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Characterization of the Promoter of Avirulence Gene D from *Pseudomonas syringae* pv. tomato

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The avirulence gene D (*avrD*) from *Pseudomonas syringae* pv. tomato comprises the first open reading frame (ORF) of a putative operon consisting of at least five tandem ORFs. The promoter of the *avrD* operon was localized to a 150-bp DNA fragment occurring 5′ to the *avrD* gene by using the Tn*7-lux* and *gus* reporter systems. The *avrD* promoter in *P. syringae* pv. tomato and *P. syringae* pv. glycinea was poorly expressed when bacteria were grown in complex culture media but was activated during bacterial growth in plants. The timing and level of induction were similar in compatible and incompatible plant-pathogen interactions. When bacteria were grown in minimal culture medium, promoter activity was repressed by certain carbon sources, high concentrations of nitrogen compounds, and pH values above 6.5. Primer extension experiments on RNA from bacteria grown in minimal medium identified two transcription initiation sites 87 and 41 nucleotides upstream from the translational start site. Only the −41 transcriptional start site was identified in bacteria grown in soybean leaves. A σ^{54} promoter consensus sequence (GG-10 bp-GC) occurred 14 bp upstream of the −41 transcriptional start, and 3′ deletions into this region completely abolished promoter activity. Little expression was observed when a *gus* fusion with the *avrD* promoter was introduced into an *ntrA* mutant strain of *P. syringae* pv. phaseolicola deficient in the σ^{54} cofactor. Expression from the *avrD* promoter also required the *hrp* regulatory genes, *hrpS* and *hrpL*. Deletions from the 5′ end of the promoter region and base substitution analyses also identified two upstream elements important for expression. Sequence comparison of these elements with other cloned avirulence genes revealed the presence of a conserved consensus sequence (GGAACC-N15/16-CCAC) in the promoters of nine different avirulence genes from *P. syringae* pathovars.

Plant pathogens harbor avirulence genes that interact with hosts carrying complementary or matching disease resistance genes to trigger active plant defense responses, collectively called the hypersensitive response (HR) (23, 48, 51). Avirulence genes have generally been studied in regard to their role at the pathogen race-plant cultivar level, but they may also function at higher taxonomic levels (7, 29, 55). Avirulence genes therefore limit the range of plants that the pathogen can successfully infect and accordingly have considerable significance in plant-microorganism interactions. Several important questions have been posed concerning the biology of avirulence genes, including the biochemical mechanisms by which they interact with host plants carrying the cognate resistance genes and their biological functions in the pathogens which harbor them.

Avirulence gene D (*avrD*) was cloned from *Pseudomonas syringae* pv. tomato (29) and is the first gene of a putative operon. Subsequently, several other *P. syringae* pathovars were also shown to contain *avrD* alleles (29, 30, 31, 58). The *avrD* gene is of particular interest because gram-negative bacteria expressing the gene produce low-molecular-weight elicitors of the plant HR. The isolated elicitors, called syringolides, trigger the HR only on soybean cultivars harboring the corresponding disease resistance gene, *Rpg4* (22, 25). The structures of the syringolides have recently been determined as two homologous C-glycoside lactones (35, 47). We previously showed that the promoter of *avrD* is not expressed when *P. syringae* pv. tomato and *P. syringae* pv. glycinea are grown on rich culture media but is highly expressed when the bacteria are inoculated into plant leaves (45). This result is consistent with the role of *avrD* in plant recognition and raises the possibility that the function of *avrD* in the bacteria is also directed to their development on or in plants.

Virulence mechanisms of bacterial plant pathogens are frequently regulated such that they are only expressed in the plant environment (1, 41, 56). Thus, environmental cues such as low pH and the presence of certain sugars and other substances result in the expression of several genes required for pathogenicity and high virulence. For example, many bacterial plant pathogens require *hrp* genes for both disease development on compatible hosts and HR induction on incompatible hosts (33). These genes are generally clustered on 20- to 30-kb DNA regions and have been identified in members of the *Erwinia* (50), *Pseudomonas* (6, 14, 33, 40, 57), and *Xanthomonas* (5, 44) genera. The expression of *hrp* genes is modulated by pH, osmolarity, and the presence of particular carbon and nitrogen sources (1, 41, 43, 54, 57). In addition, specific plant signals may induce *hrp* gene expression during bacterial growth in plants (41, 44).

