MPMI Vol. 8, No. 1 1995, pp. 49–57. © 1995 The American Phytopathological Society

Characterization of *avrE* from *Pseudomonas syringae* pv. *tomato* : A *hrp*-Linked Avirulence Locus Consisting of at Least Two Transcriptional Units

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Received 28 July 1993; Accepted 26 October 1994.

Cosmid clone pPT10E9 from *Pseudomonas syringae* pv. *tomato* caused *P. s* pv. *glycinea* to elicit the HR on leaves of all tested soybean cultivars. The avirulence function of pPT10E9, called *avrE*, occurred on an 11.3-kb DNA fragment located immediately adjacent to the *P. s.* pv. *tomato hrp* gene cluster. Tn*3-gus* saturation mutagenesis of the *avrE* locus and adjacent DNA revealed at least four transcriptional units occurring immediately adjacent to the *hrpRS* locus that were all regulated in a manner similar to *hrp* genes (induced only in minimal induction media or *in planta* and required the *hrpL* and *hrpRS* loci for expression). Transcriptional units III and IV, but not II or V, were required for *avrE* function. *P. s.* pv. *tomato* DC3000 carrying mutations in each of the four transcripts retained full virulence on tomato leaves and elicited the HR on tobacco and soybean plants. This was unlike strain PT23, where mutation of *avrE* greatly decreased virulence on tomato leaves. The promoter regions for three of the investigated transcriptional units contained a consensus sequence occurring in the promoter regions of several other *P. syringae* avirulence and *hrp* genes. The promoter region of transcriptional unit IV, required for *avrE* function, did not contain such a sequence, but included an element which may function as a sigma-54 promoter. Introduction of the cloned *P. s.* pv. *tomato avrE* locus into five other *P. syringae* pathovars did not cause them to elicit the HR on their normal host plants.

Additional keywords: avirulence genes, hypersensitive response, transcriptional regulation.

Avirulence (*avr*) genes restrict pathogen host range by specifying the hypersensitive response (HR) on plant hosts carrying complementary disease resistance genes (Flor 1955). Avirulence genes are not required for pathogenicity, but in a few cases have been associated with virulence (Kearney and Staskawicz 1990; Lorang *et al.* 1994; Swarup *et al.* 1992). The fungal, viral, and bacterial *avr* loci thus far cloned are single dominant genes with one open reading frame (Keen 1990). Several avirulence genes have been cloned from *Pseudomonas syringae* pv. *tomato* (Pst) that cause *P. s.* pv. *glycinea* to elicit a genotype-specific HR in soybean (Innes *et al.* 1993; Kobayashi *et al. 1989;* Ronald *et al.* 1992; Whalen *et al.* 1988). In this work, we report the structural characterization and regulation of the *P. s.* pv. *tomato avrE* locus, previously reported to cause the HR on several soybean cultivars (Kobayashi *et al.* 1989). We found *avrE* to be unique in that it is linked to the *P. s.* pv. *tomato hrp* gene cluster, elicits the HR on all tested soybean cultivars, and requires at least two transcriptional units.

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RESULTS

Localization of DNA required for *avrE* function.

An 11.3-kb *Bam*HI-*Eco*RI fragment was recovered from pPT10E9 by partial *Eco*RI and complete *Bam*HI digestion and cloned into pRK415 to generate pPTBE11. This was the smallest DNA fragment from pPT10E9 which caused *P. s.* pv. *glycinea* race 4 to elicit an HR on soybean leaves (Table 1 and Fig. 1). PsgR4(pPTBE11) elicited a weak HR within 24 hr after inoculation on all 10 soybean cultivars tested. The activity of pPTBE11 is unlike other cloned *P. syringae avr* genes in that it lacks cultivar specificity on soybean and requires a relatively large DNA region. Because these features are characteristic of *hrp* genes (Willis *et al.* 1991), we compared pPTBE11 to *P. s.* pv. *tomato hrp* gene DNA.

Boucher *et al.* (manuscript in preparation) isolated and characterized clones from *P. s.* pv. *tomato* (DC3000) containing *hrp* gene DNA and observed reduced virulence (pDC425) or an HR (pDC541) by *P. s.* pv. *glycinea* transconjugants harboring these clones. Comparison of restriction maps (Fig. 1A) and Southern blot analysis (data not shown) revealed that Pst DC3000 *hrp* DNA (pDC541 and pDC720 obtained from B. Staskawicz) and pPT10E9 from Pst PT23 contain similar DNA regions. Because DC3000 has a much higher frequency of conjugation than PT23 and PsgR4(pDC541) elicited a stronger HR (confluent necrosis of infiltrated tissue within 24 hr) on soybean than PsgR4(pPT10E9), subsequent analysis of the *avrE/hrp* region employed the DC3000-derived clones.

Several subclones, transposon insertion mutants and deletion derivatives of pDC541 (Table 1 and Fig. 1A) were conjugated into PsgR4 and these bacteria were tested for their ability to elicit the HR on soybean (Fig. 1A). From these data it is evident that a DNA region spanning more than 6 kb but less than 10 kb was required for *avrE* activity in PsgR4. Clones

pPTBE11 and pTn:541ΔB caused *P. s.* pv. *glycinea* to elicit the HR in soybean. These clones were also conjugated into *P. s.* pathovars *lachrymans, tabaci, phaseolicola, syringae,* and *pisi,* but none of the transconjugates elicited an HR on the tested cultivars of their normal host plants.

