MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 3, No. 2, pp. 103-111, 1990

A Gene from *Pseudomonas syringae* pv. *glycinea* with Homology to Avirulence Gene D from *P. s.* pv. *tomato* but Devoid of the Avirulence Phenotype

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Received 31 July 1989. Accepted 3 November 1989.

A gene was cloned from *Pseudomonas syringae* pv. *glycinea* that hybridized to avirulence gene D (*avrD*), previously cloned from *P. s.* pv. *tomato*. Unlike *avrD*, the hypersensitive response (HR) was not elicited when the *P. s.* pv. *glycinea* gene was reintroduced into *P. s.* pv. *glycinea* race 4 on a broad host range plasmid and the bacteria were inoculated into soybean leaves. DNA sequence data disclosed that the *P. s.* pv. *glycinea* homologue of *avrD* encoded a protein containing 86% identical amino acids to *avrD*, with substitutions distributed throughout the protein. Two ORFs immediately downstream from the *avrD* homologue were more similar in *P. s.* pv. *tomato* and *P. s.* pv. *glycinea*, with 98 and 99% identical amino acids. Expression of the wild-type *P. s.* pv. *glycinea* gene and recombinant genes constructed between the *P. s.* pv. *tomato avrD* gene and its *P. s.* pv. *glycinea* homologue in both *Escherichia coli* and *P. s.* pv. *glycinea* indicated that the *P. s.* pv. *glycinea* gene product was formed less efficiently or was less stable than was the *P. s.* pv. *tomato* protein encoded by *avrD*. The data indicated that the *P. s.* pv. *glycinea* homologue represents a recessive allele of the *P. s.* pv. *tomato avrD* gene which has been modified by mutation such that it does not lead to an avirulence phenotype on the normal host plant, soybean.

Additional keywords: gene-for-gene complementarity.

Genetic studies in plant-pathogen interactions have established a gene-for-gene relationship in which disease resistance is often governed by a single dominant gene for disease resistance in the host plant and a single dominant gene for avirulence in the pathogen (Day 1974; Ellingboe 1976; Flor 1942). This gene-for-gene complementarity is the genetic basis for specificities that occur within plant and pathogen populations, resulting in the taxonomic grouping of pathogen races according to differences in their ability to reproduce on different resistance genotypes of the host plant. The occurrence of disease resistance requires that a dominant gene for avirulence in the pathogen and its corresponding plant disease resistance gene are both present in the interacting organisms. This results in the induction of the plant hypersensitive response (HR), which is characterized by the rapid necrosis of host cells surrounding the pathogen, followed by the accumulation of antimicrobial compounds called phytoalexins (Keen and Holliday 1982; Klement 1982).

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Nucleotide and/or amino acid sequence data is to be submitted to GenBank as accession number JO3682.

Plant disease resistance genes have not been isolated and characterized, but several avirulence genes have been cloned from bacterial pathogens in both the *Pseudomonas syringae* van Hall and *Xanthomonas campestris* (Pammel) Dowson pathovar groups (reviewed by Keen and Staskawicz 1988). Three avirulence genes from *P. s.* pv. *glycinea* (Coerper) Young *et al.* have been molecularly characterized and each encodes a single polypeptide product (Napoli and Staskawicz 1987; Tamaki *et al.* 1988).

None of the avirulence genes thus far cloned from *P. s.* pv. *glycinea* have homologous sequences in other *P. s.* pv. *glycinea* races not expressing the phenotype conferred by the avirulence gene, indicating the absence of alternative alleles. However, such homology has been reported with avirulence genes cloned from *X. c.* pv. *malvacearum* (Smith) Dye (Gabriel *et al.* 1986). These observations suggest the occurrence of recessive alleles for certain avirulence genes, consistent with indications from classical genetic studies of fungal pathogens (Day 1974). Although phenotypic functions have not been associated with recessive alleles of avirulence genes, comparative studies of such alleles may provide useful information for determining the function of avirulence genes and the mechanisms of HR elicitation and pathogen escape.

Avirulence genes modulating race specificity on soybean cultivars have previously been cloned from *P. s.* pv. *tomato* (Okabe) Young *et al.*, the causal agent of bacterial speck of tomato (Kobayashi *et al.* 1989). One gene was found to be indistinguishable from *avrA* in *P. s.* pv. *glycinea* race 6, but two other avirulence genes were unique in their phenotypic patterns of the HR elicited on several soybean cultivars (Kobayashi *et al.* 1989; H. Shen and N. T. Keen, unpublished). One of these genes, designated *avrD*,

occurred on an indigenous plasmid in *P. s.* pv. *tomato* (Kobayashi *et al.* 1990) and encoded a protein with a molecular mass of 34 kDa. Southern blot analyses disclosed that this DNA fragment was conserved among several pathovars in the *P. syringae* group, including all tested races of *P. s.* pv. *glycinea*. However, hybridization data using a gene-specific probe to *avrD* indicated divergence in the sequence of a homologous gene present in four *P. s.* pv. *glycinea* races. These observations suggested that *P. s.* pv. *glycinea* contained a nonfunctional allele of *avrD*. In this research article, we report the characterization of this *P. s.* pv. *glycinea* gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used or constructed in this study are listed in Table 1. *Escherichia coli* was grown at 37° C on Luria-Bertani (LB) agar medium or in LB broth (Maniatis *et al.* 1982), and *P. syringae* strains were grown at 28° C on King's medium B (KMB) agar or in KMB broth in shaken culture (King *et al.* 1954). Antibiotics were used at the following concentrations unless otherwise noted: ampicillin, 50 μg/ml; kanamycin, 25 μg/ml; rifampicin, 100 μg/ml; and tetracycline, 25 μg/ml.

DNA manipulations, library construction, and colony hybridizations. Standard recombinant DNA methods were performed as described by Maniatis *et al.* (1982). Plasmids were generally constructed following the isolation of defined DNA fragments from low melting point agarose gels (Crouse *et al.* 1983). Total DNA from *P. s.* pv. *glycinea* race 4 was isolated as described by Staskawicz *et al.* (1984). A DNA library of *P. s.* pv. *glycinea* race 4 DNA was constructed by gel-eluting 10 μg of 4- to 6-kilobase (kb) fragments from *Hin*dIII-digested total DNA and ligating the purified DNA into the same site of pUC118.