Activation of *hrp* genes in *P. syringae* in response to environmental cues requires the *hrpS* and *hrpL* regulatory genes (12, 41). Of particular interest, *hrpS* and *hrpL* have also been shown to control the expression of certain avirulence genes, including *avrD* (15, 42, 45). Accordingly, we studied the *avrD* promoter more fully. We report here that it is novel, including a stereotypic σ^{54} promoter as well as an essential upstream element that is conserved in the promoter regions of several *P. syringae* avirulence genes.

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TABLE 1. Bacterial strains and plasmids

Designation	Relevant characteristics	Reference or source
Bacterial strains		
E. coli		
DH5α	F-*lacZ*ΔM15 *endA1 hsdR17 supE44 thi-1 relA1 l-recA1*	Bethesda Research Laboratories, Gaithersburg, Md.
S17-1	Pro res⁻ mod+, RP4-2-Tc::Mu-Km::Tn7 integrated into the genome, Tp^r Sm^r	46
P. syringae pv. glycinea		
PsgR4	Wild-type race 4	48
PsgR0	Wild-type race 0	49
E10-6	*hrpAB* mutant of PsgR0	15
E10-3	*hrpC* mutant of PsgR0	15
E10-20	*hrpD* mutant of PsgR0	15
E64	*hrpE* mutant of PsgR0	15
E67	*hrpF* mutant of PsgR0	15
E43	*hrpS* mutant of PsgR0	15
E10-18	*hrpL* mutant of PsgR0	15
P. syringae pv. phaseolicola		
NSP3121	Wild type	N. Panopoulos
ME50	*ntrA* (encoding σ^{54}) mutant of NSP3121 with Tn*3-spice* insertion	N. Panopoulos
P. syringae pv. tomato; PT23		29
Plasmids		
pUC128/129	*E. coli* cloning vectors, Ap^r	26
pBluescript KS+	*E. coli* cloning vector, Ap^r	Stratagene, La Jolla, Calif.
pLAFR6(*gus*)	pLAFR3 derivative with a promoterless *gus* reporter gene	B. Staskawicz
pHSK728/729	pUC based, Tn*7L* TT-*lux* Tn*7tnsA* Sm/Sp^r, Tn*7R*, Ap^r	45
pMON7181	IncQ, Tn*7att* Tn*7tnsB-E* Gm^r	4
pBSPD	0.15-kb *Hin*dIII-*Eco*RI fragment containing the *avrD* promoter in pBluescript KS+	This work
pAVRD33	*avrD* cloned in the broad-host-range plasmid, pDSK519	23
pHE-*gus*	Transcriptional fusion of a 0.15-kb *Hin*dIII-*Eco*RI fragment containing the *avrD* promoter to the *gus* reporter gene in pLAFR6(*gus*)	This work

MATERIALS AND METHODS

Plasmids, bacterial strains, and culture conditions. The bacterial strains and plasmids used or constructed in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C on Luria-Bertani medium (34), and *P. syringae* strains were routinely cultured at 28°C on King's medium B (KMB) (27) or MGY medium (10 g of mannitol, 2 g of glutamic acid, 0.5 g of KH_2PO_4, 0.2 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.25 g of yeast extract per liter) with or without agar at 15 g/liter. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; gentamicin, 12.5 μg/ml; kanamycin, 50 μg/ml; streptomycin, 25 μg/ml; spectinomycin, 50 μg/ml; rifampin, 100 μg/ml; tetracycline, 12.5 μg/ml in Luria-Bertani medium and 25 μg/ml in KMB.

