Characterization of *avrPphE*, a Gene for Cultivar-Specific Avirulence from *Pseudomonas syringae* pv. *phaseolicola* Which Is Physically Linked to *hrpY*, a New *hrp* Gene Identified in the Halo-Blight Bacterium

John Mansfield, Carol Jenner, Ruth Hockenhull, Mark A. Bennett and Ruth Stewart

Biological Sciences Department, Wye College, University of London, Ashford, Kent, TN25 5AH, U.K.

Received 2 May 1994. Accepted 25 July 1995.

The avirulence gene matching the *R2* gene for resistance to halo-blight disease in *Phaselous* was cloned and sequenced from race 4 strain 1302A of *Pseudomonas syringae* pv. *phaseolicola*. The predicted 41-kDa AvrPphE protein is hydrophilic, has no features that indicate function, and no similarity to other protein sequences. The promoter region of *avrPphE* contains a "*harp box*" motif. The gene was expressed more strongly in minimal than in nutrient-rich media. Lower concentrations of the phytoalexin phaseollin accumulated in tissue undergoing the hypersensitive reaction (HR) determined by *avrPphE* than by *avrPphB*. Homologs of *avrPphE* were detected in strains representing eight races of *P. s.* pv. *phaseolicola* including those virulent on cultivars with the *R2* resistance gene, and in *P. s.* pv. *tabaci* but not in *P. cichorii* or *P. s.* pvs. *coronafaciens, glycinea, maculicola, pisi,* or *syringae*. Disruption of *avrPphE* prevented induction of the HR but did not appear to affect basic pathogenicity. Transposon mutagenesis and DNA sequencing showed that *avrPphE* was linked to *hrpY* a *hrp* locus identified at the left end of the *hrp* gene cluster. Sequence analysis showed that the region linked to *avrPphE* was very similar to DNA containing *hrp* genes from *P. s.* pv. *syringae* including *hrpJ*, *hrpL*, and *hrpK*.

Additional keywords: gene-for-gene interactions, *Phaseolus acutifolius, Phaseolus vulgaris.*

The ability of *Pseudomonas syringae* pv. *phaseolicola* to cause halo-blight disease in cultivars of *Phaseolus vulgaris* L. is determined primarily by three sets of genes that control 1) basic pathogenicity (*hrp* genes), 2) toxin production, and 3) cultivar-specific avirulence (*avr* genes). The *hrp* genes determine ability to multiply within susceptible plants and to cause a hypersensitive reaction (HR) in resistant plants (Lindgren *et al.* 1986, Willis *et al.* 1991). *hrp* genes are highly conserved in pathovars of *P. syringae* (Lindgren *et al.* 1988). In *P. s.* pv. *phaseolicola* strain 3121 they have been reported to include seven contiguous loci *hrpL, hrpAB, hrpC, hrpD, hrpE, hrpF,* and *hrpRS* found in a 22-kb cluster on the bacterial chromosome, and also three unlinked genes, namely, *hrpM, hrpT,* and *hrpQ* (Grimm and Panopoulos 1989; Rahme *et al.* 1991, 1992; Fellay *et al.* 1991, Miller *et al.* 1993).

Production of the zone of chlorosis around infection sites which is the characteristic symptom of halo-blight disease in leaves requires genes for the synthesis of the phytotoxic tripeptide phaseolotoxin (Mitchell 1978, 1984; Moore *et al.* 1984; Zhang *et al.* 1993). Although toxin production is required for full symptom development, *tox*$^-$ mutants multiply at the same rate as wild-type strains within susceptible plants (De la Fuente-Martinez *et al.* 1992).

The third set, *avr* genes, comprises loci which determine cultivar specificity. Recent characterization of strains of *P. s.* pv. *phaseolicola* based on their virulence towards *Phaseolus* genotypes has demonstrated the existence of gene-for-gene interactions based, in theory, on the presence of five genes for avirulence (*A1-5*) in *P. s.* pv. *phaseolicola* which match five genes for resistance (*R1-5*) in bean as outlined in Table 1 (Jenner *et al.* 1991; Teverson 1991; Taylor *et al.* 1995a, 1995; Teverson *et al* 1994). Incompatibility is expressed by the hypersensitive reaction (HR), at inoculation sites in leaves and pods (Harper *et al.* 1987; Jenner *et al.* 1991). The HR resulting from the *R3/A3* interaction occurs more rapidly and is associated with more distinct browning than is observed with other resistance genes. Symptoms produced by different strains including eight races of *P. s.* pv. *phaseolicola*, following stab inoculation of a pod of cv. A43 are illustrated in Figure 1.

Molecular cloning of avirulence genes *avrPphA.R1* and *avrPphB.R3* (named as proposed by Vivian and Mansfield 1993) which match resistance genes *R1* and *R3*, respectively, has confirmed part of the gene-for-gene relationship proposed in bean halo-blight disease (Hitchin *et al.* 1989; Shintaku *et al.* 1989; Jenner *et al.* 1991; Taylor *et al.* 1995a, 1995b). Additional *avr* genes cloned from *P. s.* pv. *phaseolicola*; *avrPphC* and *avrPphD*, determine ability to cause the HR on certain cultivars of the non-host plants soybean and pea, respectively (Yucel *et al.* 1994; Wood *et al.* 1994). Both of the non-host *avr* genes are located on an ≈150-kb plasmid.

Avirulence genes *avrB* in *P. s.* pv. *glycinea* and *avrD*, *avrE, avrPto,* and *avrRpt2* from *P. s.* pv. *tomato* have been

Corresponding author: John Mansfield.

Nucleotide sequence data are to be submitted to GenBank as accession number U16817.

MPMI Vol. 7, No. 6, 1994, pp. 726-739

Table 1. Interaction phenotypes[a] observed between strains of *Pseudomonas syringae* pv. *phaseolicola* and genotypes of *Phaseolus* used in this work interpreted on the basis of five matching pairs of genes for avirulence and resistance

Strain, avirulence (*A*) genes and race designation	Cultivars/lines and matching resistance (*R*) genes				
	Canadian Wonder No *R* genes	Tendergreen (*R3*)	Red Mexican UI3 (*R1+R4*)	A43 (*R2+R3+R4+R5*)	*Phaseolus acutifolius* 1072 (*R2*)
1281A (*A1*) race 1	S	S	R	S	S
882 (*A2+A5*) race 2	S	S	S	R	R
1301A(*A3*) race 3	S	R	S	R	S
1302A (*A2+A3*) race 4	S	R	S	R	R
1375A (*A1+A2+A4*) race 5	S	S	R	R	R
1448A (No *A* genes) race 6	S	S	S	S	S
1449B (*A1+A2*) race 7	S	S	R	R	R
2656A (*A5*) race 8	S	S	S	R	S

[a] S or R, susceptible or resistant reactions, respectively.

found to be regulated by loci which are homologous to *hrpS* and *hrpL* from *P. s.* pv. *phaseolicola* (Huynh *et al.* 1989; Innes *et al.* 1993; Salmeron and Staskawicz 1993; Shen and Keen 1993; Lorang and Keen 1995). Conserved regions (so-called "*harp boxes*") associated with regulation by *hrp* genes, have been located within promoters of several *avr* genes from pathovars of *P. syringae* (Jenner *et al.* 1991; Dangl *et al.* 1992; Innes *et al.* 1993; Salmeron and Staskawicz 1993; Shen and Keen 1993). In *P. s.* pv. *tomato, avrE* is physically linked to *hrp*RS at the right border of the *hrp* cluster (Lorang and Keen 1995).

Here we describe the isolation and molecular characterization of the avirulence gene *avrPphE* from *P. s.* pv. *phaseolicola* race 4 strain 1302A which matches *R2* in *Phaseolus* (Table 1). Sequences hybridizing to an internal probe from *avrPphE* were found in strains representing eight races of *P. s.* pv. *phaseolicola* but gene disruption experiments showed that the *avr* gene had no obvious effect on the basic pathogenicity of the halo-blight bacterium. Serendipitously, however, sequence analysis showed that the *avrPphE* gene was closely linked to *hrpY,* a new *hrp* locus identified at the left end of the previously reported *hrp* gene cluster from *P. s.* pv. *phaseolicola* strain 3121 (Rahme *et al.* 1991). The region of the *hrp* cluster linked to *avrPphE* in strain 1302A was very similar to DNA containing *hrp* genes in *P. s.* pv. *syringae* including *hrpJ, hrpL,* and *hrpK*; there was, however, no homology to the *hrmA* locus from this bacterium (Huang *et al.* 1988, 1991; Heu and Hutcheson 1993; Xiao *et al.* 1992; Xiao and Hutcheson 1994; Xiao *et al.* 1994).

RESULTS

Avirulence genes cloned from race 4 isolate 1302A.

Attempts to isolate the *A2* gene from the genomic library of race 4 strain 1302A prepared in pLAFR3 by Jenner *et al.* (1991) were initially hampered by the absence of a suitable strain of race 6 (virulent on plants with *R1, R2, R3, R4,* or *R5*) which acted as an efficient recipient of cosmid clones. However, examination of 20 different isolates with the race 6 pathotype led to the identification of the receptive strain 1448A (Fillingham *et al.* 1992). The library was subsequently screened for *avr* genes by mobilization into a nalidixic acid resistant strain of 1448A and transconjugants tested for alteration in their virulence towards cvs. A43 (*R2 + R3 + R4 + R5*) and Tendergreen (*R3*). Four of the 960 clones examined conferred avirulence towards A43 on race 6. Two of the clones also conferred avirulence towards Tendergreen and were identified as pPPY410 and pPPY420, cosmids carrying *avrPphB.R3* (*avrPph3* in Jenner *et al.* 1991). The two novel clones (pPPY430 and pPPY440) caused an HR in A43 but not on Tendergreen. The HR induced by race 6 (pPPY430) was very similar in speed of tissue collapse and cell browning to that caused by strains of races 2, 5, and 7 in A43 (Fig. 1). Race 6 (pPPY440) caused a very similar reaction but at a few sites a mixture of sunken, brown, and water-soaked tissue was produced. Both plasmids, pPPY430 and pPPY440, also conferred avirulence towards the tepary bean (*P. acutifolius*) line 1072 but had no effect on cultivars such as Canadian Wonder, Guatemala, A53, Horsehead, and Red Mexican which are considered to lack *R2*. Analysis of F_2 progeny from a cross between A43 (proposed *R2 + R3 + R4 + R5*) and Tendergreen

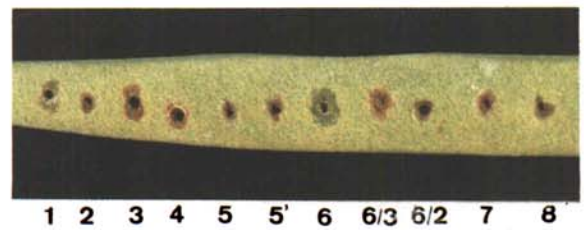

Fig. 1. Compatible and incompatible interactions in A43 (*R2 + R3 + R4 + R5*). Inoculation with strains or transconjugants containing matching avirulence (*A*) genes led to the development of hypersensitive reactions. The pod was inoculated from left to right, with strains of race 1, 1281A (*A1*); race 2, 882 (*A2 + A5*); race 3, 1301A (*A3*); race 4, 1302A (*A3 + A2*); race 5, 52 and 1375A (both *A1 + A2 + A4*); race 6, 1448A (no *A* genes); transconjugants, 1448A(pPPY310, with *A3*) and 1448A(pPPY430, with *A2*); race 7, 1449B (*A1+A2*) and race 8, 2656A (A5). *R3/A3* interactions cause a more severe HR.