Table 1. Bacterial strains, bacteriophage, and plasmids used in this study

Designation	Relevant characteristics[a]	Source or reference
Escherichia coli		
DH5α	F⁻ *lacZ* M15 *endA1 hsdR17 supE44 thi-1 gyrA relA1*⁻	Bethesda Research Laboratories, Gaithersburg, MD
MV1193	(*lac-proAB*) *thi supE44* (*srl-recA*) *306*::Tn*10* (Tc^r) (*proAB lacZ M15*)	Vieira and Messing 1987
BMH 71-18 *mutS*	K12 Δ (*lac-proAB*) *supE thi* F′ *proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15 *mutS*::Tn*10*	Kramer *et al.* 1984
Pseudomonas syringae pathovars		
P. s. pv. *glycinea* race 4	*rif*^{r}, *ap*^{r}	Kobayashi *et al.* 1989
P. s. pv. *tomato* PT23		Kobayashi *et al.* 1989
Phage		
M13K07	Helper phage	Vieira and Messing 1987
Plasmids		
pUC118/pUC119	Ap^r cloning and sequencing vectors	Vieira and Messing 1987
pUC128/pUC129	Ap^r cloning and sequencing vectors	Keen *et al.* 1988
pRK415	Tc^r broad host range vector, mob$^+$	Keen *et al.* 1988
pDSK519	Km^r broad host range vector, mob$^+$	Keen *et al.* 1988
pRK2013	Km^r, Tra$^+$, helper plasmid	Ditta *et al.* 1980
pPTD1121	*avrD* contained in deletion A20 in pUC119	Kobayashi *et al.* 1989
pPSG4000	5.6-kb *Hin*dIII fragment in pUC119 from *P. s.* pv. *glycinea* race 4 isolated by colony hybridization	This study
pPSG4001	3.2-kb *Hin*dIII-*Bam*HI fragment containing *P. s.* pv. *glycinea* ORF 1 positioned downstream from the vector *lac* promoter in pUC128	This study
pPSG4002	3.2-kb *Hin*dIII-*Bam*HI fragment in pUC129, the opposite orientation to pPSG4001	This study
pPSG4005	1.25-kb fragment from deletion GA11 containing *P. s.* pv. *glycinea* ORF 1 positioned downstream from the vector *lac* promoter in pUC128	This study
pPSG4006	Same as pPSG4005 except opposite orientation in pUC129	This study
pPAVRD5	Approximately 1.2-kb *Hin*dIII - *Eco*RI insert from pPTD1121 cloned in pUC128 in the orientation opposite to the vector *lac* promoter	Keen *et al.* 1990
pAVRD5-0	Same as pAVRD5, except an *Eco*RI site was inserted immediately before the translational start by site-directed mutagenesis	This study
pAVRD10	*Eco*RI insert from pAVRD5-0 cloned downstream of the vector promoter in pUC129	Keen *et al.* 1990
pPSGOR	Same as pPSG4006, except an *Eco*RI site was inserted immediately before the translational start by site-directed mutagenesis	This study

[a]Ap, ampicillin; Km, kanamycin; Rif, rifampicin; Tc, tetracycline; ^{r}, resistance; mob$^+$, mobility factor; kb, kilobase; and ORF, open-reading frame.

Transformed *E. coli* strain DH5α cells were plated on LB agar medium supplemented with ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and IPTG (isopropyl-β-D-thiogalactopyranoside), and white colonies were selected to comprise the library.

Oligonucleotide mutagenesis. The oligonucleotide (5'-AAGGAATGAATTCATGCCAAG-3') used for introducing an *Eco*RI site into *avrD* and the *P. s.* pv. *glycinea* homologue was synthesized by the Biotechnology Instrumentation Facility (University of California, Riverside). Site-directed mutagenesis was performed essentially as described by Kramer and Fritz (1987). Single-stranded DNA from pAVRD5 (containing *avrD*) or pPSG4006 (containing the *P. s.* pv. *glycinea* analogue, Table 1) was prepared by superinfecting *E. coli* strain MV1193 with phage M13K07 and isolating the single-stranded DNA by the method of Vieira and Messing (1987). Following annealing with the oligonucleotide and second strand production with the Klenow fragment, the DNA was transformed into *E. coli* strain BMH 71-18 *mutS* (Table 1). Colonies were miniscreened by extracting plasmid DNA by the rapid boil method (Crouse *et al.* 1983) and digesting it with *Eco*RI. Colonies that yielded plasmids containing two *Eco*RI sites (unlike the parent plasmids that contained a single site) were retained, and these plasmids were transformed into *E. coli* strain MV1193.

The mutated sites were confirmed by DNA sequencing of the appropriate regions. The sequence of *avrD* from *P. s.* pv. *tomato* was accordingly changed from GTATC-CATG (where ATG is the translational start codon) to GAATTCATG; the corresponding sequence of the *P. s.* pv. *glycinea avrD* homologue was changed from GTATG-CATG to GAATTCATG. The final plasmids were designated pAVRD5-0 (in the case of *avrD*) and pPSGOR (in the case of the homologous gene from *P. s.* pv. *glycinea*).

Sequence analysis. The 3.2-kb *Hin*dIII-*Bam*HI fragment from *P. s.* pv. *glycinea* race 4 containing the region homologous to *avrD* was recovered from the initial library clone as a *Bam*HI fragment and recloned into pUC128 in both orientations, yielding pPSG4001 and pPSG4002. Exonuclease III reactions were then performed on both ends of this fragment according to the method described by Henikoff (1984). Both strands of the fragment were sequenced by the dideoxy chain termination method (Sanger *et al.* 1977). Sequence analysis and comparisons were performed using the Bionet system supplied by

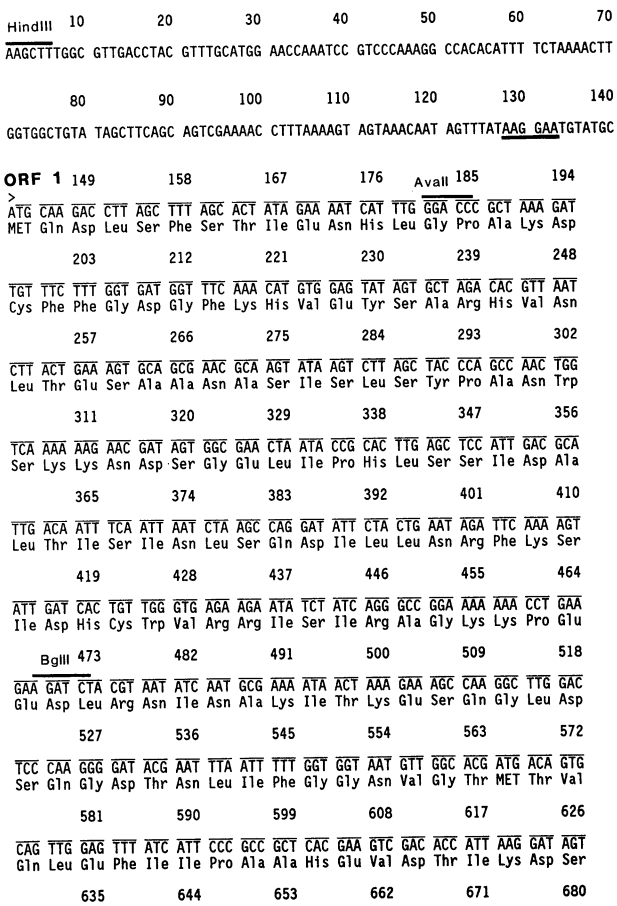

Fig. 1. Nucleotide sequence determined for the 3.2-kilobase *Hin*dIII-*Bam*HI fragment cloned from *Pseudomonas syringae* pv. *glycinea* race 4, with translated sequences of open-reading frames (ORFs) 1–3. Presumed ribosome binding sites are noted as underscores and selected restriction enzyme sites as overscores.

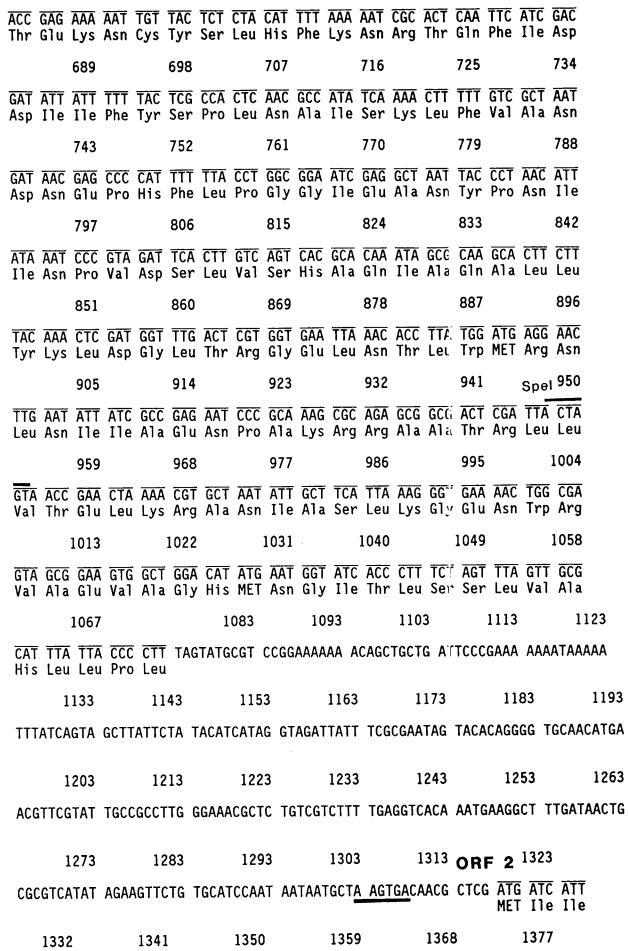

Fig. 1 *continued on next page.*

Intelligenetics Corporation, Mountain View, CA.