Recombinant DNA techniques. Plasmid isolation, restriction digestion, and ligation methods were performed as described previously (3). Plasmid DNA was introduced into *P. syringae* strains by conjugation or electroporation as described in reference 24. Double-strand DNA sequencing was employed with the Sequenase kit (U.S. Biochemicals, Cleveland, Ohio). Sequencing of *gus* fusions in pLAFR6 (*gus*) was carried out with the primer 5′-GATTTCACGG GTTGGGGTTTCT-3′ according to the method of Jefferson (19). Exonuclease III deletions of the *avrD* promoter region were performed on pBSPD (Table 1) with the Erase-a-Base system (Promega, Madison, Wis.). Reactions were carried out at room temperature and stopped at 15-s intervals. Deletion products were analyzed on 8% nondenaturing polyacrylamide gels (3), and selected clones were sequenced to map the exact deletion endpoints.

Site-directed oligomutagenesis of the *avrD* promoter was carried out on pBSPD (Table 1) by two successive rounds of polymerase chain reaction with appropriate primers as described in reference 36. The primers used for constructing OMD1, OMD2, OMD3, and OMD4 were 5′-ATCCGTCCCA AATCGAGGTCGGAGCCACAATTTT-3′, 5′-TGACCTAC GTTTGCAGATATCTAAATCCGTCCCAAG-3′, 5′-ATCC GTCCCAAAGGGATATCAGAGCCACAATTTT-3′, and 5′-CCAAAGGCCACACAGGATATCAATTTTTATAAAAA-3′, respectively. The final polymerase chain reaction products were digested with *Hin*dIII and *Eco*RI and cloned into pBluescript KS+. The entire 150-bp DNA fragments were then totally sequenced to confirm the desired base substitutions and preclude undesired mutations.

Primer extension experiments. Transcriptional start sites of *avrD* were determined with RNA isolated from *P. syringae* pv. tomato PT23 cells grown in plants or in induction medium (IM). For preparation of RNA from cells grown in plants, suspensions (A_{600} of 1.0) of PT23 or PT23::HE-*lux* were injected into soybean cotyledons as described previously (45). PT23::HE-*lux* was used to monitor *avrD* gene expression. PT23 cells were harvested 15 h after inoculation, when *avrD* gene expression was maximal as determined by scintillation counting of light production from PT23::HE-*lux*. The infected cotyledons were cut in half and infiltrated with ice-cold 1 mM $MgCl_2$ several times under vacuum (28). Bacteria were recovered from the washing fluid by centrifugation, and RNA was extracted by the rapid isolation method (3).

RNA was also isolated by the hot phenol method (53) from bacterial cultures grown for 15 h in IM. Briefly, 10 ml of culture was chilled quickly in ethanol-dry ice and then thawed on ice. Cells were pelleted by centrifugation at 4°C and resuspended in 100 ml of lysis buffer (30 mM sodium acetate, pH 5.2, and 2% sodium dodecyl sulfate). The lysates were immediately extracted twice with phenol preheated to 65°C and then were extracted twice with chloroform before precipitation with ethanol. The pellets were resuspended in 200 μl of DNase digestion buffer (30 mM sodium acetate [pH 5.2] and 10 mM $MgCl_2$) and digested with RNase-free DNase for 30 min at 37°C. After DNase digestion, the RNA was extracted twice with hot phenol and twice with chloroform before precipitation with ethanol.

Two different oligonucleotides (5′-TGCTAAAACTAAG GTCTTGCATG-3′ and 5′-CAAAGAAACGATCTTTAGC GGGTCCC-3′) complementary to different sequences in the *avrD* coding region were end labelled with ^{32}P by T4 polynucleotide kinase. The labelled primers were annealed to 15 μg of RNA in PE buffer (0.25 M KCl and 10 mM Tris-HCl [pH 8.0]) at 45°C for 1 h and were extended with Moloney murine leukemia virus reverse transcriptase at 45°C. Extension products were analyzed essentially as described in reference 3.

Preparation of plant extracts. For the isolation of intercellular fluid, fully expanded soybean leaves were infiltrated with ice-cold H_2O by vacuum infiltration (28). The washing fluids were centrifuged at 10,000 × *g* for 10 min to remove plant debris. The supernatants were dried either by lyophilization or in a rotary evaporator and then were dissolved in H_2O or M9 salts (34). For the preparation of total leaf extracts, fully expanded soybean leaves were homogenized with a mortar and pestle under liquid nitrogen. The homogenates were dissolved in H_2O or M9 salts.

