avrA and *avrE* in *Pseudomonas syringae* pv. *tomato* PT23 Play a Role in Virulence on Tomato Plants

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Four avirulence genes (*avrA, avrD, avrE,* and *avrPto*) from *Pseudomonas syringae* pv. *tomato* strain PT23 function in *P. s.* pv. *glycinea* to elicit the hypersensitive response in soybean leaves. To determine whether these genes are important for virulence on the normal host, tomato, or for resistance in nonhost plants, such as soybean, PT23 marker exchange mutants were constructed. All of the mutant strains grew normally in culture media and utilized several carbon sources, as did the wild-type bacterium. Mutations in *avrA, avrD, avrE,* and *avrPto* did not affect the ability of PT23 to cause hypersensitive reactions on leaves of tobacco and several soybean cultivars. However, mutation of *avrE* greatly reduced virulence and bacterial multiplication in tomato leaves. Mutation of *avrA* also resulted in a small but reproducible reduction in virulence on tomato leaves. These results indicate that the studied PT23 avirulence genes are not involved in nonhost resistance in tobacco and soybean but that *avrA* and *avrE* are important for high virulence on the normal host plant, tomato.

Additional keywords: disease resistance, gene-for-gene complementarity, sequential mutagenesis.

Pseudomonas syringae pv. *tomato* causes bacterial speck disease of tomato and elicits the hypersensitive response (HR) in all tested cultivars of soybean. Kobayashi *et al.* (1989) cloned three avirulence genes, *avrA*, *avrD,* and *avrE,* from *P. s.* pv. *tomato* strain PT23 that function in *P. s.* pv. *glycinea* to elicit the HR in soybean. *avrA* is virtually identical to the *avrA* gene cloned previously from *P. s.* pv. *glycinea* race 6 (Staskawicz *et al.* 1984). *avrD* has been characterized at the molecular level (Kobayashi *et al.* 1990) and shown to direct bacterial production of a cultivar-specific elicitor of the HR in soybean (Keen *et al.* 1990). *avrE* is a complex locus composed of two divergent transcripts closely linked to the *hrp* gene cluster of *P. s.* pv. *tomato* (J. M. Lorang and N. T. Keen, unpublished), but this entire avirulence locus will be referred to as *avrE* in this paper for convenience. Ronald *et al.* (1992) cloned the *P. s.* pv. *tomato avrPto* gene, which genetically complements the *Pto* disease resistance gene in tomato plants (Martin *et al.* 1993) and also causes the HR in certain soybean cultivars when transferred into *P. s.* pv. *glycinea.* Collectively, these avirulence genes elicit the HR in all tested soybean cultivars (Table 1), but it has not been established whether they have a role in restricting the host range of *P. s.* pv. *tomato* to exclude soybean. Whalen *et al.* (1991) also cloned the *avrRpt2* gene from strain JL 1065 of *P. s.* pv. *tomato,* but it is not known if this gene occurs in strain PT23.

All of the characterized PT23 avirulence genes appear to be co-regulated with *hrp* genes (Innes *et al.* 1993a; Lorang and Keen, unpublished; Salmeron and Staskawicz 1993; Shen and Keen 1993; Willis *et al.* 1994; Xiao *et al.* 1994), implying that their functions may be related to growth in or on plants. In at least two cases, *pthA* from *Xanthomonas citri* (Swarup *et al.* 1992) and *avrBs2* from *X. campestris* pv. *vesicatoria* (Kearney and Staskawicz 1990), bacterial avirulence genes are important for full virulence on a normal host plant. For all *P. syringae avr* genes thus far cloned, however, biological functions have not been determined, and mutation of single *avr* genes has not been shown to impair pathogen virulence. In this work we show that two avirulence genes, *avrA* and *avrE,* are required for full virulence of strain PT23 on tomato plants, but none of the studied *avr* genes appear to restrict host range to exclude soybean or tobacco.

RESULTS

Construction of a *recA* mutant strain of *P. s.* pv. *glycinea* race 4.

Conjugation frequencies were often low with the wild-type race 4 isolate, and occasional evidence for rearrangement of introduced plasmids was observed (unpublished data). One possible solution to these problems is to utilize a *recA* strain. Willis *et al.* (1988) cloned the *recA* gene from an isolate of *P. s.* pv. *syringae* and constructed a marker exchange mutant strain. We obtained their mutagenized *recA* cosmid clone, but unfortunately could not obtain marker exchange of the mutated gene into *P. s.* pv. *glycinea* R4. The *recA* gene was therefore isolated from a cosmid library of *Psg*R0 (Staskawicz *et al.* 1987) by its ability to complement the growth of *Escherichia coli* HB101 on mitomycin C plates. Tn*5* mutagenesis was carried out on the resulting cosmid clone, pRecA1 (Fig. 1), containing the putative *recA* gene. Mutant plasmids unable to complement the growth of *E. coli* HB101 on mitomycin C plates were isolated, and Tn*5* insertions that

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inactivated the *recA* gene were mapped to a single 6.5-kb *Eco*RI fragment. One of the mutant clones was marker-exchanged into *Psg*R4. The resulting marker exchange mutant was confirmed by its inability to grow on mitomycin C plates, unlike the wild-type strain, and by Southern blot analysis of total DNA (data not shown).