Construction of recombinant genes. An exonuclease III deletion generated for use as a sequencing template was selected; it had a 3′ end point at base 1083, 10 bases downstream from the termination codon of open-reading frame (ORF) 1 (Fig. 1). This deletion, labeled GA11, was positioned downstream from the *lac* promoter of pUC128 to generate pPSG4005, which was used for constructing recombinant genes. Similarly, pPTD1121, which contains deletion A20 of *avrD* in pUC119 (Kobayashi *et al.* 1990), was also used. Recombinant genes were then constructed by performing reciprocal exchanges at three conserved restriction sites (*Ava*II, *Bgl*II, and *Spe*I) located in both genes. Similar exchanges were performed at an *Eco*RI site introduced just ahead of the start codons (pAVRD5-0 and pPSGOR) by oligonucleotide, site-directed mutagenesis. All wild-type and recombinant genes were recloned into the broad host range plasmids pRK415 or pDSK519 before introduction into *P. s.* pv. *glycinea* race 4 by conjugation.

Bacterial conjugations, plant growth conditions, and inoculations. Bacterial conjugations were performed as previously described (Ditta *et al.* 1980; Kobayashi *et al.* 1989). Transconjugants were successively streaked on KMB agar supplemented with 100 μg/ml of rifampicin, 25 μg/ml of tetracycline, and 25 μg/ml of ampicillin. Water suspensions (10^7 cells per milliliter) of resultant single colony bacterial isolates were prepared from cells grown on KMB agar or in broth.

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GTC ATT ATC CGA CAT GGT GAA ACG CCA CAA AAT TTG CTT GGC GTT TTT CAG GGA
Val Ile Ile Arg His Gly Glu Thr Pro Gln Asn Leu Leu Gly Val Phe Gln Gly

   1386        1395        1404        1413        1422        1431

CAG TCT GAT CCT GAG CTG GAT AAC GTA GGG ATA GAT CGG TTC AAG GAC ACT GCC
Gln Ser Asp Pro Glu Leu Asp Asn Val Gly Ile Asp Arg Phe Lys Asp Thr Ala

   1440        1449        1458        1467        1476        1485

AGA ACG CTT AAG AAT GAG AAG TGG GAT GCT ATT TAT AGC TCT AAC TAT AAA CGC
Arg Thr Leu Lys Asn Glu Lys Trp Asp Ala Ile Tyr Ser Ser Asn Tyr Lys Arg

   1494        1503        1512        1521        1530        1539

TCA CTG GTT TCC GCA AAT CTT TTG ACA GTT GAC GTT AAC TTG AGA AGA TTT GTT
Ser Leu Val Ser Ala Asn Leu Leu Thr Val Asp Val Asn Leu Arg Arg Phe Val

   1548        1557        1566        1575        1584        1593

TCC ACA GAC TTT TCG GAG CGC CAC TTG GGC GCA CTG GAC GGT AAG TCT AAG GAG
Ser Thr Asp Phe Ser Glu Arg His Leu Gly Ala Leu Asp Gly Lys Ser Lys Glu

   1602        1611        1620        1629        1638        1647

CTT CTT ATA TCT GCT GAT CCT GAG TTA TCG AGA AAG TTA ATA ACA CTT GAG TAT
Leu Leu Ile Ser Ala Asp Pro Glu Leu Ser Arg Lys Leu Ile Thr Leu Glu Tyr

   1656        1665        1674        1683        1692        1701

ACC CCA TCC GGA GGG GAG TCG GGT CGT TCA GCG TTG GAG CGT TTT GTC CGT GGA
Thr Pro Ser Gly Gly Glu Ser Gly Arg Ser Ala Leu Glu Arg Phe Val Arg Gly

   1710        1719        1728        1737        1746        1755

ATA CAT ACT ATT AAA AAT AAT CAC CAA GGC CGG GTT ATT GTT GTT TCT CAT GGA
Ile His Thr Ile Lys Asn Asn His Gln Gly Arg Val Ile Val Val Ser His Gly

   1764        1773        1782        1791        1800        1809

GGT ATT GTG GCA CTT TTT GCT CAC CAC ATG TTA GGG GTG CGA CAG ACT TCC TGC
Gly Ile Val Ala Leu Phe Ala His His MET Leu Gly Val Arg Gln Thr Ser Cys

   1818        1827        1836        1845        1854        1863

CTT CTA GAG CAT GGT CAT GCC CTA ATA ATA AAG GTC TCA GGG ACT GAA ATT AGT
Leu Leu Glu His Gly His Ala Leu Ile Ile Lys Val Ser Gly Thr Glu Ile Ser

   1872        1881        1890        1899        1908        1917

TTG ATA GGC ATG AAT GTT CCA CCC AAT TCA ATA GCG GAA GCG ACG TAC TAT GGA
Leu Ile Gly MET Asn Val Pro Pro Asn Ser Ile Ala Glu Ala Thr Tyr Tyr Gly

   1926        1935        1944        1953        1962        1972        1982

AAA TAT CTT GAC AAG GGA TTC ATG GGG CAG TGG GAG AGC ATC TAGAAAAATC AGATGCCCCG
Lys Tyr Leu Asp Lys Gly Phe MET Gly Gln Trp Glu Ser Ile

      1992        2002        2012        2022        2032        2042        2052
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Fig. 1 *continued.*

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TCACCGGCTC AGCTGACACA TGACAGCGTG ATGACCAAGC AGGGACTTTA TTTCAGAGGG GTGTAAATAG

      2062        2072        2082        2092        2102        2112        2122

AGTCGGTACT CGCGTGCTTA GCCTTTGGAG CCTACGAAAG CGAACGAGCA CTCAATTAAT GGTTTTCGAT

      2132        2142        2152        2162        2172  ORF 3 2182

CATGAGGCAA TCCATGCAAG CGCTTCTAAA CTAACTTTTT TATATGGGAT AGGT ATG CAA AGC
                                                          MET Gln Ser

   2191        2200        2209        2218        2227        2236

CGA TTC AAT GGA TGG TCA ATG CAG GTT CTT GAG GTG GAT GAT ACG GCA GCG GTT
Arg Phe Asn Gly Trp Ser MET Gln Val Leu Glu Val Asp Asp Thr Ala Ala Val

   2245        2254        2263        2272        2281        2290

GGT CGA CAT ATT GAT CAG TTT GGT TTC GCG ATC GTT TCG GGG GAA TGG AGA TTC
Gly Arg His Ile Asp Gln Phe Gly Phe Ala Ile Val Ser Gly Glu Trp Arg Phe

   2299        2308        2317        2326        2335        2344

GAT GCG TCT GAT TTT GAC CGC ATG GCC GCA CTT TAC GGC TTG GGC CCA ATG TAC
Asp Ala Ser Asp Phe Asp Arg MET Ala Ala Leu Tyr Gly Leu Gly Pro MET Tyr