Plant tissue culture. A cell suspension culture of *Nicotiana glutinosa* was obtained from T. Murashige (University of California, Riverside) and grown in MS medium (38) supplemented with 3% sucrose, 0.1% casein hydrolysate, 2 mg of 2,4-dichlorophenoxyacetic acid per liter, and 0.1 mg of kinetin per liter. Callus cultures of soybean cultivars Harosoy and Acme were generated from sectors of hypocotyls on MS agar medium supplemented with 3% sucrose and 2 mg of 2,4-dichlorophenoxyacetic acid per liter. Cell suspension cultures were then initiated from callus in MS medium supplemented with 3% sucrose and 2 mg of 2,4-dichlorophenoxyacetic acid per liter. Cells were subcultured by dilution into fresh media after 1 week of growth for tobacco and 2 weeks of growth for soybeans. Cell suspensions (50 ml) were incubated in 250-ml flasks at room temperature on a shaker set at 110 rpm.

Delivery of the Tn*7-lux* system into *P. syringae* and Lux assays. Tn*7-lux* constructs were integrated into the genome of *P. syringae*, and light measurements were carried out with a scintillation counter as described in reference 45.

Gus assays. Bacterial cells from overnight cultures in KMB were washed twice in 1 mM $MgCl_2$ and inoculated into defined culture media or plant leaves. For assay of bacteria grown in culture, cells were diluted into a defined culture medium to a final concentration of an A_{600} of 0.1. This medium contained basal salts (10 mM Na_2HPO_4, 20 mM KH_2PO_4, 0.40 mg of $MgSO_4$ per ml, 0.45 mg of K_2SO_4 per ml) and micronutrients (10 μg of $FeSO_4 \cdot 7H_2O$ per ml, 0.5 μg of $CuSO_4$ per ml, 25 μg of $ZnSO_4$ per ml, 25 μg of $MnSO_4$ per ml, 60 μg of $CaCl_2$ per ml) supplemented with various carbon and nitrogen sources as indicated. The medium was adjusted to pH 5.7 unless otherwise stated. After 15 h of growth, cells were harvested by centrifugation and lysed in the extraction buffer for Gus assays (19). Bacterial cell numbers were estimated by measuring the A_{600}. For assay of bacteria grown in plants, cell suspensions (A_{600} of 0.1) were infiltrated into soybean leaves. Leaf discs (0.8 cm in diameter) were harvested 24 h postinoculation and macerated in 500 μl of ice-cold H_2O in microfuge tubes. Aliquots of 10 μl were taken for scoring CFU after dilution plating onto selective medium (MGY-tetracycline). The remaining cells were collected by centrifugation and were resuspended in extraction buffer (19). β-Glucuronidase activity was measured by fluorometric assay with a TKO 100 minifluorometer (Hoefer Scientific Instruments, San Francisco, Calif.). 4-Methylumbelliferyl glucuronide was used as the substrate, and 1 U of β-glucuronidase was defined as 1 nmol of 4-methylumbelliferone released per min as described in reference 19.

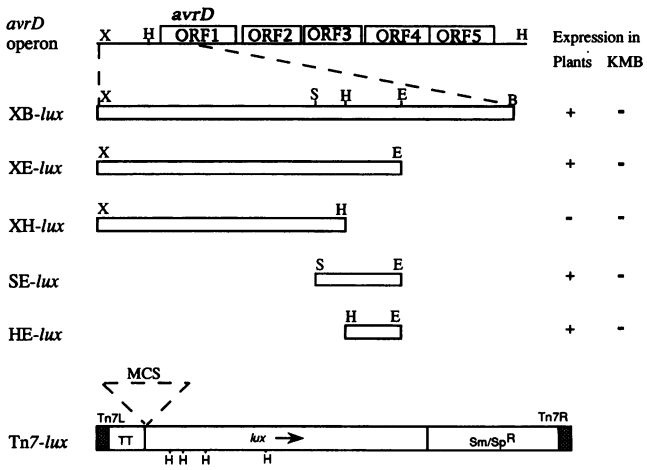

FIG. 1. Localization of the promoter for the *avrD* operon. Various DNA restriction fragments were cloned in front of the *lux* reporter genes (45). The resultant fusions were introduced into *P. syringae* pv. glycinea (PsgR4). Light production was monitored between 4 and 48 h when bacteria were grown in soybean leaves or 4 and 15 h when grown in KMB. Abbreviations: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sma*I; X, *Xho*I; ORF, open reading frame.