The *recA* mutant, *Psg*R4recA, elicited water-soaking symptoms similar to those caused by the wild-type strain when inoculated onto 10 different cultivars of soybean. However, the frequency of conjugation obtained with the *recA* mutant was more than five times greater than that of the wild-type strain when pLAFR3 or pRK415 plasmid constructs were used and no evidence of plasmid rearrangements was observed. *Psg*R4recA was subsequently used as the recipient for scoring the phenotype of *avr* gene constructs on soybean plants.

Construction of mutations in the cloned *avrA, avrD, avrE,* and *avrPto* genes.

Cosmid clone p9A11, containing the *avrA* gene, was subjected to random Tn*5* mutagenesis. Kanamycin-resistant plasmids were recovered, conjugated into *P. s.* pv. *glycinea* R4 *recA,* and screened on the normally *avrA*-incompatible soybean cultivar Harosoy. One Tn*5* mutant, pPT9A11::Tn*5,* did not elicit an HR in Harosoy or any other soybean cultivar carrying resistance gene *Rpg2,* which complements *avrA* (Keen and Buzzell 1991). The Tn*5* insertion in pPT9A11::Tn*5* mapped to a 0.95-kb *Eco*RI fragment (Fig. 1).

The *avrE* avirulence function of pPT10E9 was subcloned to an 11.3-kb *Eco*RI-*Bam*HI fragment (pPTBE11) (Lorang and Keen, unpublished). Deletion clones pPTBE11ΔKm and pPTBEl1ΔGm were constructed by replacing an internal 0.4-kb *Sac*I fragment in pPTBE11 with a kanamycin resistance or gentamicin resistance gene (Fig. 1). pPTBE11, but neither pPTBE11ΔKm nor pPTBE11ΔGm caused *Psg*R4 to elicit the HR in all tested soybean cultivars. A Tn*5* insertion mutant of the *avrD* gene has been described by Kobayashi *et al.* (1989) (Fig. 1). The deletion mutant of the *avrPto* gene, pΔ*avrPto* (Ronald *et al.* 1992), was provided by John Salmeron and Brian Staskawicz.

Generation of *P. s.* pv. *tomato avr* gene mutant strains.

The mutated genes on pPT9A11::Tn*5*, pPT4E10::Tn*5,* and pPTBEΔKm were individually marker-exchanged into *P. s.* pv. *tomato*. The resultant mutant strains, with mutations in the *avrA, avrD,* and *avrE* genes, were designated MXA, MXD, and MXE, respectively.

P. s. pv. *tomato* strain HWB is cured of the native plasmid pPT23B on which the *avrD* gene resides, but causes a normal compatible reaction on tomato and an incompatible reaction on soybean as compared to the wild-type strain PT23 (Murillo *et al.* 1994). HWB was used to make multiple *avr* gene mutant strains because it lacks the *avrD* gene without the use of a selectable marker, thus leaving several selectable markers available for construction of other *avr* gene mutations. The mutant clone pPT9A11::Tn*5* was marker-exchanged into HWB, resulting in the mutant MXAD, which has a Tn*5* insertion in the *avrA* gene and no *avrD* gene. The mutant clone pPTBEΔGm was then marker-exchanged into strain MXAD. The resultant triple mutant, MXADE, had a deletion mutation of the *avrE* gene, a Tn*5* insertion in the *avrA* gene, and no *avrD* gene, because of the absence of its resident plasmid, pPT23B. Finally, the mutant clone pΔ*avrPto* was marker-exchanged into strain MXADE, resulting in strain MXADEP. The genotypes of all mutants were confirmed by Southern blot analysis. One *Eco*RI fragment, shifted to the expected size for each mutant construct, hybridized to each gene-specific probe except for *avrD,* which was not present, as expected. Therefore, Southern blot analysis verified that each *avr* gene is present as a single copy in the PT23 genome and that each *avr* gene had in fact been mutated (data not shown).

Carbon source utilization and growth of *avr* mutant strains in culture media.

To see if mutations in *avr* genes altered the ability of *P. s.* pv. *tomato* to grow in culture media and to utilize various carbon sources, each strain was compared with wild-type PT23 for its ability to grow in a complete nutrient medium (King's medium B [KMB]) and a minimal nutrient medium (M9), and to utilize 95 different carbon sources. All of the strains grew in KMB and M9 media as determined by OD_{600} > 1 after 16 hr of growth. All strains also showed a pattern of carbon source utilization identical to that of PT23 as determined by the Biolog Gn microtiter plate assay.

Ability of *P. s.* pv. *tomato* mutant strains to elicit the HR in nonhost plants.