   2353        2362        2371        2380        2389        2398

CAG TCG GAT TTC AAC CGG CTT GAG CAT GCA GAA GGC ATA GCA TCA TCG GGA ATT
Gln Ser Asp Phe Asn Arg Leu Glu His Ala Glu Gly Ile Ala Ser Ser Gly Ile

   2407        2416        2425        2434        2443        2452

AAC CAG GTC GGA GGT CTG TCG AGC GGC AGC CAT GTC GTG TTC AAC GGC GCT ACA
Asn Gln Val Gly Gly Leu Ser Ser Gly Ser His Val Val Phe Asn Gly Ala Thr

   2461        2470        2479        2488        2497        2506

GAC GTG CCG CTT CAT ACC GAT GGT TCC TAT TTA CCT ATA GGC ACC ATC AAG ACG
Asp Val Pro Leu His Thr Asp Gly Ser Tyr Leu Pro Ile Gly Thr Ile Lys Thr

   2515        2524        2533        2542        2551        2560

TCG ATC CTC TTT TGT AGA GAA TCT GCG GCT CTC GGC GGG GAG TCC ATT CTG TTC
Ser Ile Leu Phe Cys Arg Glu Ser Ala Ala Leu Gly Gly Glu Ser Ile Leu Phe

   2569        2578        2587        2596        2605        2614

GAT AGC GTG TCG GCA TTT CGA GCA CTG AGC GAG GAT CAT CCT GAT CTT GCT CGG
Asp Ser Val Ser Ala Phe Arg Ala Leu Ser Glu Asp His Pro Asp Leu Ala Arg

   2623        2632        2641        2650        2659        2668

TCC TTG CTC GCC GAT AAT GCG TTC AGG CGC CGA TCT ACT AGT ACG CGT TCG GGT
Ser Leu Leu Ala Asp Asn Ala Phe Arg Arg Arg Ser Thr Ser Thr Arg Ser Gly

   2677        2686        2695        2704        2713        2722
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Fig. 1 *continued.*

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AGG CAG TAT CAA CAC ATT GGG CCG ATG TTT CTT CGT CGC GAA GAC GGA GAT ATT
Arg Gln Tyr Gln His Ile Gly Pro MET Phe Leu Arg Arg Glu Asp Gly Asp Ile

   2731        2740        2749        2758        2767        2776

GTT GGC GGC TTC ACG CTC GAT ATC ACG GCT GAC TGG GAA TAC TCG CGT CGT ATG
Val Gly Gly Phe Thr Leu Asp Ile Thr Ala Asp Trp Glu Tyr Ser Arg Arg MET

   2785        2794        2803        2812        2821        2830

GAC GCA CGG GTG ATT GAC GCA GCG GCG TAT CTC ATC CGG CTC GCC TCC GAA AAC
Asp Ala Arg Val Ile Asp Ala Ala Tyr Leu Ile Arg Leu Ala Ser Glu Asn

   2839        2848        2857        2866        2875        2884

AGC GAT TAC ACT CTG AAG TTT GGG TTG CAT AAA GGG CAG GTG CTA ATT ATA CGA
Ser Asp Tyr Thr Leu Lys Phe Gly Leu His Lys Gly Gln Val Leu Ile Ile Arg

   2893        2902        2911        2920        2929        2938

AAC GAC CAG CTG TCG CAT GGT CGA TGC TCA TAT GTC GAC GAC CCT GCC AGG CCT
Asn Asp Gln Leu Ser His Gly Arg Cys Ser Tyr Val Asp Asp Pro Ala Arg Pro

   2947        2956        2965        2974        2983        2992

CGA ATC CTG TTT CGA GGA CTC TTT CTG TCC TCA CCA TGC GAT TCT GGT GCA CCA
Arg Ile Leu Phe Arg Gly Leu Phe Leu Ser Ser Pro Cys Asp Ser Gly Ala Pro

   3001        3010        3019        3028        3038        3048        3058

ACA GAC TTG GTC TGT ACC CGA GGT AGC CAA TCT TGACTGAGGG AATGTCACAT ATGCCGGAGC
Thr Asp Leu Val Cys Thr Arg Gly Ser Gln Ser

      3068        3078        3088        3098        3108        3118  BamHI

AAGATGCGGG GGCGCAGTGG GCCTCATGGA TATCGTGTCG CGCCAGTCAG CTTAAGTCGC GGATCC
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Fig. 1 *continued.*

Soybean plants were grown from seed as previously described (Long *et al.* 1985). Inocula were infiltrated into fully expanded primary leaves of soybeans using the device of Hagborg (1970), and the plants were incubated at 21° C for a 16-hr photoperiod. Plants were screened daily from 1 to 5 days for the appearance of a visible HR or water-soaked lesions.

Antibodies to the *avrD*-encoded protein. *E. coli* strain DH5α cells carrying pAVRD10 (Keen *et al.* 1990) were grown in shaken LB broth cultures amended with 50 μg/ml of ampicillin and 1 mM IPTG for 16 hr at 28° C. Cells were pelleted, washed, and finally resuspended in 2 ml of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA per 15 ml starting culture. The cell suspension was lysed and boiled in an equal volume of 2× sample buffer, and 25 μl was then applied to 12% polyacrylamide gels and run according to Laemmli (1970). Proteins from cells containing pAVRD10 or pUC129 were electrophoresed for 6 hr at 35 mA on a Bio-Rad (Richmond, CA) Protein II unit at room temperature. Gels were then negatively stained with 4 M sodium acetate. Clear protein bands were visualized against a milky background, and the 34-kDa *avrD*-encoded protein product was identified by comparison with the vector only negative control. The *avrD*-encoded protein band was excised from all but one lane, and the remaining gel was stained with Coomassie Brilliant Blue R 250 to confirm that the correct band had been removed. The pooled polyacrylamide slices were rinsed for 10 min in distilled water and stored at −20° C for future use.

Gel segments containing approximately 50 μg of protein were thawed and finely ground in 1 ml of phosphate-buffered saline. An emulsion of equal parts polyacrylamide slurry and Freund's adjuvant was then produced. Freund's complete adjuvant was used in the primary injection, followed by incomplete adjuvant in all subsequent inoculations. Preimmune serum was collected from a female New Zealand white rabbit at the time of the primary inoculation. Intramuscular injections were performed weekly for 10 wk and bleedings were made biweekly.

Antibody production and specificity were followed by the standard indirect ELISA procedure (Engvall and Perlmann 1972). Antigen was prepared from lysed JM109 cells containing pAVRD10 or pUC129 (vector control) and coated onto microtiter dishes. Following washing and exposure to sera, goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) was used, and the resulting color was read at 405 nm. The results showed that serum collected at 10 wk after the initial inoculation of rabbits was most specific when diluted 1:6,400.

Electrophoresis of whole cell proteins and western blots. *E. coli* or *P. s.* pv. *glycinea* cells containing the desired plasmids were grown as described above, and whole cell proteins were run on 10 or 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels. The gels were blotted onto nitrocellulose membranes to transfer proteins, and these were exposed to the anti-AvrD antibody diluted 1:1,000 for 45–90 min at room temperature. Blots were washed twice with 10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.1% Tween 20 (TBS-Tween). Following final washes with 10 mM Tris-HCl, pH 7.4, 1 M NaCl, 0.5% Tween 20 (HST), and TBS-Tween, the blots were exposed to anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, A-8025) for 45–90 min at room temperature. The blots were washed and developed according to the manufacturer's recommendations and dried.