RESULTS

The promoter of the *avrD* operon is located within a 150-bp DNA sequence located 5′ to the *avrD* gene and is plant inducible. Various DNA restriction fragments from the 5′ region of the *avrD* gene were cloned into pHSK728 or pHSK729 (Table 1) to create transcriptional fusions with the *lux* reporter genes (Fig. 1). These constructs were delivered into the chromosome of PsgR4 with the helper plasmid pMON7181 (4, 45). Southern blot analyses showed that all fusion constructs were integrated into the single chromosomal Tn*7* insertion site, as expected (reference 45 and data not shown). PsgR4 or Pst cells carrying various *lux* fusions were assayed for light production when bacteria were grown for 15 h either in soybean leaves or in the rich culture medium, KMB. As shown in Fig. 1, little expression was observed from any of the *lux* fusions when cells were grown in KMB, whereas high levels of expression were observed from all fusions except XH-*lux* when cells were inoculated into soybean leaves. The HE-*lux* fusion gave levels of expression as high as the other three fusions, indicating that the 150-bp *Hin*dIII-*Eco*RI fragment contains the full pro-

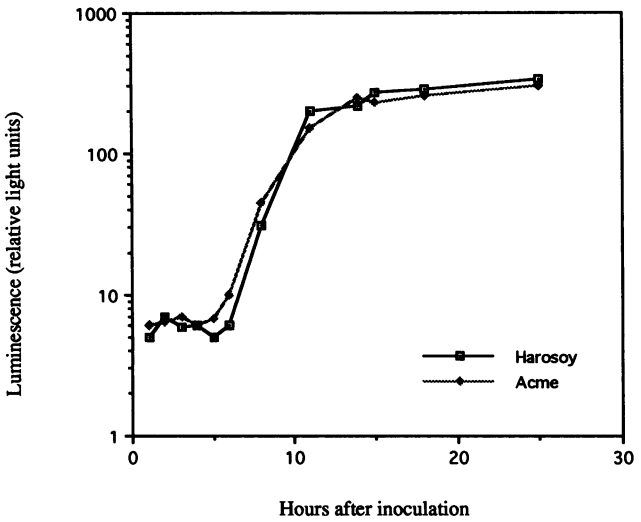

FIG. 2. Time course of *avrD* gene expression during compatible and incompatible interactions. PsgR4::HE-*lux*(pAVRD33) cells from overnight cultures in KMB were suspended in water and infiltrated into leaves of soybean cultivars Harosoy and Acme, and light production was measured when leaves were removed at different time points for scintillation counting. The levels of gene expression are the averages of three independent experiments and are reported in relative light units (scintillation cpm divided by 10^4) normalized to 8×10^8 CFU as described in reference 10.

moter. The HE-*lux* fusion was also introduced into *P. syringae* pv. tomato PT23, and the expression observed was similar to that in PsgR4 (data not shown).

The timing and level of *avrD* induction are similar in compatible and incompatible bacterium-plant interactions. It has been well documented that physiological conditions in plant tissue differ during compatible and incompatible interactions (2). Bacteria, therefore, receive different signals from the surrounding environment and may differentially regulate genes as an adaptive response. The *avrD* gene construct pAVRD33 was introduced in PsgR4::HE-*lux*; the resulting transconjugants elicited an HR on the soybean cultivar Harosoy but a susceptible reaction on cultivar Acme. We monitored *avrD* gene induction over a time course in which cell suspensions of PsgR4::HE-*lux* (pAVRD33) were infiltrated into leaves of these cultivars. In both cases, *avrD* induction occurred 6 to 8 h after inoculation and reached a maximum after ca. 15 h (Fig. 2).