Each *P. s.* pv. *tomato* mutant strain was assayed for its ability to elicit the HR in a nonhost plant, tobacco, and in 10 soybean cultivars (see Materials and Methods). MXA, MXD, and MXE elicited a visible HR in all test plants within 16 hr, which was indistinguishable from that caused by the wild-type strain, PT23. In some experiments, MXADE and MXADEP caused less intense hypersensitive reactions in the

Table 1. Production of the hypersensitive response in 10 cultivars of soybean by *Pseudomonas syringae* pv. *glycinea* R4 (Psg R4) containing cloned avirulence genes from *P. s.* pv. *tomato*[a]

Avirulence gene in Psg R4	Soybean cultivar									
	Acme	Flambeau	Keburi	Hardee	Harosoy	Merit	Lindarin	Norchief	Hurrelbrink	Peking
avrA[b]	+	−	−	−	+	+	+	−	+	+
avrD[c]	−	+	−	−	+	−	+	+	+	−
avrE[d]	+	+	NT	+	+	+	+	+	NT	+
avrPto[e]	+	+	−	+	+	+	+	+	−	±

[a] +, Hypersensitive response; −, disease symptoms; ±, intermediate response; NT, not tested.
[b] pPT9A11 (Kobayashi *et al.* 1989).
[c] (pPT4E10) (Kobayashi *et al.* 1989).
[d] (pPT10E9) (Kobayashi *et al.* 1989; J. Lorang and N. T. Keen, unpublished).
[e] (pPTE2) (Ronald *et al.* 1992; R. Ransom, J. Lorang, and N. T. Keen, unpublished).

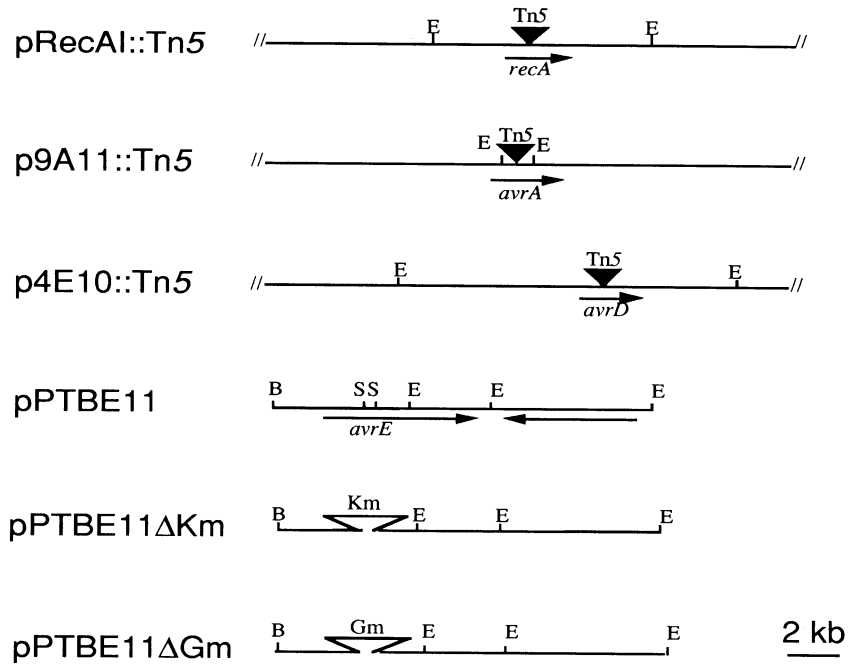

Fig. 1. Restriction maps of mutations constructed in cloned avirulence genes from *Pseudomonas syringae* pv. *tomato*. B = *Bam*HI; E = *Eco*RI; S = *Sac*I; Km = kanamycin resistance gene; Gm = gentamicin resistance gene. Closed triangles represent Tn*5* insertions; open triangles represent insertion of the designated antibiotic resistance gene into a 0.4-kb *Sac*I deletion site.

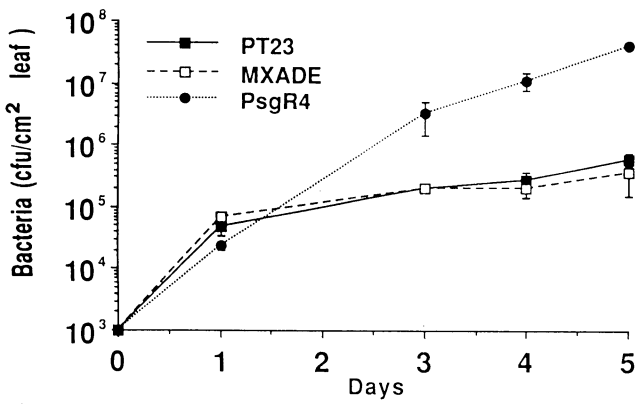

Fig. 2. Growth of incompatible *Pseudomonas syringae* pv. *tomato* strain PT23, mutant strain MXADE, and compatible *P. s.* pv. *glycinea* R4 (PsgR4) in primary leaves of the soybean cultivar Peking. The data are means of three replications, and the experiments were repeated at least twice. Error bars are not visible for some data points because they are smaller than the data point symbols.

soybean cultivar Peking inoculated with 10^7 cfu/ml than did PT23 or the other mutant strains. However, no differences were observed when Peking was inoculated with MXADE or MXADEP at 10^8 cfu/ml. Monitoring bacterial populations also showed that strain MXADE exhibited a growth curve similar to that of the wild-type PT23 whether inoculated alone (Fig. 2) or co-inoculated with strain PT23 (data not shown). Both of these strains attained populations at least 100-fold less than that of the compatible pathogen, *Psg*R4.