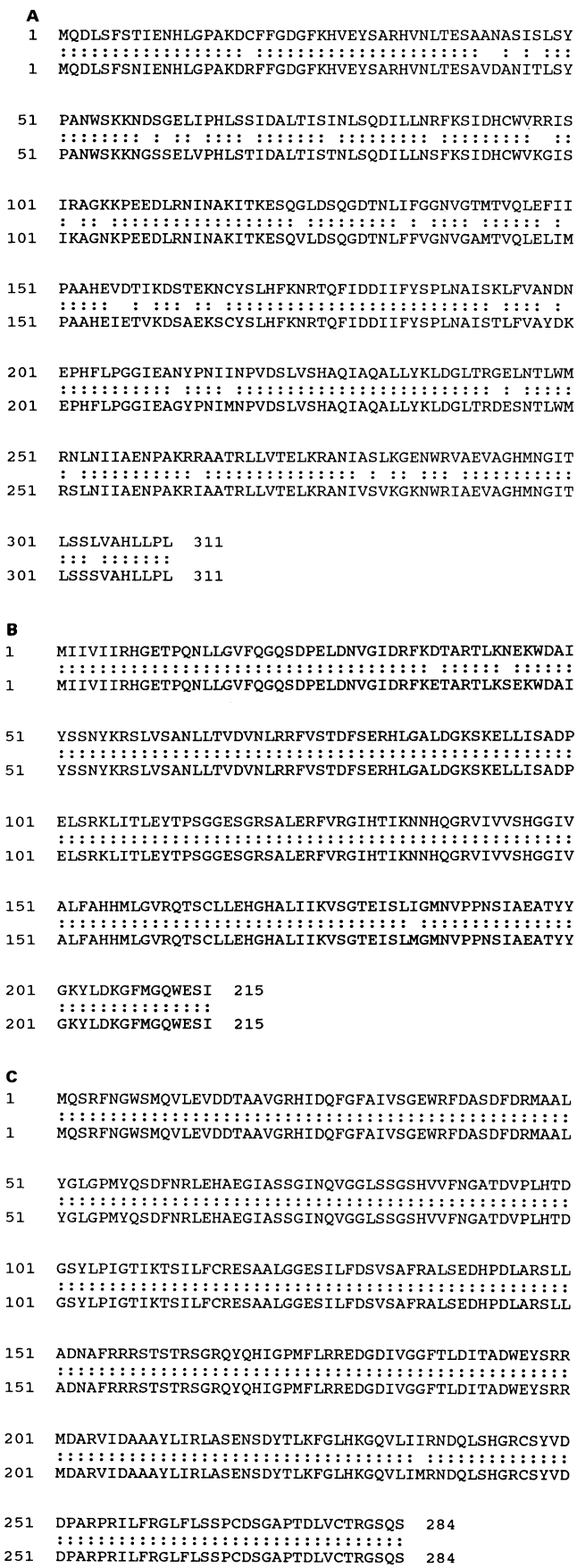
RESULTS

Cloning from *P. s.* pv. *glycinea* race 4 of a 5.6-kb *Hin*dIII fragment homologous to *avrD*. Three hundred ampicillin-resistant *E. coli* colonies were selected to comprise the library of 4- to 6-kb *Hin*dIII fragments from *P. s.* pv. *glycinea* race 4 cloned in pUC118. Colony hybridizations using the 5.6-kb *Hin*dIII fragment of *P. s.* pv. *tomato* DNA containing the *avrD* gene (Kobayashi *et al.* 1989) as a probe identified a single hybridizing colony that contained a cloned 5.6-kb *Hin*dIII fragment. Characterization of the hybridizing clone by Southern blot analyses and restriction digests indicated that this clone was similar to the 5.6-kb *Hin*dIII fragment from *P. s.* pv. *tomato* (data not shown).

DNA sequence analysis of the *Hin*dIII-*Bam*HI fragment. To further compare the cloned fragment from *P. s.* pv. *glycinea* race 4 to *avrD* from *P. s.* pv. *tomato*, a 3.2-kb *Hin*dIII-*Bam*HI fragment from *P. s.* pv. *glycinea* containing the region homologous to *avrD* was sequenced. These data revealed the presence of three ORFs, designated ORFs 1, 2, and 3 according to their order on one strand (Fig. 1). Each ORF was homologous to ORFs previously observed in the corresponding DNA fragment from *P. s.* pv. *tomato* (Kobayashi *et al.* 1990), and all three ORFs were preceded by sequences expected to function as ribosome binding sites (Shine and Dalgarno 1974). No ORF of significant length was observed on the opposite reading strand. The three ORFs did not show significant homology to known proteins when data base searches were performed using Bionet.

The translational start codon for ORF 1 was located at base 141 and terminated at base 1073. It could encode a protein of 311 amino acids with a molecular mass of 34,521 Da and a computer-calculated pI of 6.6. The presumed ATG start codon for ORF 2 was located at base 1318, 245 bases from the translational terminator of ORF 1; ORF 2 could encode a protein of 215 amino acids with a calculated molecular mass of 23,843 Da. ORF 3 began at base 2177, 215 bases from the termination of ORF 2, and could encode a protein of 284 amino acids with a molecular mass of 31,248 Da. Signal peptide sequences were not present in any of the three ORFs, and no significant regions of hydrophobicity indicative of membrane-spanning regions were evident from hydropathy plots using the algorithm of Kyte and Doolittle (1982) (data not shown).

Comparison of ORFs 1, 2, and 3 and untranslated 5′ DNA from *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*. Sequence comparisons of ORF 1 from *P. s.* pv. *glycinea* to *avrD* (Kobayashi *et al.* 1990) indicated that the two genes were greater than 93% homologous on the DNA level and shared



86% identical amino acids with mismatch substitutions dispersed throughout the proteins (Fig. 2A). Comparison of ORFs 2 and 3 indicated that the sequences from *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* shared 98% (ORF 2) and 99% (ORF 3) identical amino acids (Fig. 2, parts B and C). This high degree of homology showed that less divergence had occurred between ORFs 2 and 3 of *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* than was observed between ORF 1 and *avrD*. Furthermore, a deletion of approximately 100 base pairs occurred in the intergenic DNA between ORFs 1 and 2 in the *Hin*dIII-*Bam*HI fragment from *P. s.* pv. *glycinea* relative to that from *P. s.* pv. *tomato* (Figs. 1 and 2, Kobayashi *et al.* 1990).

A 10-base deletion, relative to the *P. s.* pv. *tomato avrD* gene, occurred in the 5′ noncoding DNA sequence of ORF 1 from *P. s.* pv. *glycinea* race 4, beginning at base 57 (Fig. 3). The deletion occurred in an area that has been implicated in regulating expression of the *P. s.* pv. *tomato avrD* gene (Kobayashi *et al.* 1990). This region in the *P. s.* pv. *tomato avrD* gene is characterized by an AT-rich palindrome of 18 base pairs that is not present in the *P. s.* pv. *glycinea* DNA (Fig. 3). However, the sequence GCCACACA, which occurs immediately 5′ to the AT-rich palindrome in *avrD* and also occurs in the 5′ regions of *avrA* and *avrB* (Kobayashi *et al.* 1990), was present in the 5′ DNA of the *P. s.* pv. *glycinea* gene.