Transcriptional organization of the *hrp/avrE* region.

pDC541ΔB, pDC70H, and pDC88HB (Table 1) were subjected to random transposon mutagenesis and the position and orientation of 140 Tn*3*-gus insertions were determined. Each clone having a Tn*3*-gus insertion was conjugated into Pst DC3000 and screened for GUS activity. A composite map of representative Tn*3*-gus insertions and their GUS activity (Fig. 1B) revealed a minimum of five putative transcriptional units in a 18-kb region. The position of the first transcript corresponds to the *hrpRS* transcript which Boucher *et al.* (manuscript in preparation) mapped to this precise location. Since relatively few transposon insertions occurred in transcriptional unit III as compared to others, it is possible that more than one transcriptional unit may occur in this region. Putative transcriptional units II and V mapped to DNA regions not required for *avrE* activity. By comparing *avrE* active clones (Fig. 1A) with the transposon map (Fig. 1B), it was concluded that transcriptional units III and IV contain the entire DNA region required for *avrE* activity.

Construction and phenotypes of *P. s.* pv. *tomato* DC3000 *hrp/avrE* mutant strains.

Tn*3*-gus insertions 1–6 (Fig. 1B) were chosen for marker-exchange mutagenesis into the strain DC3000 chromosome because they occur in each of the five putative transcriptional units. The resulting mutant strains were called MX1 to 6 (Table 1). Because of the uncertainty that transcriptional unit III represents only one transcriptional unit, two different marker exchange mutations (MX3 and MX4) were constructed in this DNA region. The genotypes of all mutant

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics	Reference or source
Bacterial strains		
Escherichia coli		
DH5α	*F⁻ lacZ* ΔM15 *endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1 l⁻*	Bethesda Research Lab., Gaithersburg, MD
HB101	*F⁻ hsdS20* [*hsdR hsdM recA13 ara-14 proA2 lacYI galK2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44 l-*]	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*R0	Wild-type	Staskawicz *et al.* 1987
*Psg*R0*hrp*	Tn*5* insertion in *hrp AB,D,E,F,L,M,* or *S*	Huyhn *et al.* 1989
*Psg*R4	Wild-type	Kobayashi *et al.* 1989
pv. *tomato*		
PT23	Wild-type	D. Cooksey
DC3000	Wild-type	B. Staskawicz
MX1	*hrpS*::Tn*3-gus* insertion 1 in DC3000 transcript I	This work
MX2	Tn*3-gus* insertion 2 in DC3000 transcript II	This work
MX3	Tn*3-gus* insertion 3 in DC3000 transcript III	This work
MX4	Tn*3-gus* insertion 4 in DC3000 transcript III	This work
MX5	Tn*3-gus* insertion 5 in DC3000 transcript IV	This work
MX6	Tn*3-gus* insertion 6 in DC3000 transcript V	This work
Plasmids		
pBluescriptKS+	*E. coli* cloning vector, Ap^R	Strategene, La Jolla, CA
pBsPv9	0.9-kb *Pvu*II fragment from pTn:541ΔB2 cloned in pBluescriptKS+	This work
pBsEP12	1.2-kb *Eco*RI-*Pvu*II fragment from pTn:541ΔB2 cloned in pBluescriptKS+	This work
pBsP71	0.7-kb *Pst*I fragment from pDC541 cloned in pBluescriptKS+	This work
pDC541	pLAFR3 clone of *P.s.* pv. *tomato* DC3000 *hrp* region	B. Staskawicz
pDC720	pLAFR3 clone of *P.s.* pv. *tomato* DC3000 *hrp* region	B. Staskawicz
pDC541ΔBK	pDC541derivative with a an 11-kb *Bam*HI-*Kpn* I deletion	This work
pDC541ΔB	pDC541derivative with a deletion 3 kb 5′ of the *Bam*HI site to a *Kpn* I site	This work
pDC11H	11-kb *Hin*dIII subclone of pDC541in pRK415	This work
pDC70H	7.0-kb *Hin*dIII subclone of pDC541in pRK415	This work
pDC88HB	8.8-kb *Hin*dIII-*Bam*HI subclone of pDC541in pRK415	This work
pDC108PB	10.8-kb *Pvu*II-*Bam*HI fragment in pRK415	This work
pLAFR3	Cosmid derivative of RK2, Tc^R	Staskawicz *et al.* 1987
pPT10E9	Cosmid clone containing the *avrE* locus	Kobayashi *et al.* 1989
pPTBE11	11.3-kb *Bam*HI-*Eco*RI subclone of pPT10E9 in pRK415	This work
pRK415	RK2 derived broad host range vector, Tc^r	Keen *et al.* 1988
pTn:541ΔB	Tn*3-gus* insertion derivatives of pDC541ΔB	This work
pTn:541ΔB(4)	Tn*3-gus* insertion #4 derivative of pDC541ΔB	This work
pTn*3-gus*	Km^r Ap^r *tnpA⁻*; Tn*3*HoHo derivative containing a promoterless B-glucuronidase gene	B. Staskawicz

strains were confirmed by Southern blot analyses which revealed only the expected DNA fragments shifted to the predicted size for each mutant construct (data not shown). This verified that each gene is present as a single copy in the DC3000 genome and that each gene had been mutated.

To see if mutations in putative transcriptional units I–V (Fig. 1B) altered the normal growth of *P. s.* pv. *tomato* in culture media or its ability to utilize various carbon sources, strains MX1 to 6 were compared with wild-type DC3000 for the ability to grow in complete (KMB) and minimal (MM) nutrient media and to utilize 95 different carbon sources. All of the strains grew in KMB and MM as evidenced by an A600 of >1 after 16 hr of growth. All strains also showed a pattern of carbon source utilization identical to that of DC3000 as determined by the Biolog Gn microtiter plate assay.