Since all soybean cultivars tested showed hypersensitive reactions to *P. s.* pv. *glycinea* R4recA carrying the cloned *avrPto* gene, it was possible that this gene accounted for the observed hypersensitive reactions of the PT23 mutants. We accordingly screened several additional soybean cultivars for their reactions to *P. s.* pv. *glycinea* R4recA carrying the cloned *avrPto* gene. Of these, cultivars Hurrelbrink and Keburi did not exhibit a hypersensitive reaction, indicating that they do not carry a functional *Pto* resistance gene (Table 1). It was accordingly of interest to see if *P. s.* pv. *tomato* PT23 strains MXADE and MXADEP elicited hypersensitive reactions similar to that caused by PT23 in these cultivars. No significant differences were observed in the hypersensitive reactions elicited by the mutant strains and PT23 at several different inoculum concentrations (data not shown). Plants showed little or no visible HR in reaction to an inoculum concentration of 10^6 cfu/ml, and a concentration of 10^7 cfu/ml produced temporally delayed hypersensitive reactions, relative to 10^8 cfu/ml, but the reactions were consistently the same for all strains at the same inoculum concentration. Thus, no evidence was obtained indicating that the four avirulence genes mutated in strain PT23 are involved with the nonhost HR in soybean.

Strain MXADEP retained the ability to induce the HR in tomato cultivar Peto 76R, which contains the *Pto* disease resistance gene (data not shown). This unexpected result was also observed by Ronald *et al.* (1992) with *P. s.* pv. *tomato* strain JL 1065.

Virulence of PT23 mutant strains on tomato plants.

The degree to which the various *avr* gene mutant strains of *P. s.* pv. *tomato* cause disease on tomato was evaluated by visual analysis of disease symptoms and by monitoring the growth of bacterial populations in tomato leaves over time. MXD produced disease symptoms and a growth curve similar to those of the wild-type strain PT23, but MXA caused slightly reduced symptoms (Fig. 3) and had a lower rate of bacterial multiplication (Fig. 4). Strains carrying a mutation in *avrE* (MXE, MXADE, and MXADEP) produced much less