Recombinant gene constructions. Recombinant genes between *avrD* and ORF 1 from *P. s.* pv. *glycinea* race 4 were constructed at three different restriction sites (*Ava*II, *Bgl*II, and *Spe*I) that were conserved within the coding regions of each gene (Figs. 1 and 4). Since the 5′ regions of *avrD* and ORF 1 diverged, an *Eco*RI site was introduced adjacent to the start codon of each gene by site-specific mutagenesis using an oligonucleotide of the sequence indicated previously in the text. The avirulence phenotype was not affected by the introduction of the *Eco*RI site into *avrD*, as indicated by soybean inoculations of *P. s.* pv. *glycinea* R4 transconjugants containing the construct in pRK415. Introduction of the *Eco*RI site permitted exchange of the DNAs located 5′ to the coding regions of *avrD* and *P. s.* pv. *glycinea* ORF 1 to determine if transcriptional differences contributed to the two avirulence phenotypes.

Each recombinant gene was subcloned into pRK415 and, with the exception of TG12, positioned downstream from the vector *lac* promoter. However, it was previously determined that the avirulence phenotype conferred by *avrD* was expressed when positioned in the antiorientation to the vector *lac* promoter of pRK415 (Kobayashi *et al.* 1990). Therefore, the positioning of TG12 relative to the *lac* promoter should not have precluded its expression.

Fig. 2. Amino acid sequence comparisons of homologous open-reading frames (ORFs) from *Pseudomonas syringae* pv. *glycinea* race 4 and *P. s.* pv. *tomato*. **A**, ORF 1 (*P. s.* pv. *glycinea*) and *avrD* (ORF 1) from *P. s.* pv. *tomato*. **B**, ORF 2 from *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*. **C**, ORF 3 from *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*. Upper lines represent *P. s.* pv. *glycinea* genes and lower lines, *P. s.* pv. *tomato*. Two dots indicate identical amino acids.

P. s. pv. *glycinea* race 4 transconjugants harboring the various recombinant constructs were inoculated into soybean cultivar Harosoy leaves that were then screened for the occurrence of the HR. The avirulence phenotype was observed only with constructs TG11 and TG13 (Fig. 4).

Expression of ORF 1 from *P. s.* pv. *glycinea* and recombinant genes in *E. coli* and *P. s.* pv. *glycinea*. SDS-polyacrylamide gels of proteins from whole *E. coli* cells containing pPSG4005 (deletion GA11 in pUC128) indicated that a new protein band of approximately 34 kDa was produced (Fig. 5), in close agreement with the molecular mass calculated from the DNA sequence of ORF 1. Although the calculated molecular mass of 34,521 Da for ORF 1 is only slightly larger than that of 34,115 Da calculated for *avrD* (Kobayashi *et al.* 1990), the observed protein size difference between bands in SDS-polyacrylamide gels associated with ORF 1 compared to the protein produced by *avrD* appeared to be somewhat greater.

To determine if the recombinant gene constructs produced detectable proteins in *E. coli* cells, each was positioned downstream from the vector *lac* promoter in either pUC118/pUC119 or pUC128/pUC129. Whole cell proteins from *E. coli* cells harboring the clones were then extracted and run on SDS-polyacrylamide gels, and the approximately 34-kDa bands were visualized by Coomassie blue staining. Production of the wild-type and recombinant proteins in *E. coli* cells, as indicated by staining with Coomassie blue, was confirmed by western blotting with detection by the anti-AvrD antibodies. The relative intensities were observed to be the same (data not shown), indicating that the proteins encoded by *avrD* and ORF 1 reacted similarly to the two detection methods. High expression of a unique, approximately 34-kDa protein in *E. coli* was directed by all recombinant genes containing 3′ DNA of *avrD* (Fig. 5). However, very weak protein bands were observed from cells containing constructs with the 3′ end of ORF 1 from *P. s.* pv. *glycinea*.

P. s. pv. *glycinea* race 4 cells carrying plasmids with the various recombinant genes were lysed and electrophoresed on SDS-polyacrylamide gels. Because the approximately 34-kDa protein bands were not clearly visible on gels of *P. s.* pv. *glycinea* cells stained with Coomassie blue, the proteins were electroblotted onto membranes and detected with antibodies. As shown in Figure 6, *P. s.* pv. *glycinea* race 4 cells carrying A20, TG11, and TG13 produced readily detectable 34-kDa bands in the western blots, but GOR, TG12, TG14, and TG15 did not. TG16 and TG17 also did not reveal detectable bands in other blots (data not shown). *P. s.* pv. *tomato* PT23 yielded a strong protein band, but the *avrD* mutant strain did not (data not shown).

Recombinant TG13 was of additional interest because initial constructs made in shuttle plasmid pRK415 gave variably weak hypersensitive reactions in soybean leaves; however, cloning of recombinant TG13 in shuttle plasmid pDSK519, which is believed to attain a higher copy number in *P. s.* pv. *glycinea* (unpublished observations), resulted in a consistently stronger HR in soybean leaves as well as readily detectable protein bands in western blots.

The data for the recombinant gene constructs in *P. s.* pv. *glycinea* were, therefore, similar to observations in *E. coli* since only clone A20 (wild-type *avrD*) and recombinants TG11 and TG13 accumulated significant amounts

```
  1  AAGCTTTGGCGTTGACCTACGTTTGCATGGAACCAAATCCGTCCCAAAGGC
     ::::::::::::::::::::::::::::::::::::::::::::::::::
  1  AAGCTTTGGCGTTGACCTACGTTTGCATGGAACCAAATCCGTCCCAAAGGC

 52  CACACA----------TTTTCTAAAACTTGGTGGCTGTATAGCTTCAGCAG
     ::::::          :::: ::::: ::::: :::::::::::::: ::::
 52  CACACAGAGCCACAATTTTTATAAAAATTGGTAGCTGTATAGCTTCCGCAG

 93  TCGAAAACCTTTAAAAGTAGTAAACAATAGTTTATAAGGAATGTATGCATG 143
     :::::::: ::: ::: : :: ::: ::: :::::::::::::::::: ::::
103  TCGAAAAACTTCAAAGGCAGCAAAGAATGGTTTATAAGGAATGTATCCATG 153
```

Fig. 3. Untranslated 5′ DNA sequences of *Pseudomonas syringae* pv. *glycinea* open-reading frame 1 (upper lines) and the *P. s.* pv. *tomato* *avrD* gene (lower lines) from the leftward *Hin*dIII sites (base 1) to the predicted translational ATG start codons denoted by overscoring. Two dots indicate identical nucleotide bases. Underscoring indicates the region previously implicated in expression of the *avrD* gene (Kobayashi *et al.* 1990) and (—) denotes deleted bases.

