *P. s.* pv. *glycinea* R4 *avr*D allele. It was previously shown (Kobayashi *et al.* 1990b) that the *P. s.* pv. *tomato* and *P. s.* pv. *glycinea* R4 alleles are 86% identical at the amino acid level with 41 amino acid substitutions. In the present study, we identified two new functional alleles, from *P. s.* pv. *phaseolicola* and *P. s.* pv. *lachrymans* allele 2, with considerably greater identity to the *P. s.* pv. *glycinea* protein (Table 3).

Comparison of all five alleles revealed that the nonfunctional *glycinea* R4 allele contained only four unique amino acid substitutions relative to the functional *P. s.* pv. *phaseolicola* and *P. s.* pv. *lachrymans* 2 alleles. Three of these changes occurred within the last 60 amino acids, consistent with earlier indications (Kobayashi *et al.* 1990b) that the carboxyl terminus of the *P. s.* pv. *glycinea* protein contributes to its lack of avirulence phenotype. The fourth amino acid substitution in the *P. s.* pv. *glycinea* allele was an arginine to cysteine change at position 19 that was not previously identified through recombinant gene constructions. This substitution could result in a major change in polarity and charge. The results accordingly suggest that arginine 19 in the functional allele is required for the putative enzymatic activity of the *avr*D protein. In the accompanying paper (Yucel and Keen, 1994), we further document the role of amino acid changes in the nonfunctional *glycinea* protein through recombinant gene constructions and oligonucleotide site-directed mutagenesis.

Avirulence gene D is plasmid-borne in all tested *P. syringae* pathovars that contain the gene (Fig. 1). *P. s.* pv. *lachrymans* was previously shown to contain two different *Hin*dIII fragments with high degrees of sequence similarity to *avr*D (Kobayashi *et al.* 1990a). We have determined that these DNA fragments, of 3.8 kb (*avr*D allele 1) and 5.6 kb (*avr*D allele 2), occur on indigenous plasmids of 75 and 90 kb, respectively. Surprisingly, DNA sequence data showed that the *P. s.* pv. *lachrymans* alleles were also the most diverged of the five *avr*D alleles compared (Table 3). This result is consistent with their observed disparate hybridization patterns and indicates that the *avr*D alleles of *P. s.* pv. *lachrymans* or

the plasmids harboring them may have been introduced from different sources.

P. s. pv. *phaseolicola* cosmid clone Psp01 was shown to harbor a functional *avr*D allele but also contains a second closely linked avirulence gene (I. Yucel and N. Keen, unpublished data). This gene functions in *P. s.* pv. *glycinea* R4 to elicit the HR on soybean cv. Acme, which does not contain the *Rpg*4 resistance gene complementing *avr*D. While the new gene is closely linked to *avr*D, its function does not appear to require the presence of *avr*D.

Table 4. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Source or reference[b]
Escherichia coli		
DH5α	F^- *lacZ* M15 *endA1 hsdR17 supE44 thi-1 gyrA relA1*$^-$	BRL
Pseudomonas syringae pathovars		
glycinea R4	Rif^r, Ap^r	Kobayashi *et al.* 1989
glycinea 2214		CFBP
tomato PT23		Kobayashi *et al.* 1989
tomato 10862		T. Denny
phaseolicola	Strain 3121, race 1	N. Panopoulos
phaseolicola	Strain G50, race 2	S. Patil
apii 1089-6		D. Cooksey
apii 0790-4		D. Cooksey
apii 0890-9		D. Cooksey
apii 1089-5		D. Cooksey
cilantro 0788-9		D. Cooksey
cilantro 0790-2		D. Cooksey
impatiens 0689-1		D. Cooksey
lachrymans		D. Cooksey
maculicola 4326		K. Davis
Plasmids		
pUC128/pUC129	Ap^r cloning and sequencing vectors	Keen *et al.* 1988
pRK415	Tc^r broad host range vector, *mob*$^+$	Keen *et al.* 1988
pRK2013	Km^r, Tra^+, helper plasmid	Ditta *et al.* 1980
pRK2073	Sm^r, Tra^+, helper plasmid	Ditta *et al.* 1980
pLAFR3	Tc^r cosmid cloning vector	Staskawicz *et al.* 1987
pMTL23	Ap^r *E. coli* cloning plasmid	Chambers *et al.* 1988
pINIII A-2	Ap^r *E. coli* expression plasmid	Masui *et al.* 1983
pPsp01	pLAFR3 cosmid clone containing ~26-kb DNA fragment from *P. s.* pv. *phaseolicola* 3121	D. Dahlbeck and B. Staskawicz
pAVRD12	*avr*D coding region from *P. s.* pv. *tomato* PT23 in an in-frame translational fusion with the *lac* promoters of pINIIIA-2	Keen *et al.* 1990
pMTLK4	3.8-kb *Hin*dIII fragment from pPsp01 in pMTL23 oriented downstream of the vector *lac* promoter	This study
pMTLK44	Same as pMTLK4 except with *avr*D positioned downstream of the *lac* promoter in pRK415	This study
pPAD1	1.8-kb *Hin*dIII-*Xba*I fragment subcloned from pMTLK4 with *avr*D positioned downstream of the *lac* promoter in pUC129	This study
pPAD2	Same as pPAD1 except in opposite orientation in pUC128	This study
pPAD5	1.8-kb *Hin*dIII-*Xba*I fragment from pPAD1 with *avr*D positioned downstream of the *lac* promoter in pRK415	This study
pLADA	3.8-kb *Hin*dIII fragment containing *avr*D allele 1 from *P. s.* pv. *lachrymans* cloned downstream of the *lac* promoter in pUC129	This study
pLAD3	1.8-kb *Hin*dIII-*Xba*I fragment subcloned from pLADA with *avr*D positioned downstream of the *lac* promoter in pUC129	This study
pLAD4	Same as pLAD3 except in pUC128	This study
pLAD5	1.8-kb *Hin*dIII fragment from pLAD3 with *avr*D positioned downstream of the *lac* promoter of pRK415	This study
pLADB	5.6-kb *Hin*dIII fragment from *P. s.* pv. *lachrymans* containing *avr*D allele 2 cloned into pUC129	This study
pLLAD1	1.8-kb *Hin*dIII-*Xba*I fragment subcloned from pLADB with *avr*D positioned downstream of the *lac* promoter in pUC129	This study
pLADB4	5.6-kb *Hin*dIII fragment from pLADB with *avr*D positioned downstream of the *lac* promoter in pRK415	This study
pLADB5	Same as pLADB4, except *avr*D is positioned in the anti-orientation to the *lac* promoter in pRK415	This study
pGA11	Cloned *avr*D allele from *P. s.* pv. *glycinea*	Kobayashi *et al.* 1990b