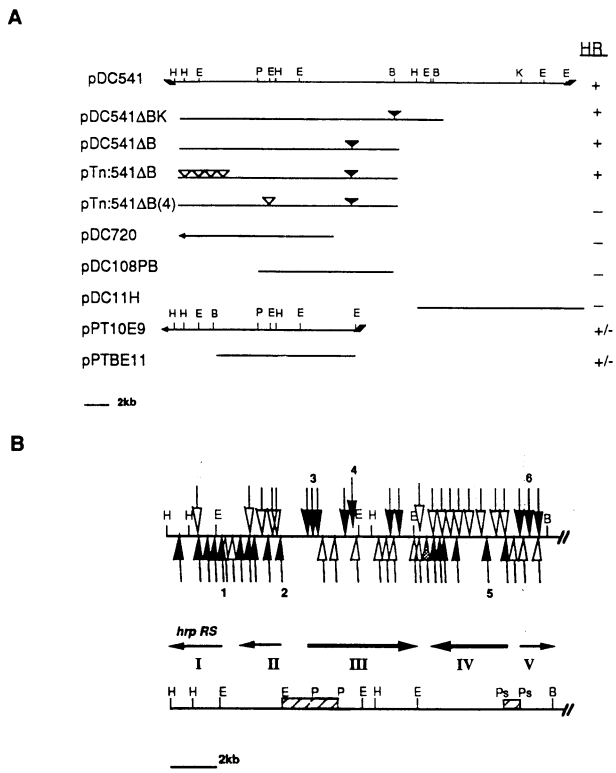

Fig.1. Structural characterization of *Pseudomonas syringae* pv. *tomato* *hrp* gene/*avrE* DNA. **A,** Localization of DNA required for *P. s.* pv. *glycinea* race 4 to elicit the hypersensitive response (HR) on soybean. Closed triangles = deletions, open triangles = Tn*3-gus* insertions, hatched lines = pLAFR3, arrows = additional insert DNA not shown; B = *Bam*HI, E = *Eco*RI, H = *Hin*dIII, P = *Pvu*II (not all sites shown), K = *Kpn*, (+) = HR on soybean, (–) = no HR on soybean, (±) = weak HR on soybean. **B,** Saturation transposon mutagenesis map of pDCΔX541ΔB revealing transcriptional organization of the *avrE* region. Arrows indicate Tn3-*gus* insertions. Arrows oriented down are inserted with the GUS reporter gene reading from left to right and those oriented up are inserted with the GUS reporter gene reading from right to left. Solid, open, and stippled arrows indicate detectable, not detectable, or inconsistent GUS activity, respectively, of Pst DC3000 transconjugates after growth on MM with 10 mM fructose for 14 days. Solid lines with arrowheads (I–V) represent putative transcriptional units. Thicker lines (III and IV) denote region required for *avrE* activity. Open bars with hatched lines show the sequenced DNA regions that contain *avr* gene promoter consensus sequences. Ps = *Pst*I (not all sites shown). Other restriction sites are abbreviated as in A.

Each *P. s.* pv. *tomato* (DC3000) mutant strain was also assayed for its ability to elicit the hypersensitive response on the non-host plant, tobacco, and on 10 different soybean cultivars. MX2, MX3, MX4, MX5, and MX6 elicited a visible HR on all test plants within 16 hr as did the wild-type strain, DC3000. However, as expected for a *hrp* mutant, MX1 did not elicit an HR on any test plant.

The degree to which *P. s.* pv. *tomato* mutant strains caused disease on tomato and competed with the wild-type strain for *in planta* growth was evaluated by both visual analysis of disease symptoms and by monitoring the growth of bacterial populations in tomato leaves. MX1 exhibited greatly reduced virulence on tomato, as expected for a *hrp* mutant, but strains MX2 through MX6 produced symptoms and growth indistinguishable from the wild-type strain (Table 2).

Regulation of *hrp/avrE* transcripts in culture and in compatible and incompatible host plants.

To study the regulation of putative transcriptional units I, II, III, IV, and V (Fig. 1B), clones pDC70H and pDC88HB (harboring Tn*3-gus* insertions 1, 2, 3, 5 and 6) were conjugated into Pst DC3000 and assayed for GUS activity after growth in KMB broth, MM broth, or *in planta.* Only tran-

Table 2. Growth and competitive ability of *Pseudomonas syringae* pv. *tomato* DC3000 *hrp/avrE* mutants in tomato leaves

			CFU × 10^{-6}/leaf area (cm^2)[a] [sd][b]			
Inoculum	Inoculated alone		Total cfu		Kanamycin^R cfu only	
PstDC3000 wild-type	14	[0.2]	–		–	
MX1[d]	0.5[c]	[0.32]	8	[0.07]	0.01[c]	[0.0]
MX2	11	[8.9]	5	[0.38]	9	[3.8]
MX3	18	[18]	7	[4.1]	12	[4.1]
MX4	14	[12]	14	[0.1]	6	[3.3]
MX5	17	[16]	18	[4.5]	12	[8.0]
MX6	29	[25]	12	[6.2]	4	[1.3]

[a] Values represent the mean of three replicates from separate leaf disks excised 6 days after inoculation with 10^4 cfu/ml.
[b] Standard deviation.
[c] Plants were inoculated with equal mixtures of wild-type PT23 and the indicated MX strain.
[d] All MX strains are kanamycin resistant.
[e] Statistically significant difference from wild-type based on a *t* test at *P* (0.05). All other values are not statistically different from wild-type.