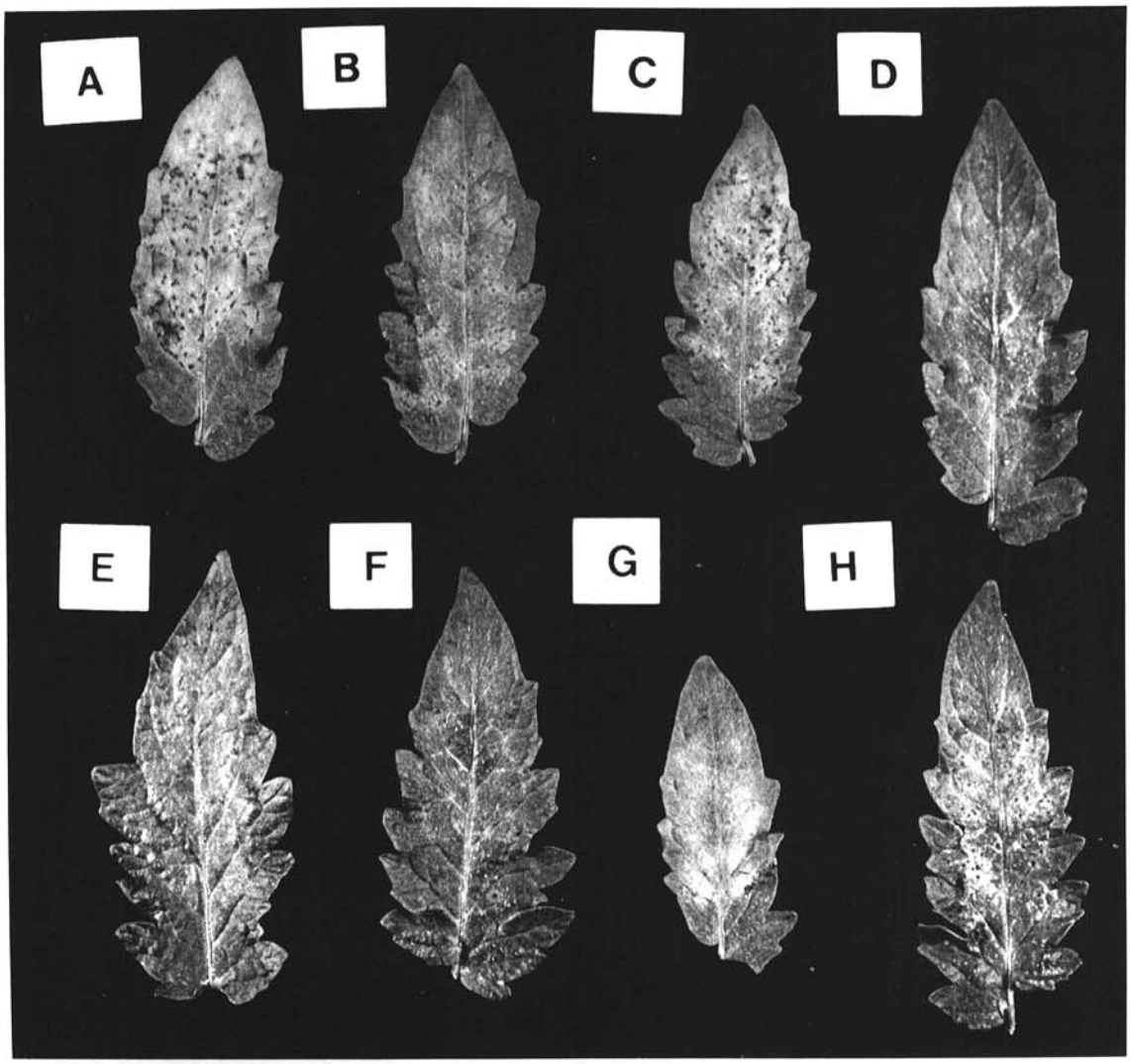

Fig. 3. Symptoms on tomato leaves caused by *Pseudomonas syringae* pv. *tomato* strain PT23 and mutant strains. **A,** PT23; **B,** MXA; **C,** MXD; **D,** MXE; **E,** MXADE; **F,** MXADE(p415A); **G,** MXADE(pPTBE11); **H,** MXE(pPTBE11).

severe symptoms (Fig. 3) and had greatly reduced populations in tomato leaves relative to PT23 (Fig. 4).

Complementation of avirulence gene mutations.

The cloned *avrE* locus (pPTBE11) was conjugated into mutant strains MXE, MXA, and MXADE as well as wild-type strain PT23 in order to determine if the *avrE* mutation could be complemented and if additional copies of *avrE* increased the virulence of PT23. *avrE* complemented the reduced virulence phenotype on tomato plants (Fig. 3) and restored bacterial multiplication of MXE and MXADE to near wild-type levels (Fig. 5). However, additional copies of *avrE* did not entirely increase the virulence of MXA or PT23 (data not shown). The cloned *avrA* gene (p415A) was conjugated into strains MXA and MXADE, and growth of the transconjugant strains in tomato leaves was monitored. This plasmid did not significantly complement disease symptoms (Fig. 3) or the growth of MXA (Fig. 5) and MXADE in tomato leaves (data not shown). However, p415A cured at a higher rate than the other RK2-based plasmid constructs, since approximately 95% of the bacteria isolated from inoculated tomato leaves were tetracycline-sensitive at 2 days after inoculation.

DISCUSSION

Avirulence (*avr*) genes presumably have two functions. The first is hypothesized to be selected functions which maintain these genes in pathogen populations (Van der Plank 1968). The second function is serendipitous, a consequence of plant recognition of pathogens expressing avirulence genes to trigger defense responses. Cloning and mutation of several *P. syringae avr* genes have verified that they specify the pathogen host range at the race-cultivar level (Keen *et al.* 1990) but have not suggested selected, beneficial functions in the bacteria. We have now observed that mutagenesis of two different *avr* genes in *P. s.* pv. *tomato* reduces its virulence on tomato plants. This finding has considerable significance for our understanding of avirulence gene functions in *P. syringae*.

We were not surprised that the PT23 mutant strains MXA, MXD, MXE, MXADE, and MXADEP were indistinguishable from wild-type PT23 in their ability to grow in minimal and rich culture media and to utilize different carbon sources. These findings are in agreement with studies of other cloned *avr* genes, none of which appear to be essential for basic

bacterial metabolism (Keen 1990). Furthermore, transcription of the *P. syringae avrD, avrE, avrRpt2,* and *avrPto* genes is induced both *in planta* and in *hrp*-inducing minimal media, but not in strains having mutations in *hrp* regulatory loci (Innes *et al.* 1993a; Lorang and Keen, unpublished; Salmeron and Staskawicz 1993; Shen and Keen 1993; Xiao *et al.* 1994). This also suggests specialized rather than basic housekeeping functions and argues that avirulence genes may be important when pathogens encounter plants. The evolution of plant surveillance mechanisms to recognize such pathogen factors expressed during plant colonization is also plausible. As a consequence, pathogens may have evolved multiple approaches to host survival and virulence, so that the loss of any one virulence function by mutation might not be detrimental. Experimental exercises involving the mutation of only one or a few *avr* genes therefore might not be informative. Furthermore, the contribution of a particular *avr* gene to survival on plants and virulence may be subtle. Carefully controlled quantitative experiments under several environmental regimes may be required to reveal such functions.

In agreement with the above theoretical predictions, we found that PT23 strains carrying individual or multiple *avr* gene mutations caused normal disease symptoms when relatively high inoculum concentrations (10^7 cfu/ml) were rubbed on or infiltrated into tomato leaves. However, when inoculum concentrations of 10^4 cfu/ml were infiltrated into leaves (a concentration more similar to natural inoculum levels), strains carrying mutations in the *avrE* locus were considerably less virulent than wild-type PT23 (Figs. 3 and 4), but complete virulence was regained when the mutant strains were complemented by the cloned *avrE* locus. We conclude that *avrE* is required for full virulence of PT23 on its normal host, tomato. Avirulence locus E in PT23 is therefore the first *P. syringae avr* gene for which clear pleiotropic functions in virulence and elicitation of the plant HR have been identified. Similar dual functions, however, have been noted for two *Xanthomonas* genes, *pthA* (Swarup *et al.* 1992) and *avrBs2* (Kearney and Staskawicz 1990). Our previous work revealed that, unlike strain PT23, the *avrE* homolog of *P. s.* pv. *tomato* strain DC3000 is not required for full virulence on tomato plants (Lorang and Keen, unpublished). This strain-dependent behavior is similar to that of *lemA,* which is essential for full virulence in *P. s.* pv. *syringae* but not required for virulence in *P. s.* pv. *phaseolicola* (Rich *et al.* 1992).