[a] Ap = ampicillin, Km = kanamycin, Rif = rifampicin, Sm = streptomycin, Tc = tetracycline, Tra^+ = transfer-competent, ^{r} = resistance.
[b] BRL = Bethesda Research Laboratories, Gaithersburg, MD; CFBP = Collection Francaise de Bacteries Phytopathogenes, INRA, Angers, France; D. Cooksey, University of California, Riverside; D. Dahlbeck and B. Staskawicz, University of California, Berkeley; K. Davis, Ohio State University, Columbus; T. Denny, University of Georgia, Athens; N. Panopoulos, University of California, Berkeley; S. Patil, University of Hawaii, Honolulu.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media.

Bacterial strains and plasmids used or constructed in this paper are shown in Table 4. *E. coli* was grown at 37° C on Luria-Bertani (LB) medium (Maniatis *et al.* 1982) in liquid culture or on agar plates. *P. syringae* pathovars were grown at 28° C on King's medium B (KB) in liquid culture or on agar plates (King *et al.* 1954). Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; rifampicin, 100 μg/ml; and tetracycline, 15 μg/ml in LB and 30 μg/ml in KB. For elicitor studies, *E. coli* or *P. syringae* cells were grown in shaken 15-ml liquid cultures of M9 medium (Maniatis *et al.* 1982) amended with thiamine at 4 μg/ml and appropriate antibiotics. *P. syringae* cultures were also grown on 50 ml of induction medium (IM) composed of: NH_4Cl, 0.3 g/L; $FeSO_4$, 1 mg/L; NaCl, 1 g/L; K_2SO_4, 0.5 g/L; $MgSO_4$, 0.4 g/L; casamino acids (Difco technical), 0.8 g/L; fructose, 3 g/L; mannitol, 3 g/L; sodium citrate, 3 g/L; $CaCl_2$, 0.05 g/L. The medium was adjusted to pH 5.5 with HCl and autoclaved; then, sterile potassium phosphate at pH 5.5 was added to 0.03 M. *E. coli* cells carrying plasmids with *lac* promoters were induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM at the time of culture initiation. *P. syringae* M9 cultures contained twice the normal glucose concentration and were supplemented with 0.1% casamino acids. All cultures for elicitor studies were grown at 28° C for 16–24 hr.

DNA manipulations.

Standard recombinant DNA methods were generally performed as described by Maniatis *et al.* (1982). A variation of the 10-min miniprep method of Zhou *et al.* (1990) was used to isolate the indigenous plasmids of *P. syringae* pathovars and for miniscreen analyses of recombinant plasmids in *E. coli.* These plasmids were constructed by isolating desired DNA fragments from low-melting-point agarose gels (Crouse *et al.* 1983) for ligation into vectors and transformation of *E. coli* strain DH5α. Generally, DNA fragments were cloned into either pUC128 or pUC129 and recloned into the broad host range plasmid, pRK415. These constructs were then introduced into *P. s.* pv. *glycinea* R4 and inoculated into several soybean cultivars to test for occurrence of the HR on those containing the *Rpg*4 resistance gene. Table 4 lists the plasmids and constructs used in this study.