(*R3*) revealed that resistance to race 6 (pPPY430) segregated in a manner as expected for regulation by a single dominant gene in A43. The observed ratio of 29 susceptible to 16 resistant plants was not significantly different from the expected 3:1 ratio ($\chi^2 = 2.674$, $P = 0.1–0.2$). Results obtained therefore indicated that the cloned avirulence gene in pPPY430 (designated *avrPphE*) matched the *R2* gene as described by Teverson (1991) and Taylor *et al.* (1995b).

Comparison of the HR phenotype determined by *R2* and *R3*.

The *avrPphE/R2* and *avrPphB/R3* interactions gave visibly different responses in A43 (Fig. 1). Phytoalexin accumulation and bacterial multiplication within lesions in pod tissue undergoing the different forms of HR to transconjugants of race 6 were compared. Higher levels of phaseollin were recovered during the expression of *R3* than *R2* (Fig. 2), but bacterial populations reached after 4 days were very similar. Bacterial growth was clearly restricted during expression of the HR (Table 2).

Location of *avrPphE* next to the *hrp* cluster.

The genomic clones pPPY430 and pPPY440 were analyzed by restriction mapping and hybridization experiments. The clones shared a region of about 8.5-kb of insert DNA. There was an obvious similarity between the map of pPPY430 and the *hrp* cluster reported by Rahme *et al.* (1991). Hybridization experiments using a probe from pPL11 which contains several *hrp* loci (Lindgren *et al.* 1988, 1989), confirmed homology to sequences in pPPY430 but not pPPY440 (data not shown). The relationship between pPPY430 and the *hrp* cluster as reported by Rahme *et al.* (1991) is summarized in Figure 3.

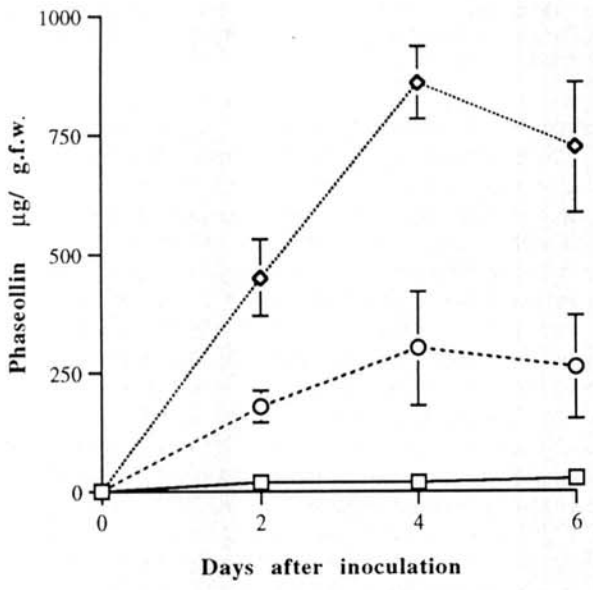

Fig. 2. Phytoalexin accumulation at infection sites in pods of A43. Phaseollin concentrations were determined following inoculation with the virulent race 6 strain 1448A (square), and with transconjugants race 6 [pPPY 310] (diamond), and race 6 [pPPY430] (circle), which induce resistance determined by the *R3* and *R2* genes respectively. Data are the mean ± SEM determined from three or four samples.

Tn*3-gus* mutagenesis was performed on pPPY430 to locate *avrPphE*. The position and orientation of transposon insertions studied are shown in Figure 3. The 5.5-kb of DNA at the left end pPPY430 had a high frequency of insertion events. Five insertions in a 1.1-kb region spanning *Eco*RI and *Hin*dIII sites completely abolished HR-inducing activity in bean cv. A43 and *P. acutifolius 1072*. Insertions 148 and 150 mapping to identical positions to the right of the 1.1-kb region did not abolish avirulence activity completely but led to a mixed phenotype with some water-soaking. Sub-cloning confirmed that the region defined by transposon mutagenesis contained the functional avirulence gene (Fig. 4).

Production of β-glucuronidase (Gus) from the transposon insertions in and around *avrPphE* was determined in a simple qualitative plate assay. Transconjugants of 1448A containing mutagenized pPPY430 were patched onto King's medium B or minimal agar (+ 0.2% glucose or fructose) containing the chromogenic Gus substrate. Colonies producing β-glucuronidase were indicated by blue coloration as summarized in Table 3. Strong activity was found from inserts 46 and 168 from within *avrPphE* when *P. s.* pv. *phaseolicola* was grown on minimal media but not on nutrient rich King's B. The direction of transcription of *avrPphE* was determined to be from right to left as indicated in Figure 3. Medium composition appeared to have no effect on *gus* expression from insert 103 downstream of *avrPphE*.

Sequences hybridizing to *avrPphE* occur in eight races of *P. s.* pv. *phaseolicola*.

The 0.55-kb *Hin*dIII/*Eco*RI internal fragment of *avrPphE* was used as a probe to Southern blots of total DNA from eight races of *P. s.* pv. *phaseolicola* and strains of a range of pathovars of *P. syringae*. Although only races 2, 4, 5, and 7 have the *A2* phenotype conferred by *avrPphE*, hybridizing fragments were found in all strains of *P. s.* pv. *phaseolicola* with no polymorphism revealed after digestion with *Eco*RI (6 kb), *Hin*dIII (23 kb), or *Pst*I (1.5-kb fragment). Similar hybridizing fragments were also detected in DNA from *P. s.* pv. *tabaci* but no hybridization was detected to DNA from strains of *P. cichorii* or *P. syringae* pvs. *coronafaciens, glycinea, maculicola, pisi,* or *syringae* (Fig. 5).

Marker exchange experiments reveal a new *hrp* gene.

Several transposon insertions in pPPY430 including number 46 which inactivated *avrPphE,* were transferred by marker gene exchange into strains of races 6 and 7. Attempts to transfer insertions to strain 1302A, race 4, the source of the genomic library, failed because of our inability to mate or electroporate clones into this strain (Jenner *et al.* 1991). The results of pathogenicity tests done with the marker exchange derived mutants are summarized in Table 4 and Figures 6 and 7. Table 2 includes data on the recovery of bacteria from inoculation sites. Genomic disruption of the *avrPphE* gene prevented induction of the HR in A43 and *P. acutifolius.* As indicated by symptom development and bacterial multiplication, disruption of *avrPphE* in races 6 and 7 did not appear to reduce pathogenicity.

Interestingly, insertion 148, which modified the HR caused by pPPY430 and was thought to be outside the *hrp* gene cluster in *P. s.* pv. *phaseolicola*, when transferred into the genome of races 6 and 7 resulted in greatly reduced pathogen-

icity and failure to cause the HR in tobacco (Tables 2 and 4, Figs. 6 and 7). The Tn*3-gus* 148 mutants were, however, not completely Hrp⁻ in phenotype in bean as very small water-soaked lesions were produced in pods of susceptible cultivars such as Tendergreen and a very localized HR developed around sites in pods of A43 inoculated with race 7::148 (Fig. 6). Tn*3-gus* 173 mutants in races 6 or 7 also caused very reduced symptoms in bean pods, but insertions 83 or 143 in both races caused a completely Hrp⁻ phenotype, as summarized in Table 4. Exchange of transposon 141, which lies just outside *avrPphE* did not affect the virulence of race 7 but rendered race 6 avirulent on A43 presumably because of the transfer of a functional *avrPphE* gene. The reactions illustrated in Figures 6 and 7 show how insertions modified responses in bean and tobacco, respectively. The Hrp⁻ mutants all multiplied as well as the wild-type strains in minimal, LB, and KB media (data not given).

Based on the map of the *hrp* region from *P. s.* pv. *phaseolicola* published by Rahme *et al.* (1991) as outlined in Figure 3, insertions 83 and 143 were considered to be in *hrpL* and 173 in *hrpF*. Insertion 148 had, however, revealed the presence of a new *hrp* gene, designated *hrpY*, outside the proposed limits of the *hrp* cluster (Rahme *et al.* 1991). To define the location of *hrpY*, the subclone pPPY437 and genomic clones pPPY430 and pPPY440, were mobilized into 1448::148 in an attempt to complement the *hrp*⁻ mutation. The 1448::148 transconjugants containing pPPY430 were fully pathogenic to Canadian Wonder, Red Mexican, and Tendergreen and caused a rapid HR in pods of A43 but the 148 mutation was not complemented by the other plasmids. This result confirmed that pPPY437 does not contain a functional *hrpY* gene. The genomic clone pPPY430 also complemented the 1448::143 and 1448::173 mutants.

Sequence analysis of *avrPphE* and linked *hrp* genes.