Table 3. Induction of *Pseudomonas syringae* pv. *tomato hrp/avrE* ranscripts in rich and minimal media and in compatible and incompatible host plants

	Gus activity(units/10^9 cfu)[a]			
	In planta		In culture	
Pst DC3000 transcriptional unit	Tomato	Soybean	KMB	MM
I (Tn3-*gus* 1)	95.2 [20.6][b]	81.3 [20.8]	23.8 [2.6]	77.7 [11.0]
II (Tn3-*gus* 2)	ND[c]	ND	0.00	42.2 [8.4]
III (Tn3-*gus* 3)	56.3 [3.0]	54.4 [13.7]	18.9 [5.1]	175.3 [8.2]
IV (Tn3-*gus* 5)	ND	ND	0.00	35.7 [5.8]
V (Tn3-*gus* 6)	ND	ND	0.00	88.3 [6.4]

[a] Values represent mean value of three replicated samples.
[b] Standard deviation.
[c] ND = not tested.

scriptional units I and III were expressed to detectable levels in KMB, but all transcripts were greatly induced in MM when fructose was used as a carbon source (Table 3). Transcript IV was expressed at a considerably lower level under inducing conditions than the other transcriptional units. Transcriptional units I and III were expressed to the highest levels and were therefore used for *in planta* studies. These transcripts were induced to about the same level in compatible and incompatible interactions with tomato and soybean plants, respectively (Table 3).

Table 4. Expression of *hrp/avrE* transcripts in *hrp* mutant strains of *Pseudomonas syringae* pv. *glycinea* R0

Pst DC3000 transcriptional unit	PsgR0 strain			
	wildtype	*hrpL*	*hrpS*	*hrpABDEF*or *M*
I (Tn3-*gus* 1)	+[a]	–[b]	+	ND[c]
II (Tn3-*gus* 2)	+	–	–	+
III (Tn3-*gus* 3)	+	–	–	+
IV (Tn3-*gus* 5)	+	–	–	+
V (Tn3-*gus* 6)	+	–	–	+

[a] Color indication of GUS activity on MM X-gluc plates within 14 days.
[b] Colonies remained white on MM X-Gluc plates after 14 days.
[c] ND = not tested.

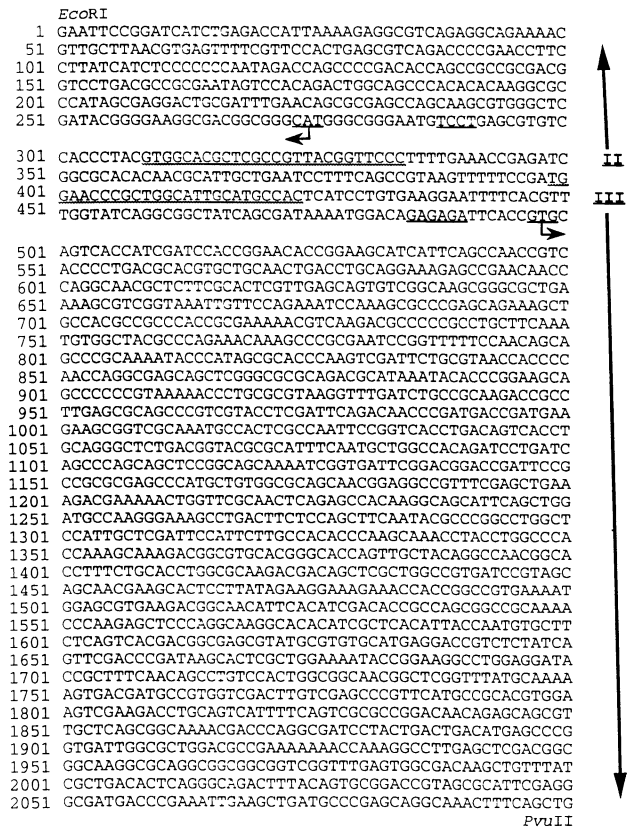

Fig. 2. DNA sequence of *Pseudomonas syringae* pv. *tomato* strain DC3000 region containing putative promoters of *hrp* gene/*avrE* transcriptional units II and III. The *Eco*RI site at base 1 originates from the right border of transposon insertion 2 (Fig. 1); the first 92 bases of the sequence are from Tn-*3gus.* Conserved promoter elements found upstream of other *P. syringae avr* and *hrp* genes are underlined. Possible translational start sites and Shine-Dalgarno sequences are underlined. Arrows indicate direction of open reading frames extending through the sequenced region.

Expression of *avrE* and adjacent transcripts require the *hrpL* and *hrpS* genes.

Tn*3*-gus insertion clones 1, 2, 3, 5, and 6 were conjugated into *P. s.* pv. *glycinea* (PsgR0) and a collection of PsgR0 *hrp* mutant strains (PsgR0 *hrpAB, D, E, F, M, L,* and *S*) to determine if transcriptional units I, II, III, IV, and V require *hrp* genes for their expression. All wild-type PsgR0 and *hrpAB,D, E, F,* and *M* transconjugants expressed *gus* as determined by the GUS plate assay (Table 4). However, PsgR0 *hrpL* transconjugants had no detectable GUS activity and only the PsgR0 *hrpS* transconjugant harboring Tn*3*-*gus* insertion 1 (in *hrpRS* transcript I) had GUS activity. The activity of this transconjugant was visible several days later than the activity of Tn*3*-gus insertion 1 in the wild-type PsgR0 strain, indicating that a functional *hrpS* gene is required for its own full expression.