The PT23 strain MXA consistently exhibited reduced bacterial multiplication in tomato leaves, compared with the wild-type strain PT23, but these small differences were not always statistically significant, because of the errors inherent in *in planta* growth experiments. Furthermore, the *avrA* clone (p415A) used to complement the *avrA* mutation was lost from PT23 strains *in planta* after about 2 days in the absence of tetracycline selection. This may have accounted for the observed partial complementation of the *avrA* mutation by p415A. The relatively small contribution of *avrA* to the virulence of *P. s.* pv. *tomato* was also epistatic to *avrE,* suggesting that these loci may contribute to a common physiological function.

Under no tested conditions were PT23 strains carrying mutations in *avrD* or *avrPto* affected in the degree of symptom formation on tomato leaves. While it might be of interest to determine if these genes have a role in other stages of the pathogen life cycle, *avrD* and *avrPto* do not appear to have a role in virulence. The result with *avrPto,* however, was complicated by the fact that these mutants unexpectedly retained the ability to elicit the HR in tomato plants carrying the cognate resistance gene, *Pto*. This result is similar to that of Ronald *et al.* (1992), who found that an *avrPto* mutation in *P. s.* pv. *tomato* DC3000 also did not eliminate the *avrPto* phenotype on tomato plants carrying the *Pto* gene. Both bacterial strains must therefore have another functional but nonhybridizing *avrPto* homolog, as postulated for strain DC3000 by Ronald *et al.* (1992).

In addition to their role in conferring specificity at the race-cultivar level, several avirulence genes also function in heterologous bacterial taxa, causing them to elicit defense reac-

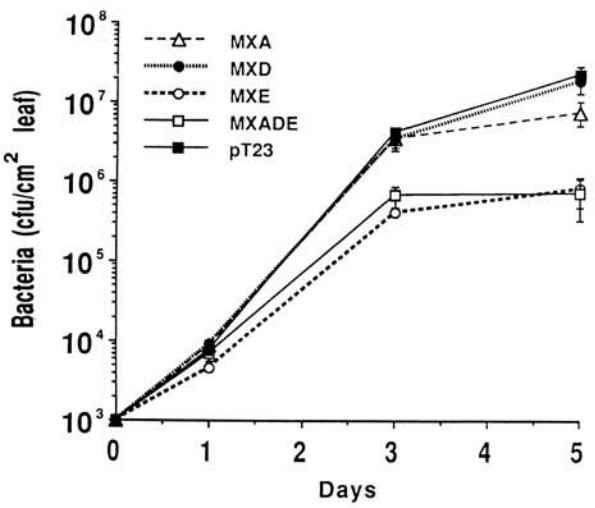

Fig. 4. Growth of *Pseudomonas syringae* pv. *tomato* strain PT23 and mutant strains MXA, MXD, MXE, and MXADE in tomato leaves. The data are means of three replicates, and the experiments were repeated at least twice. Error bars are not visible for some data points because they are smaller than the data point symbols.

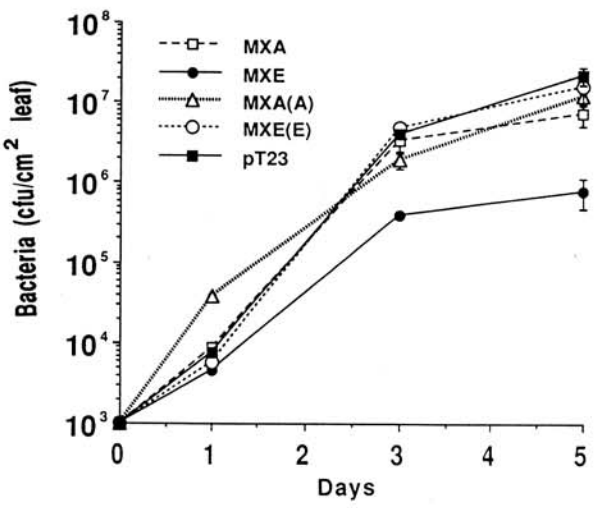

Fig. 5. Complementation of *Pseudomonas syringae* pv. *tomato* (PT23) *avr* gene mutant strain MXE by the cloned *avrE* locus, pPTBE11 (E), and of strain MXA by the cloned *avrA* gene, p415A (A). The data are means of three replicates, and the experiments were repeated at least twice. Error bars are not visible for some data points because they are smaller than the data point symbols.

tions on normally compatible hosts (Carney and Denny 1990; Fillingham *et al.* 1992; Kobayashi *et al.* 1989; Ronald *et al.* 1992; Whalen *et al.* 1988, 1991). Novel plant disease resistance genes which are functionally conserved among diverse plant taxa have also been described (Dangl *et al.* 1992; Innes *et al.* 1993b; Keen and Buzzell 1991; Wanner *et al.* 1993), raising the possibility that avirulence genes may play a role in determining host range at the pathovar–plant species level. The results in this paper, however, do not indicate such a role for the studied avirulence genes of *P. s.* pv. *tomato*.