The nucleotide sequence was determined for the 5,360-bp region of DNA containing *avrPphE* and flanking genes in the *hrp* cluster. Four open reading frames (ORFs) were identified

Table 2. Bacteria recovered from infection sites in pods of A43 4 days after inoculation

Strain	Feature	Plant reaction[a]	Log_{10} cfu recovered ± SEM[b]
Derivatives of 1448A			
1448A	Race 6 wild type	S	8.9 ± 0.2
1448A (pPPY310)	With *avrPphB*	HR	7.5 ± 0.6
1448A (pPPY430)	With *avrPphE*	HR	7.6 ± 0.1
1448A::46	Insertion in *avrPphE* homologue	S	9.2 ± 0.2
1448A::148	Insertion in *hrpY*	N/S–	6.2 ± 0.3
1448A::143	Insertion in *hrpL*	N	4.1 ± 0.2
Derivatives of 1449B			
1449B	Race 7 wild type	HR	6.9 ± 0.1
1449B::46	Insertion in *avrPphE*	S	8.5 ± 0.3

[a] S = susceptible, large water-soaked lesion, HR = hypersensitive reaction, N = no symptoms, N/S– = no symptoms or severely limited water-soaking. Reactions are illustrated in Figure 6.
[b] Pods were inoculated with approximately 10^6 cells at each site. The values are recoveries from sites in three separate pods.

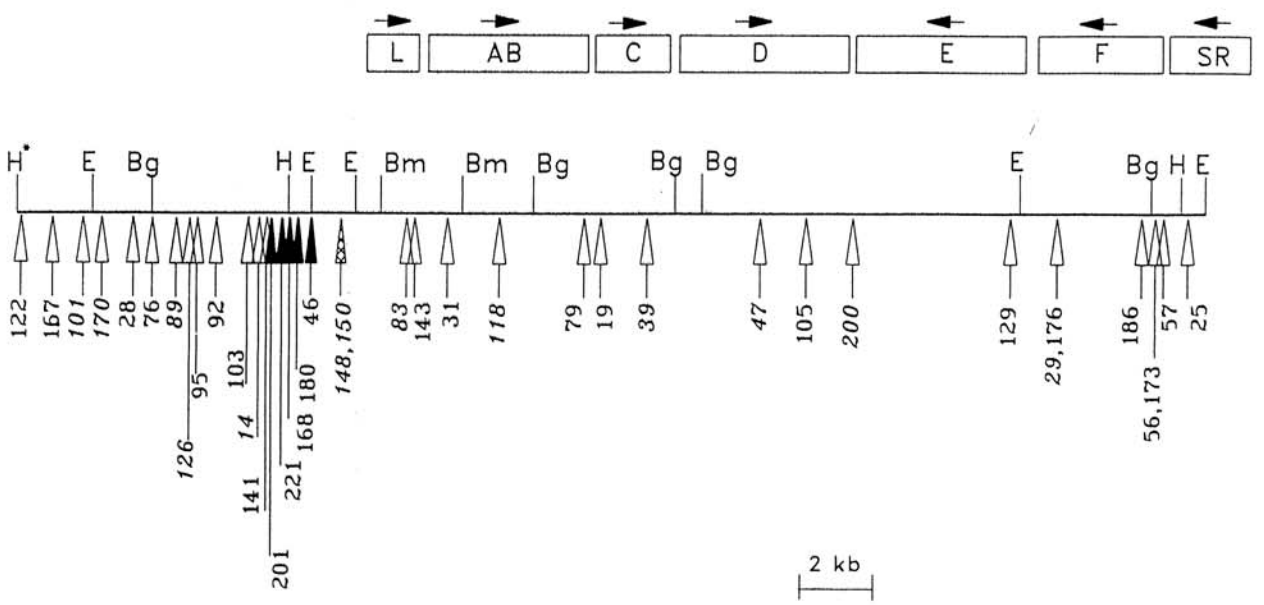

Fig. 3. Restriction map of pPPY430, cloned from race 4, showing the position of Tn*3-gus* insertions which prevent (↑) or have no effect on (↑) ability to cause the HR in cultivars with the *R2* gene. Insertions 148 and 150 modify but do not prevent the HR. Italicized numbers indicate insertion of the transposon giving transcription of *gus* from left to right and other numbers right to left. *avrPphE* is located to the left of the cluster of *hrp* loci L, AB, C, D, E, F, and RS shown as defined by Rahme *et al.* (1991). Restriction sites for *Bam*HI (Bm), *Bgl*II (Bg), *Eco*RI (E), and *Hin*dIII (H) are marked; * indicates site in the vector.

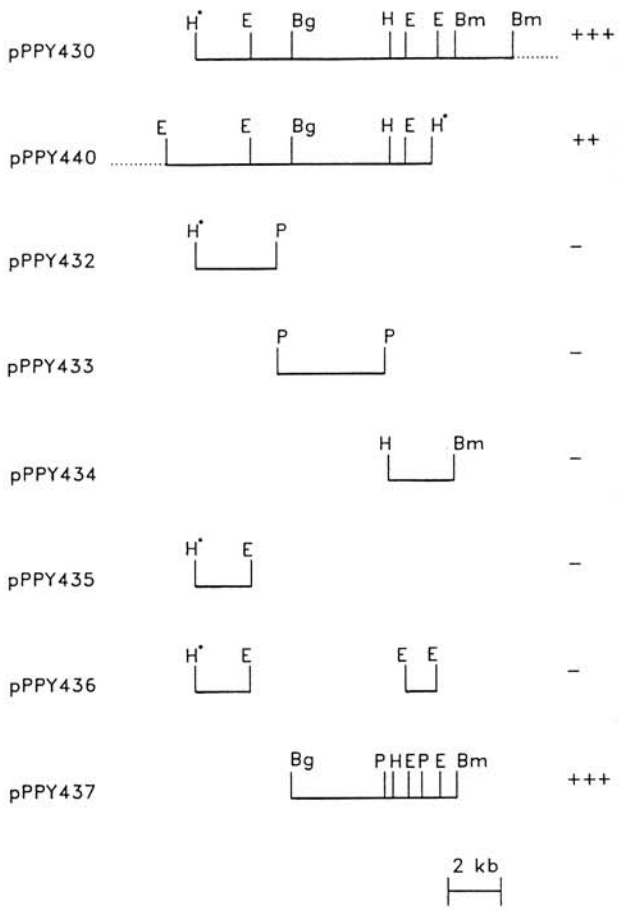

Fig. 4. Subcloning of the inserts in pPPY430 and pPPY440 to locate the *avrPphE* gene. The reaction of *P. acutifolius* and A43 to transconjugants of race 6 strain 1448A containing the clones is indicated.. The genomic clone, pPPY440 gave a slightly weaker HR,(++) than pPPY430, (+++). The *Bgl*II/*Bam*H1 fragment in pPPY437 gave the same phenotype as pPPY430, (both scored as HR+++), whereas none of the other subclones affected the virulence of race 6 (HR-). Restriction sites for *Bam*HI (Bm), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), and *Pst*I (P) are marked in pPPY437, only selected sites are shown in the other clones. Only parts of the inserts in the genomic clones are illustrated; * indicates site in the vector.

Table 3. Histochemical estimation of ß-glucuronidase (Gus) production by transconjugants of strain 1448A containing pPPY430 mutagenized with Tn*3-gus*

Transposon insertion in pPPY430[a]	Intensity[b] of blue staining indicating Gus activity in colonies growing on agar media		
	King's B	Minimal + 0.2% glucose	Minimal + 0.2% fructose
103	++	+++	++
14 or 141	0	0	0
168[c]	+	++	+++
46[c]	+	+++	++++
148,83 or 143	0	0	0

[a] Selected transposons are listed as located from left to right in pPPY430 (Figure 3).
[b] 0 = no blue color; + to ++++ increasing pigment production.
[c] Insertions within *avrPphE*.

which, based on published sequences (Huang *et al.* 1993; Xiao *et al.* 1994) and our genetic analysis, were most likely to correspond to the *hrpJ*, *hrpL*, *hrpY,* and *avrPphE* genes (Figure 8). As predicted from complementation experiments, the *hrpY* ORF extended beyond the *Bam*H1 site of pPPY437 (Figure 4). The sequence is presented in Figure 9 in the opposite orientation to the restriction map (Figure 8) as all genes except *hrpJ* were transcribed from right to left. Special features indicated include the sites of key transposon insertions 141, 46, 148, 83, and 143 and the end of insert DNA in pPPY440, all of which were determined by sequencing.

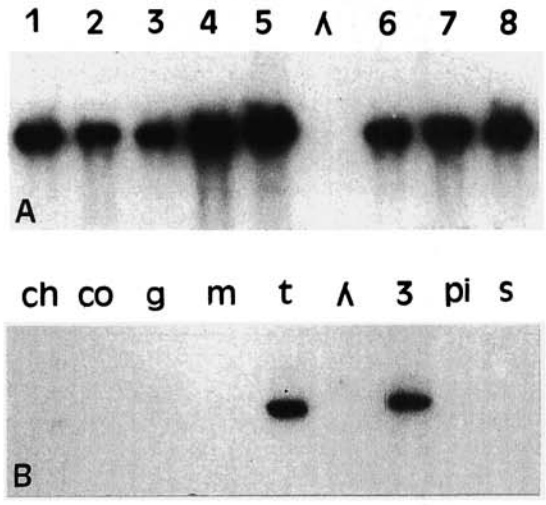

Fig. 5. Southern hybridization (high stringency) of *Hin*dIII digested total DNA from different strains of *Pseudomonas syringae* using the 0.55-kb *Eco*RI/*Hin*dIII fragment from within *avrPphE* (see Fig. 3) as a probe. **A,** Races 1, 2, 3, 4, 5, 6, 7, and 8 of *P. s.* pv. *phaseolicola* and, **B,** *Pseudomonas cichorii* (ch), *P. s.* pvs. *coronafaciens* (co), *glycinea* (g), *maculicola* (m), *tabaci* (t), *phaseolicola* race 3 (3), *pisi* (pi), and *syringae* (s). *P. s.* pv. *tabaci* and all races *P. s.* pv. *phaseolicola* but not the other pathovars contain a single hybridizing fragment (23-kb).

Table 4. Pathogenicity of mutants derived by marker exchange of Tn*3-gus* insertions in pPPY430

Strain	Interaction on pods of bean cultivar			
	A43	Canadian Wonder	Red Mexican	Tendergreen
1448A race 6 wild type	S	S	S	S
1448A::141	HR	S	S	S
1448A::46	S	S	S	S
1488A::143	N	N	N	N
1488A::83	N	N	N	N
1488A::148	N/S-	N/S–	N/S–	N/S–
1488A::173	N	N	N	N
1449B race 7 wild type	HR	S	HR	S
1449B::141	HR	S	HR	S
1449B::46	S	S	HR	S
1449B::148	N/HR-	N/S–	N/HR-	N/S–
1449B::173	N/HR-	N	N/HR-	N

[a] S = susceptible, large water-soaked lesion, HR = hypersensitive reaction, N = no symptoms, N/S– = no symptoms or severely limited water-soaking, N/HR– = no symptoms or very localized weak HR. Reactions are illustrated in Figure 6.