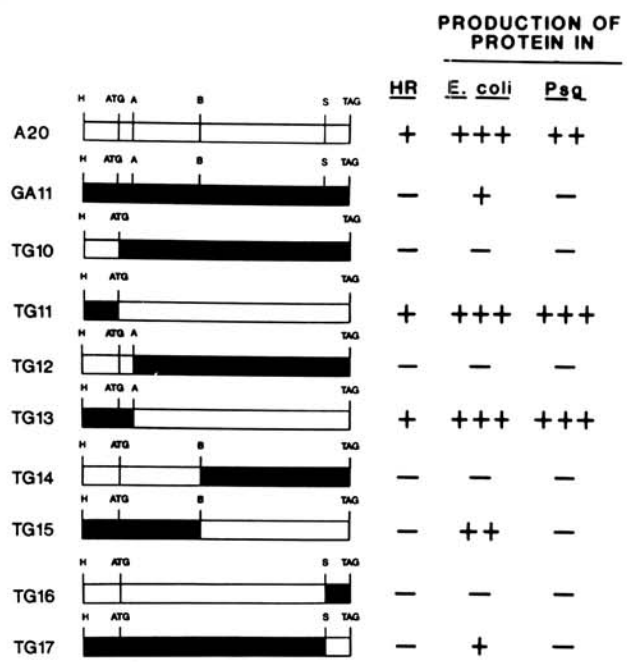


Fig. 4. Production of protein products and hypersensitive reactions by *Escherichia coli* or *Pseudomonas syringae* pv. *glycinea* race 4 cells carrying *avrD*, *P. s.* pv. *glycinea* open-reading frame (ORF) 1, or various recombinant genes constructed as denoted by the bars. Amino acid coding and 5′ untranslated regions of *avrD* from *P. s.* pv. *tomato* (light bars) and ORF 1 from *P. s.* pv. *glycinea* (dark bars) were compared. Sequences extend from the leftward, upstream *Hin*dIII sites to the protein termination codons (TAG). Presumed start codons are indicated by ATG; *Eco*RI restriction sites present immediately before the ATG codons permitted interchanges of the 5′ untranslated DNAs in TG10 and TG11. H, *Hin*dIII; A, *Ava*II; B, *Bgl*II; and S, *Spe*I. A (+) under the HR column denotes that a visible hypersensitive reaction was observed when *P. s.* pv. *glycinea* race 4 cells carrying plasmids with the noted construct were inoculated into soybean cultivars incompatible to *avrD*; a (—) indicates that no visible hypersensitive reaction was observed. Production of proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole cells and detection with Coomassie Brilliant Blue R 250 (*E. coli*) or western blotting (*P. s.* pv. *glycinea* race 4). (—) no protein detected; (+) to (+++) denote progressively greater quantities of protein detected on the gels.

of protein and elicited an HR (summarized in Fig. 4). The results therefore indicated that the protein encoded by ORF 1 from *P. s.* pv. *glycinea* is either translated less efficiently or is less stable in *P. s.* pv. *glycinea* and *E. coli* cells than is the protein encoded by *avrD*. This observation may be related to the failure of ORF 1 to elicit the HR phenotype in soybean.

DISCUSSION

Although ORF 1 from *P. s.* pv. *glycinea* reads colinearly and shares 86% identical amino acids with the *avrD*-encoded protein from *P. s.* pv. *tomato* (Fig. 2A), the *P. s.* pv. *glycinea* gene does not elicit the soybean HR when reintroduced into *P. s.* pv. *glycinea* on plasmids. The *P. s.* pv. *glycinea* gene encoding ORF 1 therefore appears to be a recessive allele of *avrD* with respect to the avirulence phenotype. Recessive alleles of avirulence genes have been identified in genetic studies with fungal pathogens (Day 1974) and by the occurrence of homologous but nonfunctional DNA sequences to cloned avirulence genes in *X. c.* pv. *malvacearum* (Gabriel *et al.* 1986) and *X. c.* pv. *vesicatoria* (Doidge) Dye (Kearney *et al.* 1988; Swanson *et al.* 1988). Historically, recessive alleles to avirulence genes have been referred to as "virulence" genes (see Day 1974); however, as pointed out by Gabriel (1986), the phenotypes for such recessive alleles have not been determined and therefore the terminology is misleading.

Surprisingly, deletions, insertions (for example, Kearney *et al.* 1988), or frame shift mutations were not observed in the *P. s.* pv. *glycinea avrD* analogue that would abolish production of a functional protein product. The absence of the avirulence phenotype by bacteria expressing ORF 1 from *P. s.* pv. *glycinea* also does not appear to involve differences in its transcriptional regulation. Despite sequence differences in the 5′ DNA in the region previously implicated in expression of *avrD* (Fig. 3; Kobayashi *et al.* 1990), *lux* reporter gene experiments have not detected down regulation of ORF 1 from *P. s.* pv. *glycinea* (H. Shen and N. T. Keen, unpublished).

The results in this study indicate that altered translation or stability of the *P. s.* pv. *glycinea* ORF 1 gene product may be involved in the absence of an avirulence phenotype (Figs. 4, 5, and 6). However, the complete conservation of reading frame integrity between *P. s.* pv. *glycinea* ORF 1 and *P. s.* pv. *tomato avrD* (Fig. 2A) raises the possibility that the *P. s.* pv. *glycinea* gene retains a bacterial function. Significantly, *E. coli* cells expressing ORF 1 from *P. s.* pv. *glycinea* have been shown to produce small quantities of the *avrD* elicitor (Keen *et al.* 1990). This result implies

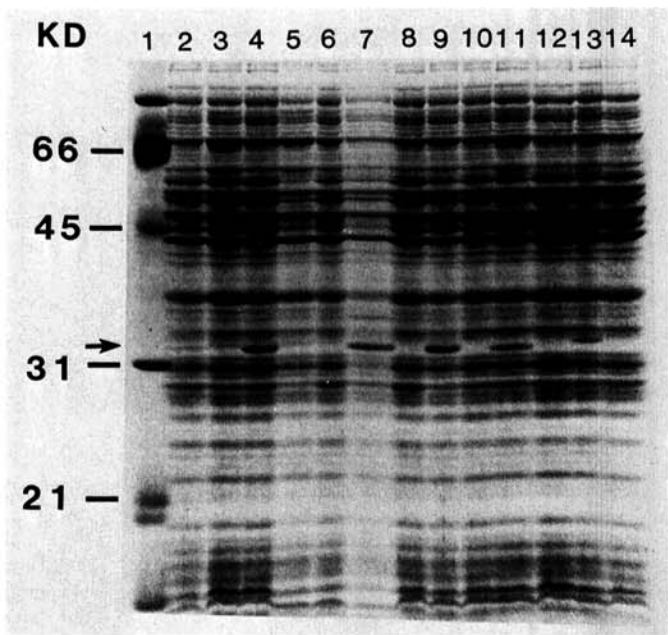


Fig. 5. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% gel) of whole *Escherichia coli* strain DH5α cells containing plasmids with open-reading frame (ORF) 1 or *avrD* or desired recombinant constructs (shown in Fig. 4) positioned downstream from the vector *lac* promoter of pUC118/pUC119 or pUC128/pUC129. Cells were grown at 28° C for 14 hr in Luria-Bertani medium plus 1 mM isopropyl-β-D-thiogalactopyranoside and 50 μg/ml of ampicillin, and electrophoresed as described in the text. Ten microliters was loaded into each well. Lane 1, size standards; lane 2, A20 (*avrD*); lane 3, GA11 (ORF 1); lane 4, pAVRD10 (A20 with an introduced *Eco*RI site); lane 5, pPSGOR (GA11 with an introduced *Eco*RI site); lane 6, TG10; lane 7, TG11; lane 8, TG12; lane 9, TG13; lane 10, TG14; lane 11, TG15; lane 12, TG16; lane 13, TG17; and lane 14, pUC119. The arrow indicates the approximate position of new proteins produced. KD, kilodaltons.