A promoter sequence conserved in *P. syringae avr* genes is also present in three promoters of the *avrE* region.

A 2.05-kb DNA fragment from the *Eco*RI site in the 5′ border repeat of Tn*3*-gus insertion 2 to a *Pvu*II site 2.05-kb downstream and a 0.7-kb *Pst*I fragment located approximately 400 bp 5′ of Tn*3*-gus insertion 6 (Fig. 1A and B) were cloned into pBluescriptKS+ (pBsEP12, pBsPv9, and pBsP71, Table 1) and sequenced. These fragments were chosen for sequencing because transcriptional mapping data (Figs. 1B, 2, and 3) predicted that they contain promoter regions for transcripts II, III, IV, and V. Three sequences similar to required elements in the promoters of other *P. syringae avr* and *hrp* genes were identified in these regions (Figs. 2 and 3, Table 5). The sequences are properly located for the promoters of transcriptional units II, III, and V (Fig. 1B), but data are not available to test their physiological role. The first conserved sequence is located approximately 300 bp to the right of Tn*3*-gus insertion 2 and reads from left to right. The second sequence is on the opposite strand, 64 bp to the right of the first sequence, approximately 600 base pairs left of Tn*3*-gus in-

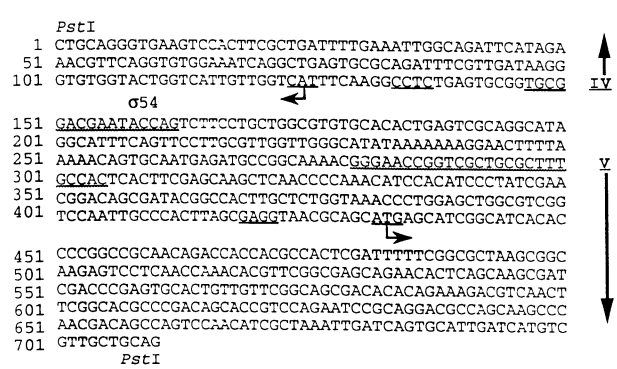

Fig. 3. DNA sequence of *Pseudomonas syringae* pv. *tomato* strain DC3000 region containing putative promoters of *hrp* gene/*avrE* transcriptional units IV and V. A conserved DNA element found upstream of several other*P. syringae avr* genes and a putative sigma-54 promoter are underlined. Possible translational start sites and Shine-Dalgarno sequence are underlined. Arrows indicate direction of open reading frames extending through the sequenced regions shown.

sertion 3 and reads from right to left. The third conserved sequence is located about 640 bp left of Tn*3-gus* insertion 6 and reads from left to right, where the promoter for transcript V should be located. A similar sequence was not found in the predicted promoter region of transcriptional unit IV, an observation that may be related to the observed lower expression level of this transcript relative to the other four transcriptional units (Table 3). However, a sequence similar to promoters of genes which require σ^{54} for transcription was found in the predicted promoter region of transcript IV (Fig. 3).

Start codons downstream of each putative promoter initiate open reading frames that extend through the sequenced regions (Figs. 2 and 3). An open reading frame in transcriptional unit III, with a GTG initiation codon at base 497, potentially encodes a 494 amino acid protein (Fig. 2). A Hopp and Wood hydropathy plot of this deduced peptide sequence revealed a hydrophilic 54-kDa protein with no obvious leader peptide. This peptide sequence had no significant similarity to any protein sequence in the Swiss-Prot database. All other possible open reading frames of significant length in the sequenced regions were analyzed for similarity to known proteins without success.

DISCUSSION

We localized the DNA region required for function of the *P. s.* pv. *tomato* (Pst) *avrE* locus to the right external border of the *hrp* gene cluster (Fig. 1). The *avrE* locus and adjacent DNA is organized into at least four transcriptional units having regulatory as well as physical linkage to the bacterial *hrp* genes. None of the new transcriptional units was required for *hrp* gene function, but putative transcriptional units III and IV (Fig. 1), comprising about 9 kb of DNA, were required for *avrE* function when conjugated into *P. s.* pv. *glycinea*. Except for *hrp* gene clusters, this is the first case in which more than one gene or one transcriptional unit is required for elicitation of the HR on a nonhost plant. We previously showed that mutation of the *avrE* locus in Pst strain PT23, unlike strain DC3000, greatly reduced the virulence of PT23 on tomato plants (Lorang *et al.* 1994). These characteristics make it difficult to strictly define *avrE* as either an *avr* or a *hrp* locus. *avrE* has common features with two *hrp* genes, *hrpZ* of *P. s.* pv. *syringae* and *hrpN* of *Erwinia amylovora*. Mutation of *hrpZ* in *P. s.* pv. *syringae* (He *et al.* 1993) and *avrE* in *P. s.* pv. *tomato* PT23 reduced but did not eliminate the virulence of these pathogens. However, whereas *hrpZ* mutant strains exhibited generally reduced HR on nonhost plants, *avrE* mutant strains elicited the HR on tobacco and several soybean cultivars as did the wild-type bacteria (Lorang *et al.* 1994). *hrpN* encodes a protein elicitor (harpin) of the HR in all tested genotypes of several plant species (Wei *et al.* 1992a). Elicitation of the HR by the *avrE* locus is also genotype-nonspecific in soybean. Unlike *hrpN, avrE* may be plant species specific since it did not cause five other *P. syringae* pathovars to elicit the HR on their normal host plants. *avrE* may therefore represent a link between *hrp* genes and *avr* genes. Its characterization has extended our interest to include other bacterial loci linked to the *hrp* cluster proper.