Database searches revealed striking similarity with recently published sequences from the left end of the *P. s.* pv. *syringae hrp* cluster. The incomplete ORF at the beginning of our sequence encoded part of a protein with 95% identity to the corresponding region of HrpJ from *P. s.* pv. *syringae* (Huang *et al.* 1993). Transposons 83 and 143 which confer a clear Hrp⁻ phenotype, were inserted into a small ORF transcribing from right to left. This ORF was designated the structural gene of *hrpL* as the predicted protein product was 93% identical to HrpL from *P. s.* pv. *syringae* (Xiao *et al.* 1994); the two protein sequences are aligned in Figure 10. No ORFs were found which agreed with the proposed location of *hrpL* in strain 3121 (Figure 3 and Rahme *et al.* 1991). The predicted HrpY protein sequence was 96% identical to HrpK from *P. s.* pv. *syringae* over the first 615 amino acids but our data indicated that the *hrpY* ORF extended 383 bp beyond *hrpK* to generate a different C terminus (Heu and Hutcheson 1993; Xiao *et al.* 1994). Overall, the homology with DNA sequences from *P. s.* pv. *syringae* broke down only at the end of the *hrpY* ORF, 617 bp downstream of the end of the coding region of *hrmA*, which was located in the same position as *avrPphE*. The breakdown in similarity is located in Figure 9. The 2.1-kb *Bam*HI fragment containing *hrpL* and parts of *hrpJ* and *hrpY* was also found to be virtually identical to DNA from *P. s.* pv. *glycinea* (R. Innes, personal communication).

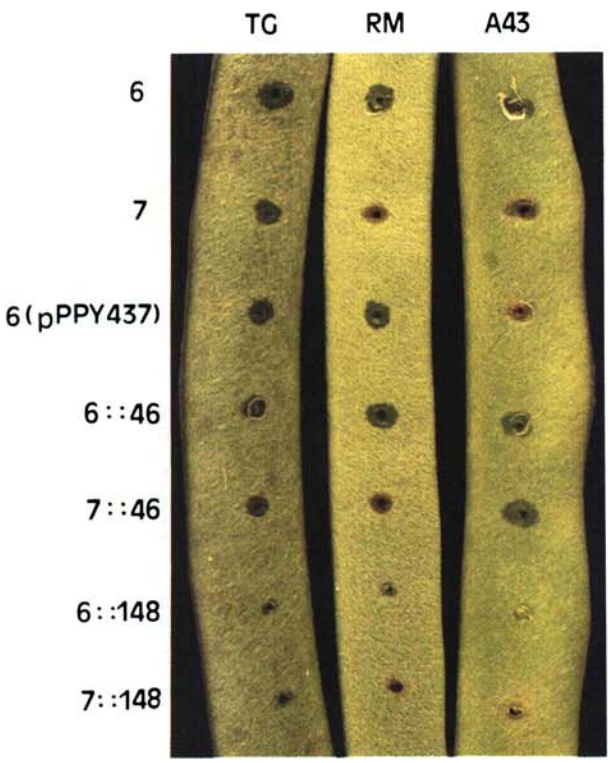

Fig. 6. Reactions in pods of bean cultivars Tendergreen, TG, (*R3*), Red Mexican, RM, (*R1* + *R4*) and A43 (*R2* + *R3* + *R4* + *R5*), 3 days after inoculation from top to bottom with the race 6 strain 1448A, race 7 strain 1449B, the transconjugant of race 6 1448A(pPPY437) which expresses *avrPphE*, marker exchange mutants 1448A::46, 1449B::46, 1448A::148, and 1449B::148. The race 6 strain 1448A is virulent on all cultivars. Race 7 strain 1449B is predicted to contain avirulence genes matching *R1* and *R2*. The transposons 46 and 148 are in *avrPphE* (which matches *R2*) and *hrpY*, respectively. As predicted, the insertion in *avrPphE* in 1449B prevents the HR in A43 but not in Red Mexican.

A sequence conforming to the original "*harp box*" motif as described by Fellay *et al* (1991) was located upstream of *avrPphE* but within the coding region of *hrpY* (Fig. 9). The *hrpY* and *hrpJ* genes were preceded by the revised "*harp box*" which has been found upstream of most *avr* genes from pathovars of *P. syringae* (Innes *et al.* 1993). The proposed *hrpL* gene did not contain such a motif in its putative promoter. A potential σ^{54} promoter consensus for *Pseudomonas* (Deretic *et al.* 1989; Shen and Keen 1993) was found upstream of *hrpL* (as reported for *hrpL* from *P. s.* pv. *syringae* by Xiao *et al.* 1994) and also before an alternative ATG start of *hrpY*.

Hydrophilicity plots for the predicted proteins encoded by the genes *avrPphE*, *hrpL*, and *hrpY* are given in Figure 11. AvrPphE encodes a 41-kDa hydrophilic protein with no membrane spanning regions or structural motifs indicative of function. No homologies were found between AvrPphE or HrpY and proteins in databases. The *hrpY* product is predicted to be a predominantly hydrophilic protein of 80-kDa with a hydrophobic domain at the C terminus. *HrpL* is pre-

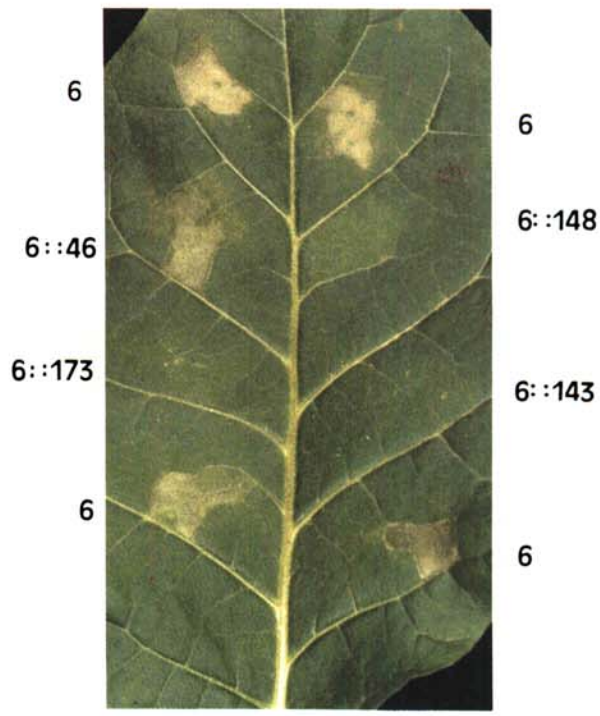

Fig. 7. Reactions at interveinal infiltration sites in tobacco leaves 3 days after inoculation with race 6 strain 1448A, or marker exchanged mutants of race 6 with Tn*3-gus* insertions 148, 143, and 173 giving the Hrp⁻ phenotype or 46 which does not affect the HR. Each of the *hrp*⁻ mutants has caused very slight yellowing.

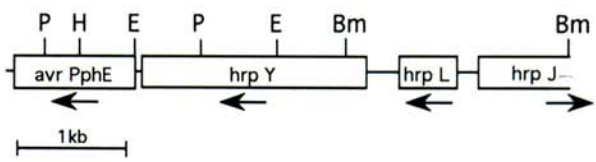

Fig. 8. Location of open reading frames for *avrPphE*, *hrpY*, *hrpL*, and part of *hrpJ* within the 5,360-bp fragment of DNA sequenced. Restriction sites for *Bam*HI (Bm), *Pst*I (P), *Eco*RI (E), and *Hin*dIII (H) are marked. The directions of transcription are indicated by arrows.

Fig. 9. Nucleotide sequence of the region of DNA from strain 1302A containing *avrPphE* and the border of the *hrp* cluster including *hrpJ*, *hrpL*, and *hrpY*. Amino acids in the translation products from open reading frames are given in three-letter abbreviations. Features marked include selected restriction sites, the position of Tn*3-gus* insertions 143, 83, 148, 46, and 141 and the end of insert DNA in pPPY440 (all of which were determined by sequencing), consensus motifs for the original "*harp box*" (shaded and overlined), revised "*harp box*" (shaded and underlined), potential σ^{54} promoters (boxed and underlined) and ribosome binding sites (underlined). The asterisk at nucleotide 4021 indicates the position at which similarity with DNA sequence from *P. s.* pv. *syringae* (Hutcheson and Xiao 1993) breaks down. The line of sequence from *P. s.* pv. *syringae* is given above that from *P. s.* pv. *phaseolicola* for nucleotides 3991 to 4060, comparison of the two sequences emphasizes the abrupt loss of similarity.

```
          ↓10        ↓20        ↓30        ↓40        ↓50        ↓60        ↓70
MFPNLVILDAPQPRQDSSSAGIRQLTADQIQMLRAFIQKRVMNPDDVDDILQCVFLEALRNEHKFQHASK
M:PNLVILD..:PR: SSSAGIRQLTADQIQMLRAFIQKRV.N:DDVDDILQCVFLEALRNEHKFQHASK
MLPNLVILDVTEPRKPSSSAGIRQLTADQIQMLRAFIQKRVKNADDVDDILQCVFLEALRNEHKFQHASK

          ↓80        ↓90        ↓100       ↓110       ↓120       ↓130       ↓140
PQTWLCGIALNLIRNHFRKMYRQPYQESWEDDVHSDLEWNGHITHQVHGHRQLARVIPAIDCLPSNMQKV
PQTWLCGIALNLIRNHFRKMYRQPYQESWEDDVH:DLEW:G:ITHQV:GHRQLARVI AIDCLP:NMQKV
PQTWLCGIALNLIRNHFRKMYRQPYQESWEDDVHTDLEWHGDITHQVDGHRQLARVIEAIDCLPTNMQKV

          ↓150       ↓160       ↓170       ↓180
LEVSLEMDGNYQDTANTLGVPIGTVRSRLSRARVQLKQQIDPFA
LEVSLEMDGNYQ:TANTLGVPIGTVRSRLSRARVQLKQQIDPFA
LEVSLEMDGNYQETANTLGVPIGTVRSRLSRARVQLKQQIDPFA
```

Fig. 10. Alignment of HrpL proteins from *Pseudomonas syringae* pvs. *phaseolicola* (top line) and *syringae* (bottom line, from Xiao *et al.* 1994). Identical amino acids are given in single-letter code. Dots show intermediate nonconservative exchanges; colons indicate conservative changes.

dicted to encode a 21-kDa protein which is hydrophilic and has no distinct structural features. As shown by Xiao *et al.* (1994), HrpL has similarity to AlgU which regulates the *algD* operon in *P. aeruginosa*, and also to a subset of group III sigma factors related to the *Bacillus* SigH ,σ^{30} (Deretic *et al.* 1987; Dubnau *et al.* 1988; Martin *et al.* 1993).