The indigenous plasmids of *P. syringae* pathovars were resolved on 20-cm-long 0.8% agarose gels using 1× TBE and run at 100 V for 10 hr at 4° C. For Southern blots, DNA fragments on gels were transferred to 0.45-μm nylon membranes (Micron Separations, Inc., Westboro, MA). Hybridizations with a ^{32}P-labeled *avr*D-specific probe (1.1-kb *Eco*RI fragment from pAVRD12 [Keen *et al.* 1990]) were performed in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt's solution, 0.02 M sodium phosphate, pH 6.7, and salmon sperm DNA (0.1 mg/ml) at 42° C with gentle shaking for at least 8 hr. Blots were then washed at 42° C for 30 min with two changes of 2× SSC, 0.1% sodium dodecyl sulfate and exposed to X-ray film.

In some experiments, *P. s.* pv. *lachrymans* plasmids were resolved on 0.6% low-melting-point agarose gels, and the two largest plasmids were excised separately and digested with *Hin*dIII (Crouse *et al.* 1983). The independently digested plasmid fragments and a total plasmid preparation also digested with *Hin*dIII were resolved on a 1% agarose gel and transferred to a nylon membrane for Southern analysis as described above.

A plasmid-specific library of *P. s.* pv. *lachrymans* DNA was constructed by isolating *Hin*dIII-digested plasmid DNA fragments (3–6 kb) from a 0.8% low-melting-point agarose gel and cloning them into the same site of pUC129. *E. coli* transformants were selected on LB plates containing ampicillin, X-Gal, and IPTG. White colonies were selected and picked onto master plates. Colony hybridization experiments were used to identify clones containing the 3.8- and 5.6-kb *Hin*dIII fragments with homology to an *avr*D-specific probe. Positive clones identified through this procedure were reconfirmed by miniscreens and Southern analysis to obtain pLAD1 and pLLAD1, which contain the 3.8- and 5.6-kb *Hin*dIII fragments, respectively.

DNA sequence analysis.

Exonuclease III deletions were performed on DNA fragments to be sequenced using the Erase-a-base protocol (Promega, Madison, WI). Both strands were sequenced by the dideoxy chain termination method (Sanger *et al.* 1977) using Sequenase (U.S. Biochemical, Cleveland, OH). Sequence analysis and comparisons were made using the Genetics Computer Group (GCG), University of Wisconsin, package.

Bacterial conjugations, plant growth conditions, and inoculations.

Bacterial conjugations were performed based on the method described by Ditta *et al.* (1980). Overnight cultures were used to make a mating mixture containing about 106 cells per milliliter each of donor and helper bacteria and 108 cells per milliliter of the recipient, *P. s.* pv. *glycinea* R4. The mating mixture was spotted onto nonselective KB plates, incubated at 28° C for 10–12 hr, and then streaked onto KB plates containing rifampicin and tetracycline and incubated at 28° C. The resulting transconjugants were successively single-colony purified two or three times on selective KB before inoculation of soybean plants. Water suspensions containing about 5×10^7 cells of *P. s.* pv. *glycinea* R4 transconjugants per milliliter were prepared from overnight cultures grown on plates and used to inoculate fully expanded primary leaves as described previously (Keen *et al.* 1990). Soybean plants were grown from seed (Long *et al.* 1985) and maintained in a growth chamber (10,000 lux, 16-hr photoperiod) at 21° C with 90% relative humidity after inoculation. Inoculations were scored daily for the appearance of a visible HR or water-soaked lesions, which occurred typically after 24–30 or 48–72 hr, respectively.

Elicitor assays.

Supernatant M9 or IM culture fluids from *E. coli* or *P. syringae* cells expressing *avr*D were desalted with Sep-Pak C18 cartridges (Waters Associates, Milford, MA) and further processed according to Keen *et al.* (1990). Primary leaves of soybean cultivars Harosoy, Acme, Flambeau, Merit, Norchief, or Peking were infiltrated with the elicitor preparations and scored for appearance of the HR which occurred 12–20 hr after infiltration of active preparations only into cultivars Harosoy, Flambeau, and Norchief.

Nucleotide sequence and accession numbers.

The nucleotide sequences of the three *avr*D alleles from *P. s.* pvs. *lachrymans* and *phaseolicola* have been submitted to GenBank and assigned accession numbers L11334 (*P. s.* pv. *lachrymans* 1), L11335 (*P. s.* pv. *lachrymans* 2), and L11336 (*P. s.* pv. *phaseolicola*).

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