Preliminary results suggested that PT23 strains with mutations in *avrA, avrE,* and *avrPto* failed to give hypersensitive reactions on the soybean cultivar Peking. However, these results were only observed at relatively low inoculum concentrations, and no differences were noted in multiplication of the mutant strains, compared to strain PT23 (Fig. 2). Furthermore, Peking shows reactions that are more difficult to reliably score than those of other cultivars. When the cultivars Keburi and Hurrelbrink were identified as appearing to lack a functional *Pto* resistance gene, no evidence was obtained for differences in elicitation of the HR by the various *avr* gene mutant strains at several inoculum levels. Thus, there is no indication that *avrA, avrD, avrE,* or *avrPto* contribute to the nonhost nature of soybean and tobacco plants to *P. s.* pv. *tomato* PT23. The fact that these strains retain the ability to elicit an HR in soybean and tobacco plants may be a consequence of *hrp* gene activity (Willis *et al.* 1994), but it is also possible that *P. s.* pv. *tomato* lacks positive-acting functions required for pathogenicity on these plants. Evidence for the occurrence of such factors at the plant species–pathogen level has been found in several systems (Ma *et al.* 1988; Mellano and Cooksey 1988; Salch and Shaw 1988; Swarup *et al.* 1992; Waney *et al.* 1991).

MATERIALS AND METHODS

Plasmids, bacterial strains, and culture conditions.

Bacterial strains and plasmids used or constructed in this study are listed in Table 2. *E. coli* strains were grown at 37° C on Luria-Bertani (LB) medium (Maniatis *et al.* 1982), and *P. syringae* strains were grown at 28° C on KMB (King *et al.* 1954). When appropriate, antibiotics were used at the follow-

Table 2. Bacterial strains and plasmids used in this study

	Relevant characteristics[a]	Reference or source
Escherichia coli		
DH5α	*F⁻ lacZ* ΔM15 *endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1* λ⁻	Bethesda Research Laboratories (Gaithersburg, MD)
HB101	*F⁻ hsdS20* [*hsdR hsdM recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44* λ⁻]	Maniatis *et al.* 1982
S17-1	*Pro res⁻ mod⁺*, RP4-2-Tc::Mu-Km::Tn*7* integrated into the chromosome, Tp^r Sm^r	Simon *et al.* 1983
Pseudomonas syringae pv. *glycinea*		
*Psg*RO	Wild type	Staskawicz *et al.* 1987
*Psg*R4	Wild type	Kobayashi *et al.* 1990
*Psg*R4recA	*recA*::Tn*5*	This work
P. s. pv. *tomato*		
PT23	Wild type	Bender and Cooksey 1987
HWB	PT23 cured of native plasmid pPT23B	Murillo *et al.* 1994
MXA	PT23, *avrA*::Tn*5*	This work
MXD	PT23, *avrD*::Tn*5*	This work
MXE	PT23, *avrE*::Km	This work
MXAD	PT23, *avrA*::Tn*5* derivative of HWB	This work
MXADE	PT23, *avrE*Δ::Gm derivative of MXAD	This work
MXADEP	PT23, Δ*avrPto* derivative of MXADE	This work
Plasmids		
pUC128/129	*E. coli* cloning vector, Ap^r	Keen *et al.* 1988
pBluescriptKS+	*E. coli* cloning vector, Ap^r	Stratagene (La Jolla, CA)
pRK415	RK2-derived broad-host-range vector, Tc^r	Keen *et al.* 1988
pLAFR3	Cosmid derivative of RK2, Tc^r	Staskawicz *et al.* 1987
pRecAI	Cosmid clone containing the *Psg recA* gene	This work
pRecAI::Tn*5*	Tn*5* insertion into the *recA* gene in pRecAI	This work
pRARI	6.5-kb *Eco*RI subclone of the *recA* gene in pRK415	This work
pPT9A11	Cosmid clone containing the *avrA* gene	Kobayashi *et al.* 1989
pPT9A11::Tn*5*	Tn*5* insertion into *avrA* in p9A11	This work
p415A	Approximately 3-kb *Sal*I fragment carrying *avrA* cloned into pRK415	This work
pPT4E10	Cosmid clone containing the *avrD* gene	Kobayashi *et al.* 1989
pPT4E10::Tn*5*	Tn*5* insertion into *avrD* in pPT4E10	Kobayashi *et al.* 1989
pPT10E9	Cosmid clone containing the *avrE* locus	Kobayashi *et al.* 1989
pPTBE11	11.3-kb *Bam*HI-*Eco*RI subclone of pPT10E9	J. Lorang and N. T. Keen, unpublished
pPTBE11ΔKm	*avrE* mutant, pPTBE11 with a Km^r gene replacing a 0.4-kb *Sac*I fragment	This work
pPTBE11ΔGm	*avrE* mutant, pPTBE11 with a Gm^r gene replacing a 0.4-kb *Sac*I fragment	This work
pΔ*avrPto*	Mutant of the *avrPto* gene with a 2.0-kb Ω fragment replacing a 2.5-kb exoIII deletion	Ronald *et al.* 1992

[a] Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance.

ing concentrations: ampicillin, 50 μg/ml; gentamicin, 12.5 μg/ml; kanamycin, 25 μg/ml; mitomycin C, 1 μg/ml; spectinomycin, 50 μg/ml; rifampicin, 100 μg/ml; and tetracycline, 12.5 μg/ml in LB and 25 μg/ml in KMB.