DISCUSSION

Incompatibility determined by *avrPphE* fits the pattern of interactions predicted for an avirulence gene matching the *R2* gene in *P. vulgaris*. The full designation of the avirulence gene according to Vivian and Mansfield (1993) should therefore be *avrPphE-1.R2*, as the fifth (*E*) *avr* gene cloned from *P. s.* pv. *phaseolicola* (*Pph*), the first allele (*1*) characterized for this gene which matches *R2*. Effectively using *avrPphE* as a probe for *R2* has clearly demonstrated the presence of the *R2* gene in line 1072 of *P. acutifolius* which has not been crossed successfully with *P. vulgaris*. Clones harboring *avrPphE* were also conjugated into *P. s.* pv. *maculicola* and *P. s.* pv. *pisi* and transconjugants tested for ability to cause the HR in a range of genotypes of their hosts *Arabidopsis thaliana* and pea, respectively, but no significant alterations in virulence were observed (Fillingham *et al.* 1992; Dangl *et al.* 1993; unpublished results).

The HR determined by *avrPphE* in *P. s.* pv. *phaseolicola* is macroscopically different from that caused by *avrPphB* which matches *R3* (Jenner *et al.* 1991; Vivian and Mansfield 1993). The different phenotypes of *R2* and *R3*-based resistance are particularly clear in cv. A43, which carries both genes. The more rapid HR determined by *R3* is associated with higher levels of phytoalexin accumulation at inoculation sites. The appearance of pod inoculations as illustrated in Figure 1, suggests that more plant cells are affected from the same initial inoculum during the expression of *R3* than of *R2* and that affected cells also respond differently. Clearly, detailed microscopical studies are needed to examine these responses and to differentiate between qualitative and quantitative effects as discussed by Brown and Mansfield (1988). The induction of different forms of HR has also been described for other *avr/R* gene interactions involving cloned *avr* genes, for example, in soybean, *avrB* causes a more rapid response than *avrC* from *P. s.* pv. *glycinea* (Staskawicz *et al.* 1987; Tamaki *et al.* 1988). Overexpression of *avrBs3* leads to

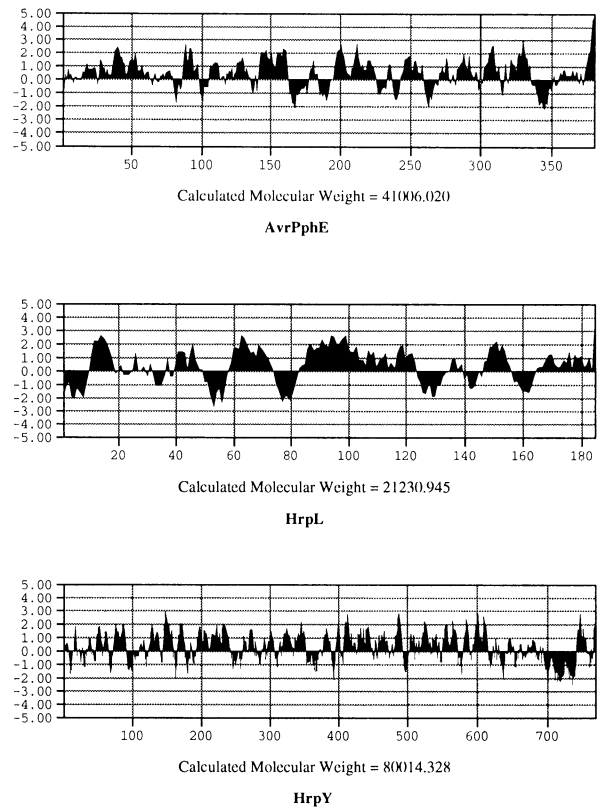

Fig. 11. Hydrophilicity plots prepared with the MacVector program using the Kyte-Doolittle scale for predicted proteins from *P. s.* pv. *phaseolicola avrPphE, hrpL,* and *hrpY* (hydrophilicity window = 7).

a more rapid HR in pepper cultivars with the *Bs3* gene for resitance to *Xanthomonas campestris* pv. *vesicatoria* (Brown *et al.* 1993). Whether or not these differences are due to the operation of differing signal transduction pathways leading to the plant's response or different levels of activation of the same pathway (Lamb *et al.* 1989; Lindsay *et al.* 1993) will only become clear when molecules involved in recognition (putative elicitors and receptors) have been identified. The modulating effects of transposons 148 and 150 on the phenotype conferred by *avrPphE* may be explained by a quantitative relationship with levels of AvrPphE protein. The transposons, possibly being located in the promoter, may reduce

Table 5. Bacterial strains and plasmids used in this study[a]

Strain/Plasmid	Relevant properties	Source or reference
Bacteria		
Pseudomonas syringae pv. *phaseolicola*		
Principal isolates used		
1302AN	Race 4 Nal^R from 1302A	Jenner *et al.* 1991
1448AR	Race 6 Rif^R from 1448A	Fillingham *et al.* 1992
1448AN	Race 6 Nal^R from1448A	Fillingham *et al.* 1992
1449BR	Race 7 Rif^R from 1449B	D. Teverson[b]
Additional isolates		D. Teverson
1281A Race 1		D . Teverson
882 Race 2		D . Teverson
0001301A Race 3		
0001375A Race 5		D . Teverson
52 Race5		Jenner *et al.* 1991
1299A Race6		D . Teverson
2656A Race8		D . Teverson
P. cichorii		
2379	Lettuce pathogen	NCPPB[c]
P.s. pv. *coronafaciens*		
1354	Oat pathogen	Harper *et al.* 1987
P.s. pv. *glycinea*		
1416A	Soybean pathogen	
P.s. pv. *maculicola*		J. D. Taylor[b]
1820	Brassica pathogen	NCPPB
P.s. pv. *pisi*		
974B	Pea pathogen	J.D.Taylor
P.s. pv. *syringae*		
281	Lilac pathogen	NCPPB
P.s. pv. *tabaci*		
11528	Tobacco pathogen	J. Turner[d]
E. coli		
C2110	Nal^R, *polA1*	Leong *et al.* 1980
DH5α	Nal^R, *recA*, *lacZΔM15*	Bethesda Research Labs
ED8767	*recA*	Murray *et al.* 1977
HB101	Sm^R, *recA*	Boyer and Roulland-Dussoix 1969
Helpers and Vectors		
pBluescriptII SK^+	Ap^R, ColE1 replicon, multiple cloning and priming sites	Stratagene
pHoKmGus	Ap^R, Km^R, *tnpA*, promoterless β-glucuronidase gene in Tn*3*, pWB15A replicon	Bonas *et al.* 1989
pIJ3200	pLAFR1 containing pBluescriptII polylinker	Liu *et al.* 1990
pLAFR3	Tc^R, IncP1 replicon, Tra^-, Mob^+, cosmid	Staskawicz *et al.* 1987
pRK2013	Km^R, ColE1 replicon, Tra^+, Mob^+, helper plasmid	Figurski and Helinski 1979
pSShe	Cm^R, $tnpA^+$, pACYC184 replicon	Stachel *et al.* 1985
Clones containing the avirulence gene *avrPphE*		
pPPY430	pLAFR3-based genomic clone from race 4	This study
pPPY437	6.5-kb *Bam*HI-*Bgl*II fragment from pPPY430 in pIJ3200	This study
pPPY440	pLAFR3-based genomic clone from race 4	This study
pPPY4012	6.5-kb *Bam*HI-*Bgl*II fragment from pPPY430 in pBluescriptII SK^+	This study
pPPY4013	As pPPY4012 but in opposite orientation	This study
Insert DNA from pPPY430 cloned into pLAFR3 (see Figure 3)		
pPPY432	2.9-kb *Hin*dIII-*Pst*I fragment	This study
pPPY433	2.9-kb *Pst*I fragment	This study
pPPY434	2.4-kb *Hin*dIII-*Bam*HI fragment	This study
pPPY435	2.0-kb *Hin*dIII-*Eco*RI fragment	This study
pPPY436	2.0-kb *Hin*dIII-*Eco*R1 and 1.3kb *Eco*RI fragments	This study
Additional plasmids		
pPPY310	pLAFR1 based genomic clone containing *avrPphB*	Jenner *et al.* 1991
pPL11	19-kb section of *hrp* cluster from *P.s.* pv. *phaseolicola*, lacking *hrpL*, cloned in pWB5A, Tc^R	Lindgren *et al.* 1989

[a] Nal^R, Rif^R, Sm^R, Ap^R, Km^R, Tc^R, Cm^R indicate resistance to nalidixic acid, rifampicin, streptomycin, ampicillin, kanamycin, tetracyline, and chloramphenicol, respectively.
[b] Horticulture Research Internation, Wellesbourne, U.K.
[c] National Collection of Plant Pathogenic Bacteria, Harpenden, U.K.
[d] University of East Anglia, Norwich, U.K.

levels of transcription of *avrPphE*. A similar reduction in gene expression may explain the somewhat weaker HR caused by pPPY440.

The *avrPphE* structural gene is predicted to encode a hydrophilic protein. There are no obvious features of the AvrPphE protein that can be related to function. The lack of similarity to sequences in databases searched at the DNA or amino acid levels is disappointing but in agreement with findings for other *avr* genes from pathovars of *P. syringae* (Keen 1990; Long and Staskawicz 1993). The absence of common Sec dependent or independent secretory signals (Salmond *et al.* 1993, Pogliano and Beckwith 1994) does not of course preclude export of the AvrPphE protein via alternative secretory pathways perhaps involving *hrp* gene products as discussed by Fenselau *et al.* (1992).