Table 5. Conserved sequence in putative promoters of *Pseudomomas syringae hrp* and avirulence genes.

Gene(s)		Sequence
avr consensus[a]		g/tGGAACC-N15/16-CCAC
avrE transcript	II	GGGAACC-N15- CCAC
	III	TGGAACC- N15- CCAC
	V	GGGAACC-N16- CCAC

[a] Innes *et al.* 1993; Shen and Keen 1993; Willis *et al.* 1994.

Expression of the four putative transcriptional units occurring in the Pst *avrE* region as well as the adjacent *hrpRS* transcript are induced in bacteria grown in MM. A similar regulation pattern has been shown for all *hrp* gene clusters investigated to date (Arlat *et al.* 1991, 1992; He *et al.* 1993; Rahme *et al.* 1992; Schulte and Bonas 1992a; Wei *et al.* 1992b; Xiao *et al.* 1992) and for several other *P. syringae avr* genes (Huynh *et al.* 1989; Innes *et al.* 1993; Salmeron and Staskawicz 1993; Shen and Keen 1993). We did not attempt to identify components of MM that were important for the observed induction of gene expression. Specific components of MM previously reported to induce gene expression differ for each system studied. Low osmolarity, pH, and carbon source were important for *P. s.* pv. *phaseolicola hrp* gene induction (Rahme *et al.* 1992), phosphate and sodium chloride concentrations and sulfur-containing amino acids for *Xanthomonas campestris* pv. *vesicatoria hrp* genes (Schulte and Bonas 1992a) and ammonium ions, nicotinic acid, complex-nitrogen sources, temperature and pH for *Erwinia amylovora hrp* genes (Wei *et al.* 1992b). Carbon sources which induced maximum gene expression also varied among several systems (Arlat *et al.* 1991, 1992; Huynh *et al.* 1989; Schulte and Bonas 1992a; Wei *et al.* 1992b; Xiao *et al.* 1992). However, sucrose induced gene expression in all cases. We used fructose as a carbon source in this study because it induced *hrp/avrE* gene expression to higher levels than other sugars tested, a conclusion also reached by Huynh *et al.* (1989) for *avrB* gene expression in *P. s.* pv. *glycinea*.

P. syringae avr genes *avrB* (Huynh *et al.* 1989), *avrD* (Shen and Keen 1993), *avrPto* (Salmeron and Staskawicz 1993), and *avrRpt2* (Innes *et al.* 1993) are induced in minimal-salts media (10 mM fructose) and also require functional *hrpL* and *hrpRS* loci for their expression. Our data are consistent with this conclusion since functional *hrpL* and *hrpRS* loci, but not other *hrp* loci, were required for expression of *hrp/avrE* transcripts (Table 4). Excepting *hrpL* and *hrpRS* mutant strains, the inability of *hrp* mutant strains carrying the cloned *avrE* locus to elicit the HR is therefore not determined by the failure of *avrE* gene transcription. The similarity of *Xanthomonas campestris* (Fenselau *et al.* 1992) and *P. solanacearum* (Gough *et al.* 1992) *hrp* gene products to *Klebsiella* and *Yersinia* proteins which function in extracellular secretory pathways supports the alternative hypothesis that *hrp* mutants cannot cause the HR because they are unable to export harpin elicitors (He *et al.* 1993; Wei *et al.* 1992a) into the intercellular spaces of plant hosts.

The Pst *hrpRS* transcript was dependent on *hrpL* and *hrpRS* for full expression but was expressed to some degree in a *hrpRS* mutant background (Table 4). This is an expected result given that the *hrpRS* gene products from *P. s.* pv. *phaseolicola* have similarity to the *NtrC* family of DNA-binding, bacterial regulatory proteins (Grimm and Panopoulos 1989) and *hrpS* was shown to be required for its own expression as well as that of several other *hrp* operons in the cluster (Fellay

et al. 1991). Furthermore, promoters of these *hrp* operons contain a conserved consensus sequence similar to the *avr* box motif, G/TGGAACC-N15 or 16-CCAC (Table 5), found upstream of several *P. syringae avr* and *hrp* genes (Dangl *et al.* 1992; Huang *et al.* 1993; Innes *et al.* 1993; Jenner *et al.* 1991; Kobayashi *et al.* 1990a and b; Napoli and Staskawicz 1987; Tamaki *et al.* 1988; Salmeron and Staskawicz 1993; Shen and Keen 1993; Yucel *et al.* 1994a). Recently, *hrpL* in *P. s.* pv. *syringae* has been suggested to encode an alternate sigma factor which may interact with this promoter element to activate transcription (Xiao and Hutcheson 1994). The same *avr* box sequence motif also occurs upstream of *avrE* region transcriptional units II, III, and V (Figs. 1, 2, and 3; Table 5).

We did not find the *avr* box motif in the region where we expected a promoter for putative transcript IV. However, the level of GUS expression of Tn*3-gus* inserts in putative transcript IV was lower than that of insertions in the other putative transcriptional units. While position effects of GUS gene fusions could account for this observation, it is possible that the lower expression of transcript IV results from not having the "harp box" consensus sequence. The predicted promoter region of transcript IV does contain a sequence similar to the motif CTGGNA -6 bp- TTGCA which is centered at the –12/–24 positions in promoters of genes that require the σ^{54} co-factor for transcription (Kustu *et al.* 1989). Genes requiring σ^{54} are involved in environmental adaptation or specialized metabolic functions and include *P. s.* pv. *tomato avrD* (Shen and Keen 1993) and several *P. s.* pv. *phaseolicola hrp* genes (Fellay *et al.* 1991). Like *avrE*, these genes are induced *in planta* and may require the σ^{54} co-factor for expression. Because the organization of transcriptional units in this study was based solely on transposon mapping, a more detailed analysis of the DNA region upstream of putative transcriptional unit IV will be necessary to precisely define the DNA sequences required to promote its transcription.