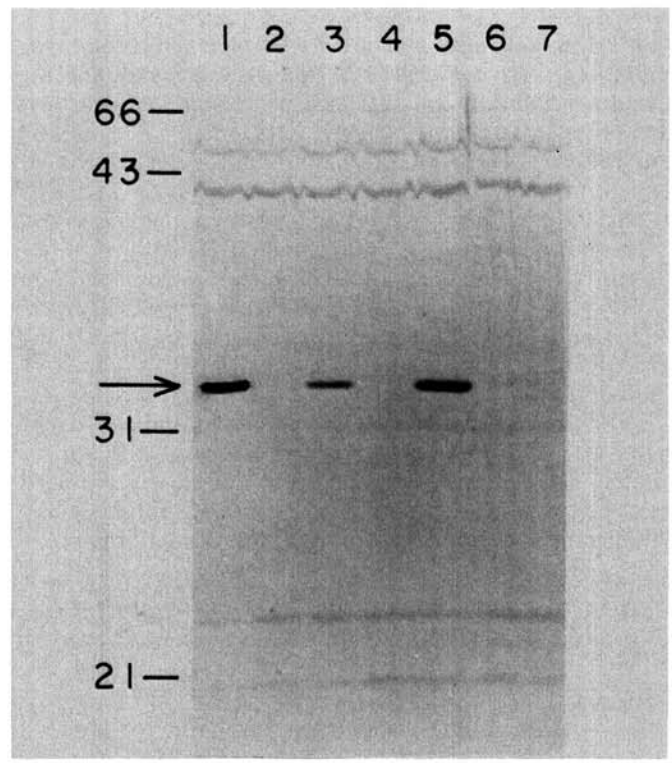


Fig. 6. Western blot of a 12% sodium dodecyl sulfate-polyacrylamide gel run with whole cell proteins from *Pseudomonas syringae* pv. *glycinea* race 4 containing *avrD*, open-reading frame (ORF) 1 from *P. s.* pv. *glycinea*, or various recombinant genes. The arrow denotes bands specifically detected by antiserum prepared against the *P. s.* pv. *tomato avrD*-encoded protein. Sizes of protein standards are shown on the left (bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor). Lane 1, A20; lane 2, GOR; lane 3, TG11; lane 4, TG12; lane 5, TG13; lane 6, TG14; and lane 7, TG15. Clone designations are shown in Figure 4.

that *P. s.* pv. *glycinea* ORF 1 may be functional, but at a lower level than the *P. s.* pv. *tomato avrD* gene.

Additional recombinant gene constructions or site-specific mutations may aid in determining why ORF 1 does not express the avirulence phenotype and inefficiently leads to production of the *avrD* elicitor. Similar studies (S. Tamaki and N. T. Keen, unpublished) have identified required regions of approximately 500 base pairs in *avrB* and in the moderately homologous gene *avrC* (Tamaki *et al.* 1988) that are responsible for the avirulence specificity of each gene. Studies with tobacco mosaic virus have also identified single or double amino acid changes in specific viral proteins that determine HR-eliciting abilities in resistant tobacco and tomato plants (Culver and Dawson 1989; Knorr and Dawson 1988; Meshi *et al.* 1988). However, an analogous region for *avrD* has not been clearly defined and may not, in fact, be determined by simple sequence changes. For example, both recombinant exchanges at the *Bgl*II site (clones TG14 and TG15) failed to elicit the soybean HR in *P. s.* pv. *glycinea* (Fig. 4). The location of the *Bgl*II site is preceded by a region of heterogeneity between ORF 1 and *avrD* that is due to amino acid mismatch substitutions which contribute to changes in the hydropathy profiles.

ACKNOWLEDGMENTS

Soybean seed was supplied by R. I. Buzzell and E. E. Hartwig.

Research was supported by National Science Foundation grant DCB-8709867.

LITERATURE CITED

Crouse, G. F., Frischauf, A., and Lehrach, H. 1983. An integrated and simplified approach to cloning into plasmids and single-stranded phages. Methods Enzymol. 101:78-89.

Culver, J. N., and Dawson, W. O. 1989. Point mutations in the coat protein gene of tobacco mosaic virus induce hypersensitivity in *Nicotiana sylvestris*. Mol. Plant-Microbe Interact. 2:209-213.

Day, P. R. 1974. Genetics of Host-Parasite Interactions. Freeman, San Francisco. 238 pp.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.

Ellingboe, A. H. 1976. Genetics of host-parasite interactions. Pages 761-778 in: Encyclopedia of Plant Pathology, New Series, Vol. 4: Physiological Plant Pathology. R. Heitefuss and P. H. Williams, eds. Springer-Verlag, Heidelberg.

Engvall, E., and Perlmann, P. 1972. Enzyme-linked immunosorbent assay, ELISA III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen coated tubes. J. Immunol. 109:129-135.

Flor, H. H. 1942. Inheritance of pathogenicity in *Melampsora lini*. Phytopathology 32:653-669.

Gabriel, D. W. 1986. Specificity and gene function in plant-pathogen interactions. ASM News 52:19-25.

Gabriel, D. W., Burges, A., and Lazo, G. R. 1986. Gene-for-gene interactions of five cloned avirulence genes from *Xanthomonas campestris* pv. *malvacearum* with specific resistance genes in cotton. Proc. Natl. Acad. Sci. USA 83:6415-6419.

Hagborg, W. A. F. 1970. A device for injecting solutions and suspensions into thin leaves of plants. Can. J. Bot. 48:1135-1136.

Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28:351-359.

Kearney, B., Ronald, P. C., Dahlbeck, D., and Staskawicz, B. J. 1988. Molecular basis for evasion of plant host defense in bacterial spot disease of pepper. Nature 332:541-543.

Keen, N. T., and Holliday, M. J. 1982. Recognition of bacterial pathogens by plants. Pages 179-217 in: Phytopathogenic Prokaryotes, Vol. 2. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.

Keen, N. T., and Staskawicz, B. 1988. Host range determinants in plant pathogens and symbionts. Annu. Rev. Microbiol. 42:421-440.

Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene 70:191-197.

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 phycocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.

Klement, Z. 1982. Hypersensitivity. Pages 149-177 in: Phytopathogenic Prokaryotes, Vol. 2. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.

Knorr, D. A., and Dawson, W. O. 1988. A point mutation in the tobacco mosaic virus capsid protein gene induces hypersensitivity in *Nicotiana sylvestris*. Proc. Natl. Acad. Sci. USA 85:170-174.

Kobayashi, D. Y., Tamaki, S. J., and Keen, N. T. 1989. Cloned avirulence genes from the tomato pathogen *Pseudomonas syringae* pv. *tomato* confer cultivar specificity on soybean. Proc. Natl. Acad. Sci. USA 86:157-161.

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

Kramer, W., and Fritz, H.-J. 1987. Oligonucleotide-directed construction of mutations via gapped duplex DNA. Methods Enzymol. 154:350-367.

Kramer, B., Kramer, W., and Fritz, H.-J. 1984. Different base/base mismatches are corrected with different efficiencies by the methyl-directed DNA mismatch-repair system of *E. coli*. Cell 38:879-887.

Kyte, J., and Doolittle, R. F. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157:105-132.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Long, M., Barton-Willis, P., Staskawicz, B. J., Dahlbeck, D., and Keen, N. T. 1985. Further studies on the relationship between glyceollin accumulation and the resistance of soybean leaves to *Pseudomonas syringae* pv. *glycinea*. Phytopathology 75:235-239.

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

Meshi, T., Motoyoshi, F., Adachi, A., Watanabe, Y., Takamatsu, N., and Okada, Y. 1988. Two concomitant base substitutions in the putative replicase genes of tobacco mosaic virus confer the ability to overcome the effects of a tomato resistance gene, *Tm-1*. EMBO J. 7:1575-1581.

Napoli, C., and Staskawicz, B. 1987. Molecular characterization and nucleic acid sequence of an avirulence gene from race 6 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:572-578.

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

Shine, J., and Dalgarno, L. 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: Complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA 71:1342-1346.

Staskawicz, B. J., Dahlbeck, D., and Keen, N. T. 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max* (L.) Merr. Proc. Natl. Acad. Sci. USA 81:6024-6028.

Swanson, J., Kearney, B., Dahlbeck, D., and Staskawicz, B. 1988. Cloned avirulence gene of *Xanthomonas campestris* pv. *vesicatoria* complements spontaneous race-change mutants. Mol. Plant-Microbe Interact. 1:5-9.

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

Vieira, J., and Messing, J. 1987. Production of single-stranded plasmid DNA. Methods Enzymol. 153:3-11.