Hybridization experiments revealed the presence of *avrPphE* homologues in races which are virulent on cultivars containing the matching *R2* gene. Marker exchange mutants of races 6 and 7 with disrupted *avrPphE* were as pathogenic as wild-type strains when assessed by the simple tests used here. Disruption of *avrPphE* in race 7 caused the expected change from avirulence to virulence in A43 and *P. acutifolius*. As observed with several other *avr* genes, e.g., *avrBs3* (Bonas *et al.* 1989); *avrPto* (Innes *et al.* 1993), and *avrRpt2* (Salmeron and Staskawicz 1993) the *avrPphE* gene does not appear to be a significant determinant of pathogenicity. The presence of apparently non-functional homologues of *avrPphE* within all strains of *P. s.* pv. *phaseolicola* examined parallels the observations made with *avrD* from *P. s.* pv. *glycinea* (Kobayashi *et al.* 1990a, 1990b; Yucel *et al.* 1994a). Our findings with *avrPphE* are in direct contrast with *avrPphB,* which is found only in races that are avirulent on bean genotypes with the matching *R3* gene (Jenner *et al.* 1991).

Experiments with Tn*3-gus* insertions in *avrPphE* show that the gene is expressed much more strongly in minimal than in rich media. Such a pattern of expression has been reported for other *avr* genes from pathovars of *P. syringae* which are regulated by *hrpS* and *hrpL* (Huyhn *et al.* 1989; Salmeron and Staskawicz 1993; Innes *et al.* 1993; Xiao *et al.* 1994). Attempts to use the *hrpS*$^-$ mutant strain LRG94 of *P. s.* pv. *phaseolicola* 3121 (kindly provided by Laurence Rahme, Rahme *et al.* 1991) to investigate regulation of *avrPphE* have so far proved unsuccessful because pPPY430 or pPPY430::46 were unexpectedly found to complement the *hrpS*$^-$ mutation. The promoters of all avirulence genes thought to be regulated by *hrpL* contain a highly conserved consensus GGAACC-15N-CCAC (Shen and Keen 1993; Xiao and Hutcheson 1994). This motif is a revised version of the "*harp box*" initially reported by Fellay *et al.* (1991). The *avrPphE* promoter contains the original version of the "*harp box.*" The significance of this finding for regulation of *avrPphE* remains to be determined.

Marker exchange experiments revealed the presence of an additional *hrp* locus to the left of the reported *hrp* cluster in *P. s.* pv. *phaseolicola*. Extending DNA sequencing upstream of *avrPphE* has revealed an ORF, designated *hrpY,* which is predicted to encode an 80-kDa protein of unknown function. The striking similarity at the DNA level between *hrpY* and sequences published for the region downstream of the *hrmA* gene in *P. s.* pv. *syringae* (Heu and Hutcheson 1993, Xiao *et al.* 1994) strongly suggests that *hrpY* and the *hrpK* gene from *P. s.* pv. *syringae* are homologous. In view of the linkage of *hrpK* with *hrmA* in *P. s.* pv. *syringae* we might have expected *avrPphE* to show some similarity with the *hrmA* gene. But the similarity between the DNA sequences from *P. s.* pv. *phaseolicola* and *P. s.* pv. *syringae* terminated at the *hrpY/avrPphE* junction. A possible explanation for the abrupt change in sequence indicated in Figure 9, is the insertion of a DNA fragment containing either *avrPphE* in *P. s. pv. phaseolicola* or *hrmA* in *P. s.* pv. *syringae*, into a generally common genome. Hybridization experiments using fragments downstream of *avrPphE* as probes (e.g., cloned DNA in pPPY432) revealed homology in all pathovars (data not shown) supporting the idea that the *avrPphE/hrmA* location is less conserved than flanking regions within pathovars of *P. syringae.* Heu and Hutcheson (1993) also reported that strains of *P. s.* pv. *phaseolicola* lack *hrmA*.

The gene recognized as *hrpL* in this work is predicted to encode a protein 93% identical to *hrpL* from *P. s.* pv. *syringae* (Xiao *et al.* 1994). The location and orientation of transcription of *hrpL* conflicts with that reported for the *hrpL* locus in *P. s.* pv. *phaseolicola* strain 3121 (Rahme *et al.* 1991). The role of the *hrpL* locus we have located in strain 1302A requires further investigation, particularly in relation to regulation of *avr* and other *hrp* genes in the halo-blight bacterium. Key regulatory roles have been proposed for the different *hrpL* loci in *P. s.* pv. *phaseolicola* 3121, *P. s.* pv. *glycinea, P. s.* pv. *syringae,* and *P. s.* pv. *tomato* (Huynh *et al.* 1989; Fellay *et al.* 1991; Rahme *et al.* 1991 1992; Innes *et al.* 1993; Salmeron and Staskawicz 1993; Xiao and Hutcheson 1994; Xiao *et al.* 1994).

The function of *avrPphE* and in particular the biochemical basis for its unique ability to confer avirulence in genotypes of *Phaseolus* with the *R2* gene remains unknown. The hydrophilic nature of the AvrPphE protein implies a cytoplasmic location. The protein may be enzymic and be involved in the synthesis of an elicitor as proposed for the AvrD protein from *P. s.* pv. *tomato* (Keen *et al* 1990; Smith *et al.* 1993). It will be interesting to examine the expression and DNA sequences of the apparently nonfunctional homologues of *avrPphE* in various strains of *P. s.* pv. *phaseolicola*. Such analysis may indicate functional domains in AvrPphE. It remains a possibility, however, that the complete structural gene may be present and expressed in strains of the halo-blight bacterium which are virulent on plants with *R2* but that suppression of the avirulence phenotype may lead to virulence.

MATERIALS AND METHODS

Bacteria and plasmids.

Principal bacterial strains, cosmids, and plasmids used are noted in Table 5. Isolates and transconjugants of *P. s.* pv. *phaseolicola* were grown routinely on King's medium B (KB) agar at 25° C and *Escherichia coli* strains on Luria-Bertani (LB) agar or in LB broth at 37° C (King *et al.* 1954; Miller 1972). To test for β-glucuronidase (GUS) production, bacteria were grown on M9 minimal medium agar supplemented with 0.2% fructose or glucose (Miller 1972) or on KB containing 25 μg ml^{-1} X-Gluc (5-bromo-4-chloro-3-indoyl-β-D-glucuronide from Sigma, Poole, Dorset). Blue pigmentation of colonies indicated Gus activity. Antibiotics, obtained from Sigma, were usually used at the following concentrations (μg ml^{-1}); rifampicin, Rif (50); nalidixic acid, Nal (50);

tetracycline, Tc (15); kanamycin, Km (25); ampicillin, Ap (50); chloramphenicol, Cm (25); streptomycin, Sm (100).

Cloning procedures.

The construction of the genomic library of race 4 was described by Jenner *et al.* (1991). The library was screened for determinants of avirulence by conjugation of individual clones into race 6 strain 1448AN with the helper plasmid pRK2013 (Figurski and Helinski 1979). Transconjugants were tested for pathogenicity in pods of Tendergreen and A43 as described by Harper *et al.* (1987).

DNA of pLAFR3, pIJ3200, and pBluescript II SK$^+$ was isolated from *E. coli* ED8767 by the alkaline lysis method (Birnboim and Doly 1979) and purified by CsCl/EtBr gradient centrifugation (Maniatis *et al.* 1982). Restriction fragments from pPPY430 were cloned into pLAFR3, pBluescriptII SK$^+$, and pIJ3200 after recovery of DNA from agarose gels using Qiaex kits (Qiagen, Hybaid, Cambridge) according to the manufacturer's instructions. Ligations were performed with T4 DNA Ligase (BRL Life Technologies, Paisley), restriction enzymes came from the same supplier. Small-scale preparations of plasmids were done with the methods of Birnboim and Doly (1979) or Kado and Liu (1981). Basic procedures for handling DNA were as described in Maniatis *et al.* (1982) or Ausubel *et al.* (1987).

Mutagenesis with Tn*3-gus*.

The method of Bonas *et al.* (1989) was followed. Purified DNA of pPPY430 was transformed into *E. coli* HB101 (pSShe, pHoKmGus). Twenty independent transformants were grown together in LB (Cm, Ap, Km, Tc) prior to a triparental conjugation into *E. coli* C2110 using ED8767 (pRK2013) as the mobilizing strain. Individual transconjugants of C2110 (pPPY430::Tn*3-gus*) were isolated. The clones were mapped in C2110, and mated into *P. s.* pv. *phaseolicola* 1448AR to test for avirulence on pods. Plasmids were recovered from transconjugants producing susceptible reactions on A43 to confirm the integrity of the insert.

Marker exchange.

Individual transconjugants of race 6 (1448AR) and race 7 (1449AR) containing selected pPPY430::Tn*3-gus* clones were subcultured on LA Km until replica plating showed loss of tetracycline resistance. Genomic DNA digested with *Bgl*II was probed with a 3-kb internal *Bam*HI fragment from Tn*3-gus* to confirm the position of the transposon in the target region.

Plants and pathogenicity tests.

Pods of cultivars of French bean (*Phaseolus vulgaris* L.) and tepary bean (*P. acutifolius* L.) were inoculated as previously described (Harper *et al.* 1987; Hitchin *et al.* 1989). The compatible interaction in pods is expressed by the development of water-soaked lesions at sites of stab inoculation. Incompatibility is expressed by the HR, observed as the formation of sunken brown lesions (Harper *et al.* 1987).

Leaves of tobacco plants cv. White Burley, were infiltrated with bacterial suspensions using a syringe and hypodermic needle and incubated at 24° C (Smith and Mansfield 1981).

Growth of strains in pods of A43 was determined using the method described by Hitchin *et al.* (1989) and Fillingham *et al.* (1992). Detached pods were swabbed with 70% ethanol and inoculated by the addition of a 5-µl droplet of suspension containing approximately 10^6 cells to a wound made with a cocktail stick. Pods were incubated in humid chambers at 25° C. Tissue containing developing lesions was dissected from around inoculation sites and homogenized in 10 mM $MgCl_2$. Serial dilutions of the homogenate were spread onto KB agar to allow colony development.

Phaseollin accumulation.