Tn*3-gus* insertions in transcriptional units I and III had the highest levels of GUS activity in MM and *in planta*. DNA clones containing these gene fusions were introduced into Pst DC3000 and expressed to equivalent levels when the bacteria were infiltrated into tomato and soybean leaves. Similar results were reported for the *avrPto* gene (Salmeron and Staskawicz 1993), the *avrB* gene (Huynh *et al.* 1989) and *P. s.* pv. *syringae hrp* loci (Xiao *et al.* 1992), indicating that host-specific induction of *hrp* or *avr* genes may not determine host species specificity. On the contrary, Wei *et al.* (1992b) found that *Erwinia amylovora hrp* genes were induced more rapidly and to higher levels in tobacco (incompatible) than in pear (compatible host). The lower levels of GUS expression we observed from transcriptional unit III *in planta* compared to MM could be due to several experimental factors including different inoculum levels and growth rates *in planta* versus MM, the absence of antibiotic selection for plasmids carrying GUS gene fusions *in planta* and components in the assay medium (e.g., plant extracts containing proteases and pigments which cause a background level of fluorescence). For *P. solanacerarum* (Arlat *et al.* 1992), *P. s.* pv. *syringae* (Xiao *et al.* 1992), *Xanthomonas campestris* pv. *vesicatoria* (Schulte and Bonas 1992b), *Erwinia amylovora hrp* loci (Wei *et al.* 1992b), and transcriptional unit I, in this work, levels of gene expression in defined minimal-salt media were comparable to those obtained *in planta*. Fellay *et al.* (1991), however, suggested that expression of *P. s.* pv. *phaseolicola hrp* loci at higher levels *in planta* than in M9 medium might be due to a specific plant signal. Although we obtained higher or comparable levels of *hrp/avrE* gene expression in MM than *in planta*, the possibility that specific plant signals regulate *in planta* transcription cannot be ruled out.

The co-regulation of *avr, hrp,* and *hrp*-linked genes raises the question of whether these genes participate in a common biological function or are simply part of a global regulatory circuit induced in minimal nutrient conditions. Arlat *et al.* (1992) noted that gene fusions resulting from transposon insertions mapping outside of the *P. solanacearum hrp* cluster were also co-regulated with *hrp* loci. One of these genes encoded PopA1, a Hrp-secreted protein that functioned as an HR elicitor in tobacco and certain cultivars of petunia (Arlat *et al.* 1994). Two other *hrp*-linked genes, the *P. solanacearum pehA* gene which encodes an extracellular polygalacturonase (Allen *et al.* 1991) and *avrPphE* which borders the *P. s.* pv. *phaseolicola hrpL* locus (Mansfield *et al.* 1994), also determine the outcome of bacterial interactions with host plants. This supports the idea that genes adjacent to but independent of the formal *hrp* cluster may be important in host-pathogen interactions. In this work we observed that Pst DC3000 strains carrying mutations in each of the four *hrp*-linked *avrE* transcripts were not detectably altered in their interactions with the host plant, tomato (Table 2), or the non-host plants tobacco and soybean. However, in another study (Lorang *et al.* 1994) we found that a deletion mutation in *avrE* transcriptional unit III of Pst strain PT23 greatly reduced virulence in tomato plants. This reinforces the hypothesis that genes co-regulated with *hrp* genes may function in the pathogenic process. We do not know why *avrE* was required for full virulence of strain PT23 but not strain DC3000. It is possible that other gene products present in Pst DC3000 but not strain PT23 compensate for the loss of *avrE* function.

Another difference between the Pst DC3000 and PT23 *avrE* loci was the relative strength of the HR caused on soybean leaves when expressed in *P. s.* pv. *glycinea*. The weaker HR of the PT23 *avrE* locus could be attributed to altered gene products and/or different levels of expression in *P. s.* pv. *glycinea*. For example, Innes *et al.* (1993) reported that *avrRpt2* and *avrPto* had considerably lower levels of expression in Pst strain JL1065 than in strain DC3000 and that JL1065 gives a much weaker HR than DC3000 on *A. thaliana*. *avrD* gene products also direct the production of low molecular weight elicitors called syringolides (Midland *et al.* 1993; Smith *et al.* 1993) and Yucel *et al.* (1994a, 1994b) reported that discrete amino acid changes in particular *avrD* gene alleles resulted in the production of structurally different syringolides.

Hendson *et al.* (1992) used clones from the Pst *avrE* region to study the relatedness of *P. s.* pv. *tomato, P. s.* pv. *maculicola,* and *P. s.* pv. *antirrhini* isolates. These isolates fell into four groups based on nutritional and RFLP analyses, but showed no polymorphisms in the 2.3- and 5.7-kb *Eco*RI fragments from the *avrE* locus (Fig. 1). We also observed DNA homologous to *avrE* in nine other *P. syringae* pathovars (unpublished data). This relatively high degree of conservation implies biological importance for the *avrE* locus, as was proven for Pst strain PT23 (Lorang *et al.* 1994). It will be of interest to determine if other *P. syringae avrE* homologues are

also linked to *hrp* gene clusters and are required for full virulence on their host plants.

MATERIALS AND METHODS

Plasmids, bacterial strains, and culture conditions.