Recombinant DNA techniques.

Standard molecular biology techniques were used (Sambrook *et al.* 1989). Plasmid DNA was isolated according to Zhou *et al.* (1990), and DNA fragments were subcloned according to the method of Crouse *et al.* (1983). For Southern blots, 4 μg of total DNA was digested with appropriate restriction enzymes and electrophoresed in 0.7% agarose gels before transfer onto nylon membranes as described by Kobayashi *et al.* (1990). Probes were ^{32}P-labeled with random primers (Boehringer Mannheim Biochemicals, Indianapolis, IN). Hybridizations were performed in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt's solution, 0.002 M sodium phosphate, pH 6.7, and salmon sperm DNA (0.1 mg/ml) with gentle shaking at 42° C. The membranes were then washed twice in 2× SSC and 0.1% sodium dodecyl sulfate (SDS) at 42° C for 15 min and then again in 0.5× SSC and 0.1% SDS at 42° C for 15 min before exposure to X-ray film.

Mutagenesis, conjugations, and marker exchange experiments.

Tn*5* mutagenesis of cosmid clones was conducted in *E. coli* DH5 by the use of phage λ::Tn*5* as described by de Bruijn and Lupski (1984). Conjugations were performed using *E. coli* S17-1 as described by Keen *et al.* (1992). Mating mixtures were incubated at 28° C overnight, and the cells were streaked on KMB agar plates supplemented with appropriate antibiotics. Following growth at 28° C, transconjugants were single-colony-purified several times.

To obtain marker exchange mutants of *P. syringae* cells carrying pRK415 or pLAFR3, transconjugants were cycled four times in 5 ml of KMB broth which was shaken for 12 hr at 28° C without selection and then one time with kanamycin, gentamicin, or spectinomycin added. Cells were plated onto KMB supplemented with kanamycin, gentamicin, or spectinomycin and replica-plated on KMB plus tetracycline. Potential marker exchange mutants were identified by screening for loss of tetracycline resistance. In cases where marker exchange mutants were not readily obtained, a round of cycloserine enrichment in the presence of tetracycline was performed. Briefly, after four successive cycles in KMB without selection, cells were diluted to 10^7 cells per milliliter in 5 ml of KMB plus tetracycline. After 2 hr of growth, cycloserine was added to 2 mM, and the cells were incubated at 28° C with shaking for 4–5 hr. Survivors were plated on KMB plus kanamycin, gentamicin, or spectinomycin agar plates and replica-plated on KMB plus tetracycline agar plates to screen for loss of tetracycline resistance.

Plant growth conditions and inoculations.

Soybean (cultivars Acme, Flambeau, Centennial, Merit, Harosoy, Hurrelbrink, Keburi, Norchief, Hardee, Lindarin, Chippewa, and Peking), tobacco (*Nicotiana tabacum* cv. Xanthi), and tomato (Peto seed cultivars 76S and 76R and cultivar Bonnie Best) plants were grown from seed in standard potting soil (UC mix) in 8-cm peat pots in the greenhouse. After inoculation, soybean plants were placed in a growth chamber at 21° C with a 16-hr photoperiod and 90% relative humidity. Bacteria for inoculation were grown overnight on KMB plates at 28° C and resuspended in water to A_{600} = 0.1. An inoculum concentration of 10^8 cfu/ml was used to score for the appearance of an HR, and 10^4 cfu/ml was used in growth curve studies. Inocula were infiltrated into fully expanded primary leaves of 7- to 10-day-old soybean plants, leaflets of the fourth or fifth leaves of tomato plants, or fully expanded tobacco leaves; 1-ml disposable syringes were used for inoculation. The plants were monitored for symptom development daily for 5 days (soybean) and 10 days (tomato). For growth curve experiments, bacterial cell populations were determined by excising leaf disks with a #4 cork borer, grinding them in 1 ml of sterile water, and plating 10 μl of appropriate dilutions on KMB agar plates, which were then incubated for 2 days at 28° C. For each strain in each experiment, a sample of two leaf disks taken from separate leaves was replicated three times. The experiments were repeated at least three times.

Nutrient utilization of *avr* gene mutants.

P. s. pv. *tomato* strains were analyzed for their ability to utilize 95 carbon sources; Biolog Gn microplates were used according to the manufacturer's instructions (Biolog, Inc., Hayward, CA). For growth comparisons, cultures were seeded with approximately 10^7 cells in 5-ml tubes of KMB or M9 broth, and the absorbance of cultures at 600 nm was measured after shaking for 16 hr at 28° C.

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