Phytoalexins were isolated from lesions dissected from inoculation sites in pods of A43. The method used was based on that described by Fillingham *et al.* (1992). Tissues (0.2–0.5 g fresh wt.) were ground in ethanol (about 10 ml), cell debris was pelleted by centrifugation and supernatants rotary-evaporated to dryness at 45° C. Extracts were resuspended in ethanol and applied to TLC plates (Merck Si gel 60 F_{254} 5715). Chromatograms were developed in chloroform:ethanol (100:3) and viewed under UV radiation at 254 nm. Phaseollin, identified as a quenching band with an R_f value of approximately 0.5, was scraped from the chromatograms and eluted in ethanol. Recoveries of phaseollin (λmax 280, 286, and 315 nm) were quantified by UV spectrophotometry using the extinction coefficient at λmax 280 nm, log ε = 4.04 (Bailey and Burden 1983).

Hybridization experiments.

Probes were recovered from digests of plasmid DNA after electrophoresis in low melting point agarose (Sigma). Purified fragments were ^{32}P-labeled in the agarose by the random priming method (Feinberg and Vogelstein 1983) using a Pharmacia oligolabeling kit (Pharmacia, Milton Keynes, Bucks.). Total DNA was obtained from races of *P. s.* pv. *phaseolicola*, pathovars of *P. syringae* and *P. cichorii* using the CTAB procedure described in Ausubel *et al.* (1987) and Jenner *et al.* (1991). DNA digested with appropriate restriction endonucleases was electrophoresed in 0.8% agarose gels before Southern transfer onto Hybond nylon membrane (Amersham International, Aylesbury, Bucks.). Hybridizations were performed in 5× SSPE (0.9 M NaCl, 0.05 M sodium phosphate, 0.005 M ethylenediaminetetra-acetic acid, pH 7.7), 5× Denhardt's solution (Maniatis *et al.* 1982), 0.5% sodium dodecyl sulfate (SDS), and 30 µg ml^{-1} salmon sperm DNA with gentle shaking for 18 hr at 65° C. Blots were washed twice in 2× SSPE, 0.1% SDS at room temperature for 10 min and then in 1× SSPE, 0.1% SDS at 65°C for 15 min before preliminary autoradiography. A subsequent high stringency wash was done with 0.1× SSPE, 0.1% SDS at 65° C for 15 min before exposure to X-ray film. Previously hybridized blots were stripped of probes in 0.4 M NaOH (30 min at 45° C) and then neutralized in 0.1× SSC (15 mM NaCl, 1.5 mM trisodium citrate) 0.1% SDS, 0.2 M Tris-HCl, pH 7.5, for 15 min at 45° C.

Sequencing.

DNA sequences were determined by the dideoxynucleotide method (Sanger *et al* 1977) usually using T7 DNA polymerase incorporating deoxyadenosine 5′-([∝-^{35}S] thio) triphosphate (1,200 Ci/mmol; Amersham, Bucks.) according to the manufacturer's protocol for CsCl gradient-purified plasmid DNA. Polyacrylamide gels contained 6% acrylamide

and 7 M urea. All sequencing mixes included 7-deaza dGTP. Compressions were resolved using dITP to replace dGTP or by conducting reactions at high temperature with the *Taq* polymerase based Gene-ATAQ sequencing kit (Pharmacia).

Internal *Bgl*11/*Eco*RI, *Bgl*11/*Pst*I, *Pst*I, *Pst*/*Bam*HI, *Eco*RI, and *Eco*RI/*Bam*HI fragments from pPPY4012 and 4013 (Fig. 4 and Table 5) and the *Bam*HI fragment containing *hrpL* from pPPY430, were subcloned (in both orientations where possible) into pBluescript II SK. Insert DNA was sequenced on both strands from M13, T3, and T7 priming sites in the vector and from oligonucleotide primers designed from derived sequences.

To determine the location of Tn*3-gus* inserts in the genomic clone pPPY430, DNA of the large pLAFR3-basid plasmid was prepared by Qiagen Tip 100 columns (Qiagen, Hybaid, Cambridge) and sequenced using the Gene-ATAQ system with the primer 5′AAAGAGGCGTCAGAGGC3′ which complements an internal sequence of the transposon. The junction of insert DNA with the *Bam*HI cloning site in pPPY440 was determined by sequencing this genomic clone in the same way using a primer 5′GCTATTACGCCAGCT3′ derived from the pUC8 polylinker in pLAFR3 (Staskawicz *et al.* 1987). Nucleotide sequences and derived proteins were analyzed with DNASTAR and MacVector programs. Database searches for similar sequences were completed using the National Center for Biotechnology Information network service and the BLAST algorithm (Altschul *et al.* 1990; Gish and States 1993).

ACKNOWLEDGMENTS

This work was supported by grants from the Agricultural and Food Research Council and Science and Engineering Research Council, and completed under the U.K. Ministry of Agriculture, Food and Fisheries licences numbers 64/114(78) and 64A/398/33. We are indebted to Laurence Rahme, John Taylor, and Dawn Teverson for the provision of strains and invaluable discussion; Jim Beynon for advice on sequence analysis, and Ulla Bonas for her comments on drafts of the manuscript. Marjorie Gibbon kindly provided the primer for locating Tn3-*gus* inserts. Thanks are also due to Roger Innes and Steven Hutcheson for provision of sequences before publication.

LITERATURE CITED

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Siedman, J. G., Smith, J. A., and Struhl, K. 1987. Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Bailey, J. A., and Burden, R. S. 1983. Biochemical changes and phytoalexin accumulation in *Phaseolus vulgaris* following cellular browning caused by tobacco necrosis virus. Physiol. Plant Pathol. 3:171-177.

Birnboim, H. C., and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.

Bonas, U., Stall, R. E., and Staskawicz, B. 1989. Genetic and structural characterisation of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria*. Mol. Gen. Genet. 218:127-136.

Boyer, H. W., and Roulland-Dussoix, D. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 41:459-472.

Brown, I. R., and Mansfield, J. W. 1988. An ultrastructural study, including cytochemistry and quantitative analyses of the interactions between pseudomonads and leaves of *Phaseolus vulgaris* L. Physiol. Mol. Plant Pathol. 33:351.

Brown, I. R., Mansfield, J. W., Irlam, I., Conrads-Strauch, J., and Bonas, U. 1993. Ultrastructure of interactions between *Xanthomonas campestris* pv. *vesicatoria* and pepper, including immunocytochemical localization of extracellular polysaccharides and the AvrBs3 protein. Mol. Plant-Microbe Interact. 6:376-386.

Dangl, J., Ritter, C., Gibbon, M., Mur, L. A. J., Wood, J. R., Goss, S., Mansfield, J., Taylor, J. D., and Vivian, A. 1992. Functional homologs of the *Arabidopsis Rpm1* disease resistance gene in bean and pea. Plant Cell 4:1359-1369.

De la Fuente-Martinez, J. M., Mosqueda-Cano, G., Alvarez-Morales, A., and Herrera-Estrella, L. 1993. Pathogen-derived strategy to produce transgenic plants resistant to the bacterial toxin phaseolotoxin. Pages 579-586 in: Advances in Molecular Genetics of Plant-Microbe Interactions. Vol. 2. E. W. Nester and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht.

Deretic, V., Gill, J. F., and Chakrabarty, A. M. 1987. Gene *algD* coding for GDP mannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. J. Bacteriol. 169:351-358.

Deretic, V., Konyecsni, W. M., Mohr, C. D., Martin, D. W., and Hibler, N. S. 1989. Common denominators of promoter control in *Pseudomonas* and other bacteria. Biotechnology 7:1249-1254.

Dubnau, E., Weir, J., Nair, G., Carter, L., Moran, C., and Smith, I. 1988. *Bacillus* sporulation gene *spoOH* codes for σ^{30} (σ^{H}). J. Bacteriol. 170:1054-1062.

Feinberg, A. P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem.132:6-13.

Fellay, R., Rahme, L. G., Mindrinos, M. N., Frederick, R. D., Pisi, A., and Panopoulos, N. J. 1991. Genes and signals controlling the *Pseudomonas syringae* pv. *phaseolicola*-plant interaction. Pages 45-52 in: Advances in Molecular Genetics of Plant-Microbe Interactions. Vol. 1. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, Netherlands.

Fenselau, S., Balbo, I., and Bonas, U. 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. Mol. Plant-Microbe Interact. 5:390-396.

Figurski, D. H., and Helinski, D. R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc. Natl. Acad. Sci. USA 76:1648-1652.

Fillingham, A. J., Wood, J., Bevan, J. R., Crute, I. R., Mansfield, J. W., Taylor, J. D., and Vivian, A. 1992. Avirulence genes from *Pseudomonas syringae* pathovars *phaseolicola* and *pisi* confer specificity towards both host and non-host species. Physiol. Mol. Plant Pathol. 40:1-15.

Gish, W., and States, D. J. 1993. Identification of protein coding regions by database similarity search. Nature Genet. 3:266-272.

Grimm, C., and Panopoulos, N. J. 1989. The predicted product of a pathogenicity locus from *Pseudomonas syringae* pv. *phaseolicola* is homologous to a highly conserved domain of several procaryotic regulatory proteins. J. Bacteriol. 171:5031-5038.

Harper, S., Zewdie, N., Brown, I. R., and Mansfield, J. W. 1987. Histological, physiological and genetical studies of the responses of leaves and pods of *Phaseolus vulgaris* to three races of *Pseudomonas syringae* pv. *phaseolicola* and to *Pseudomonas syringae* pv. *coronafaciens*. Physiol. Mol. Plant Pathol. 31:153-172.

Heu, S., and Hutcheson, S. W. 1993. Nucleotide sequence and properties of the *hrmA* locus associated with the *Pseudmonoas syringae* pv. *syringae* 61 *hrp* gene cluster. Mol. Plant-Microbe Interact. 6:553-564.

Hitchin, F. E., Jenner, C. E., Harper, S., Mansfield, J. W., Barber, C. E., and Daniels, M. J. 1989. Determinant of cultivar specific avirulence cloned from *Pseudomonas syringae* pv. *phaseolicola* race 3. Physiol. Mol. Plant Pathol. 34:309-322.

Huang, H.-C., Hutcheson, S. W., and Collmer, A. 1991. Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and Tn*phoA* tagging of genes encoding exported or membrane-spanning Hrp proteins. Mol. Plant-Microbe Interact. 4:469-476.

Huang, H.-C., Shuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel, I., Hutcheson, S. W., and Collmer, A. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco plants. J. Bacteriol. 170:4848-4756.