Bacterial strains and plasmids used or constructed in this study are listed in Table 1. *Escherichia 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 King's medium B (KMB) (King *et al.* 1954) or minimal media (MM) with 10 mM fructose (Shulte and Bonas 1992a). When appropriate, antibiotics were used at the following concentrations (μg/ml): ampicillin (Ap), 50; gentamycin (Gm), 12.5; kanamycin (Km), 25; rifampicin (Rif), 100; tetracycline (Tc), 12.5 in LB and 25 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. Southern transfer was carried out as described (Kobayashi *et al.* 1990a). Probes were ^{32}P-labeled with random primers (Boehringer Mannheim Biochemicals). 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 0.1 mg/ml salmon sperm DNA with gentle shaking at 42° C. Membranes were then washed twice in 2× SSC, 0.1% sodium dodecyl sulfate (SDS) at 42° C for 15 min, followed by 0.5× SSC, 0.1% SDS at 42° C for 15 min before exposure to X-ray film. DNA sequencing was performed by generating a series of overlapping deletions using the Erase-a-Base System (Promega Corporation, Madison, WI) and sequencing by the dideoxy chain-termination method using Sequenase (United States Biochemicals, Cleveland, OH) and deoxyadenosine 5′-α[^{35}S] thiotriphosphate (>1,000 Ci/mmol, Amersham Corp., Arlington Heights, IL). Both strands were sequenced and analyzed with the University of Wisconsin Genetics Computer Group program (Devereaux *et al.* 1984).

Transposon mutagenesis, conjugations, and marker-exchange experiments.

DNA cloned into pLAFR3 and pRK415 was subjected to random insertion mutagenesis in *E. coli* (HB101) using Tn*3-gus* as described by Bonas *et al.* (1989) and the insertions were mapped by restriction enzyme analysis. Conjugations into *P. syringae* were performed using *E. coli* S17-1 or HB101(pRK2013) as described by Keen *et al.* (1992). Mating mixtures were incubated at 28° C overnight and the cells streaked onto 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 DNA constructs in pRK415 or pLAFR3 were cycled four times in 5 ml of KMB broth with shaking for 12 hr without selection and then with Km added. Cells were plated onto KMB supplemented with Km and replica-plated onto KMB + Tc. Potential marker exchange mutants were identified by screening for loss of Tc resistance and confirmed by Southern blot analysis.

GUS assays.

For GUS plate assays, bacteria containing Tn*3-gus* insertions were grown overnight in KMB broth, centrifuged, washed once with 1 mM $MgCl_2$ and spotted onto MM plates containing 10 mM fructose and 25 μg/ml X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronide) (Diagnostic Chemicals Ltd., Charlottetown, Canada). Colonies that displayed any blue after 14 days of incubation at 28° C were considered positive. Those colonies that remained white after 14 days were considered negative.

Quantification of GUS activity in bacteria growing in culture and *in planta* used a fluorimetric assay (Jefferson 1987) using a TKO100 fluorometer (Hoefer Scientific Instruments, San Fransisco, CA) and 4-methylumbelliferyl glucuronide as substrate. In culture (KMB or MM), cells were grown for 16 hr and quantified (cfu/ml) by dilution plating on the appropriate selective media. Cell aliquots were also centrifuged and resuspended in assay buffer. For *in planta* studies, leaf disks were excised from plants using a #4 cork borer 12 hr after inoculation (10^8 cfu/ml, as described below) and pulverized in 500 μl of sterile water. After cell debris had settled for a few minutes, 10-μl aliquots were taken to determine bacterial cell densities by dilution plating, and 300-μl aliquots were centrifuged and resuspended in assay buffer. GUS activity was expressed as units (relative amount [nM] of 4-methylumbelliferone release/minute) per 10^9 cells.

Plant growth conditions and inoculations.

Soybean (cultivars Acme, Flambeau, Centennial, Merit, Harosoy, Norchief, Hardee, Lindarin, Chippewa and Peking), tobacco (*Nicotiana tabacum* 'xanthi'), tomato (cv. Bonnie Best), cucumber (cv. Wisconsin SMR 58), pea (cv. Progress #9), and bean (Red Kidney) plants were grown from seed in 8-cm peat pots in standard potting soil (U.C. mix) in the greenhouse. After inoculation, plants were incubated in a growth chamber at 21° C with a 16-hr photoperiod and 90% relative humidity, except for tomato plants, which remained in the greenhouse. Bacteria for inoculation were grown overnight on KMB plates at 28° C and resuspended in water to A_{600} = 0.1. Inoculum concentrations of 10^8 cfu/ml were used to score for the appearance of an HR and 10^4 cfu/ml were used to assess virulence on tomato. 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, cucumber, pea, or bean leaves using 1-ml disposable syringes. Plants were monitored for symptom development daily for 5 or 7 days (tomato). Bacterial populations were determined by excising leaf disks with a #4 cork borer, grinding them in 1-ml 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. Experiments were repeated at least three times.

Nutrient utilization of *avr* mutants.

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

ACKNOWLEDGMENTS

We thank Brian Staskawicz, Christian Boucher, and Doug Dahlbeck for cosmid clones pDC541 and pDC720, *P. s.* pv. *glycinea* Race0 hrp⁻ strains, pTn*3 gus* and helpful communication of information before publication. Dave Trollinger contributed preliminary information. This research was funded by a grant from the National Science Foundation grant MCB - 9005388-02. J. Lorang was partially supported by a USDA National Needs Predoctoral Fellowship. The reported DNA sequence data has been submitted to Genbank as accession no. U16118 and U16119.

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