Huang, H.-C., Xiao, Y., Lin, R.-H., Lu, Y., Hutcheson, S. W., and Collmer, A. 1993. Characterization of the *Pseudomonas syringae* pv. *syringae* 61 *hrpJ* and *hrpI* genes: Homology of HrpI to a superfamily of

proteins associated with protein translocation. Mol. Plant-Microbe Interact. 6:515-520.

Huynh, T., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.

Innes, R. W., Bent, A. F., Kunkel, B. N., Bisgrove, S. R., and Staskawicz, B. J. 1993. Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. J. Bacteriol. 175:4859-4869.

Jenner, C., Hitchin, E., Mansfield, J., Walters, K., Betteridge, P., and Taylor, J. 1991. Gene-for-gene interactions between *Pseudomonas syringae* pv. *phaseolicola* and *Phaseolus*. Mol. Plant-Microbe Interact. 4:553-562.

Kado, C. I., and Liu, S. T. 1981. Rapid procedure for the detection and isolation of large and small plasmids. J. Bacteriol. 145:1356-1373.

Keen, N. T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. Annu. Rev. Genet. 24:447-463.

Keen, N. T., Tamaki, S., Kobayashi, D., Gerhold, D., Stayton, M., Shen, H., Gold, S., Lorang, J., Thordal-Christensen, H., Dahlbeck, D., and Staskawicz, B. 1990. Bacteria expressing avirulence gene D produce a specific elicitor of the soybean hypersensitive reaction. Mol. Plant-Microbe Interact. 3:112-121.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and flurescin. J. Lab. Clin. Med. 44:301-307.

Kobayashi, D., Tamaki, S., and Keen, N. T. 1990a. Molecular characteristization of avirulence gene D from *Pseudomonas syringae* pv. *tomato*. Mol. Plant Microbe Interact. 3:94-102.

Kobayashi, D., Tamaki, S., Trollinger, D. J., Gold, S., and Keen, N. T. 1990b. A gene from *Pseudomonas syringae* pv. *glycinea* with homology to avirulence gene D from *P. s.* pv. *tomato* but devoid of the avirulence genotype. Mol. Plant-Microbe Interact. 3:103-111.

Lamb, C. J., Lawton, M. A., Dron, M., and Dixon, R. A. 1989. Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell 56:215-224.

Leong, S., Ditta, G., and Helinski, D. 1982. Heme biosynthesis in *Rhizobium*. J. Biol. Chem. 257:8724-8730.

Lindgren, P. B., Frederick, R., Govindarajan, A. G., Panopoulos, N. J., Staskawicz, B. J., and Lindow, S. E. 1989. An ice nucleation reporter gene system: Identification of inducible pathogenicity genes in *Pseudomonas syringae* pv. *phaseolicola*. EMBO J. 8:1291-1301.

Lindgren, P. B., Panopoulos, N. J., Staskawicz, B. J., and Dahlbeck, D. 1988. Genes required for pathogenicity and hypersensitivity are conserved and interchangeable among pathovars of *Pseudomonas syringae*. Mol. Gen. Genet. 211:499-506.

Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168:512-522.

Lindsay, W. P., Lamb, C. J., and Dixon, R. A. 1993. Microbial recognition and activation of plant defense systems. Trends Microbiol 1:181-187.

Liu, Y.-N., Tang, J.-L., Clarke, B. R., Dow, J. M., and Daniels, M. J. 1990. A multipurpose broad host range cloning vector and its use to characterise an extracellular protease gene of *Xanthomonas campestris* pathovar *campestris*. Mol. Gen. Genet. 220:433-440.

Long, S. R., and Staskawicz, B. J. 1993. Parasites at the table. Cell,73:921-935.

Lorang, J. M., and Keen, N. T. 1995. Characterization of *Pseudomonas syringae* pv. *tomato avrE*: a *hrp*-linked locus. Mol. Plant-Microbe Interact. In press.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Martin, D. W., Holloway, B. W., and Deretic, V. 1993. Characterization of a locus determining the mucoid status of *Pseudomonas aeruginosa*: AlgU shows sequence similarities with a *Bacillus* sigma factor. J. Bacteriol. 175:1153-1154.

Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Miller, W., Mindrinos, M. N., Rahme, L. G., Frederick, R. D., Grimm, C., Gressman, R., Kyriakides, X., Kokkinidis, M., and Panopoulos, N. J. 1993. *Pseudomonas syringae* pv. *phaseolicola*-plant interactions: Host-pathogen signalling through cascade control of *hrp* gene expression. Pages 267-274 in: Advances in Molecular Genetic of Plant-Microbe Interactions. Vol. 2. E. W. Nester and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht.

Mitchell, R. E. 1978. Halo blight of beans: Toxin production by several *Pseudomonas phaseolicola* isolates. Physiol. Plant Pathol. 13:37-49.

Mitchell, R. E. 1984. The relevance of non-host specific toxins in the expression of virulence by pathogens. Annu. Rev. Phytopathol. 22:215-245.

Moore, R. E., Niemeczura, W. P., Kwork, O. C. H., and Patil, S. S. 1984. Inhibitors of ornithine carbamoyltransferase from *Pseudomonas syringae* pv. *phaseolicola*. Revised structure of phaseolotoxin. Tetrahedron Lett. 25:3931-3934.

Murray, N. E., Brammar, W. J., and Murray, K. 1977. Lambdoid phages that simplify the recovery of *in vitro* recombinants. Mol. Gen. Genet. 150:53-61.

Pogliano, J. A., and Beckwith, J. 1994. SecD and SecF facilitate protein export in *Escherichia coli*. EMBO J. 13:554-561.

Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. 1991. Genetic and transcriptional organization of the *hrp* cluster of *Pseudomonas syringae* pv. *phaseoliocla*. J. Bacteriol. 173:575-586.

Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. 1992. Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola*. J. Bacteriol. 174:3499-3507.

Salmeron, J. M., and Staskawicz, B. J. 1993. Molecular characterization and *hrp*-dependence of the avirulence gene *avr*Pto from *Pseudomonas syringae* pv. *tomato*. Mol. Gen. Genet. 239:6-10.

Salmond, G. P. C., and Reeves, P. J. 1993. Membrane traffic wardens and protein secretion in gram-negative bacteria. Trends Biochem. Sci.18:7-12.

Sanger, F., Nicklin, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

Shen, H., and Keen, N. T. 1993. Characterization of the promoter of avirulence gene D from *Pseudomonas syringae* pv. *tomato*. J. Bacteriol. 175:5916-5924.

Shintaku, M. H., Kluepfel, D. A., Yacoub, A., and Patil, S. S. 1989. Cloning and characterization of an avirulence determinant from race 1 of *Pseudomonas syringae* pv. *phaseolicola*. Physiol. Mol. Plant Pathol. 35:313-322.

Smith, J. J., and Mansfield, J. W. 1981. Interactions between pseudomonads and leaves of oats, wheat and barley. Physiol. Plant Pathol. 18:345-356.

Smith, M. J., Mazzola, E. P., Sims, J. J., Midland, S. L., Keen, N. T., Burton, V., and Stayton, M. M. 1993. The syringolides: Bacterial C-glycosyl lipids that trigger plant disease resistance. Tetrahedron Lett. 34:223-226.

Stachel, S. E., An, G., Flores, C. W., and Nester, E. W. 1985. A Tn*31acZ* transposon for the random generation of β-galactosidase gene fusions: Application to the analysis of gene expression in *Agrobacterium*. EMBO J. 4:891-898.

Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:5789-5794.

Tamaki, S., Dahlbeck, D., Stasckawicz, B., and Keen, N. T. 1988. Characterization and expression of two avirulence genes cloned from *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 170:4846-4854.

Taylor, J. D., Teverson, D. M., Allen, D. J., and Pastor-Corrales, M. A. 1995a. Pathogenic variation in *Pseudomonas syringae* pv. *phaseolicola*. I. Identification and origin of races from Africa and other bean growing areas. Plant Pathol. In press.

Taylor, J. D., Teverson, D. M., and Davis, J. H. C. 1995b. Pathogenic variation in *Pseudomonas syringae* pv. *phaseolicola*. II. Sources of resistance in *Phaseolus vulgaris*. Plant Pathol. In press.

Teverson, D. M. 1991. Genetics of pathogenicity and resistance in the halo-blight disease of beans in Africa. PhD. thesis, University of Birmingham, U.K.

Vivian, A., and Mansfield, J. 1993. A proposal for a uniform genetic nomenclature for avirulence genes in phytopathogenic pseudomonads. Mol. Plant-Microbe Interact. 6:9-10.

Willis, D. K., Rich, J. J., and Hrabak, E. M. 1991. *hrp* genes of phytopathogenic bacteria. Mol. Plant-Microbe Interact. 4:132-138.

Wood, J. R., Vivian, A, Jenner, C. J., Mansfield, J. W., and Taylor, J. D. Detection of a gene in pea controlling nonhost resistance to *Pseudomonas syringae* pv. *phaseolicola*. Mol. Plant-Microbe Interact. 7:534-

537.

Xiao, Y., Heu, S., Yi, J., Lu, Y., and Hutcheson, S. W. 1994. Identification of a putative alternative sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. J. Bacteriol. 176:1025-1036.

Xiao, Y. and Hutcheson, S.W. 1994. A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. J. Bacteriol. 176:3089-3091.

Xiao, Y., Lu, Y., Heu, S., and Hutcheson, S. W. 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. J. Bacteriol. 174:1734-1741.

Yucel, I., Boyd, C., Debnam, Q., and Keen, N. T. 1994a. Two different classes of *avrD* alleles occur in pathovars of *Pseudomonas syringae*. Mol. Plant-Microbe Interact. 7:131-139.

Yucel, I., Slaymaker, D., Boyd, C., Murillo, J., Buzzell, R. I. and Keen, N. T. 1994. Avirulence gene *avrPphC* from *Pseudomonas syringae* pv. *phaseolicola* 3121: a plasmid-borne homologue of *avrC* closely linked to an *avrD* allele. Mol. Plant-Microbe Interact. 7:677-679.

Zhang, Y., Rowley, K. B., and Patil, S. S. 1993. Genetic organization of a cluster of genes involved in the production of phaseolotoxin, a toxin produced by *Pseudomonas syringae* pv. *phaseolicola*. J. Bacteriol. 175:6451-6458.