Expression of the *avrD* promoter is modulated by environmental conditions. Induction of the *avrD* gene in plants could be due to a specific inducing factor(s) of plant origin and/or to particular environmental conditions. To test the possibility of a specific plant signal(s), various plant extracts were analyzed for their ability to induce *avrD* gene expression. Leaf intercellular fluids or total leaf extracts were isolated from healthy soybean leaves or leaves previously inoculated with PsgR4. PsgR4::HE-*lux* cells were grown in intercellular fluids or leaf extracts, and light production was measured. While slight induction was observed in some experiments, the results were not reproducible. Leaf intercellular fluids were fractionated by being passed through a Sep-Pak C18 cartridge in an attempt to concentrate any possible inducer(s), but all experiments yielded negative results.

Light production was observed when PsgR4::HE-*lux* cells were cocultivated in tobacco or soybean cell suspension cultures. Cell suspensions from overnight cultures in KMB were inoculated at a 1/100 dilution into 25-ml plant cell suspensions and were grown at room temperature with shaking. Aliquots of 500-μl cultures were taken at 2-h intervals and assayed for light production. Induction started 6 h after inoculation and reached a maximal level (ca. 10^5 per 10^{10} CFU) after 8 h of cocultivation. However, no expression was detectable when PsgR4::HE-*lux* cells were grown in cell-free media previously used for the cultivation of plant cells. Thus, no evidence for a discrete plant signal molecule inducing *avrD* promoter activity was obtained.

The *gus* reporter gene was used for quantitative analysis of promoter activity in bacteria grown in culture media. The 150-bp *Hin*dIII-*Eco*RI fragment from pBSPD containing the *avrD* promoter was cloned into pLAFR6(*gus*) to generate a transcriptional HE-*gus* fusion. PsgR4 cells carrying the HE-*gus* fusion did not give β-glucuronidase activity above the background level when grown in KMB. Overnight cultures grown in KMB were then transferred to defined culture media with various carbon and nitrogen sources, pHs, and osmolarities. β-Glucuronidase activities were determined 15 h after transfer. Expression varied greatly when different carbon sources were used, with the highest expression on fructose or mannitol (Fig. 3A). Expression was low at pH values above 6.5 and in the presence of high concentrations of ammonium ions or Casamino Acids (Fig. 3B). Modulation of osmolarity by NaCl or the nonutilizable sugar sorbitol did not significantly affect expression (data not shown). PsgR4 [pLAFR6(*gus*)] was included as a negative control and did not give detectable β-glucuronidase activity under any of the conditions described above. On the basis of these data, an IM was formulated for maximal induction and optimal cell growth and was used in all subsequent experiments. The IM is composed of basal salts and micronutrients supplemented with 10 mM mannitol, 0.03% Casamino Acids, and 0.1% NH_4Cl (pH 5.7).

The *avrD* gene is regulated by the chromosomal *hrpS* and *hrpL* genes. The expression data described above were suggestive of *hrp* gene regulation. The *hrp* genes in *P. syringae* pv. phaseolicola are regulated by two *hrp* loci, *hrpS* and *hrpL* (12), and expression of the *avrB* gene from *P. syringae* pv. glycinea also requires these genes (15). The HE-*gus* fusion was therefore introduced into several different *hrp* mutants of *Psg* race 0, and β-glucuronidase activities were determined when the cells were grown in IM or in plants (Table 2). Little β-glucuronidase activity was detected in the *hrpS* or *hrpL* mutants when cells were grown in IM or in planta, while levels of expression in the other *hrp* mutants were comparable to that in the wild-type bacterium.

Transcription of the *avrD* gene. Primer extension experiments were carried out on *avrD* message isolated from PT23 cells grown in plants or induction medium. Two different DNA primers were used to avoid artifacts but gave similar results. RNA isolated from cells grown in plants gave a single strong extension product which terminated 41 bp upstream of the translational start of the *avrD* gene (Fig. 4). However, RNA isolated from cells grown in IM generally gave two primer extension bands (data not shown). One was 41 bp upstream of the translational start site, as found for bacteria grown in plants, and the other was 87 bp upstream of the translational start site. The latter site was not observed in all experiments, however, and does not occur downstream from any known promoter consensus sequence. The start site at position −87 was also not observed in any experiment with RNA prepared from bacteria grown in leaves. The transcriptional start site located 41 bp upstream

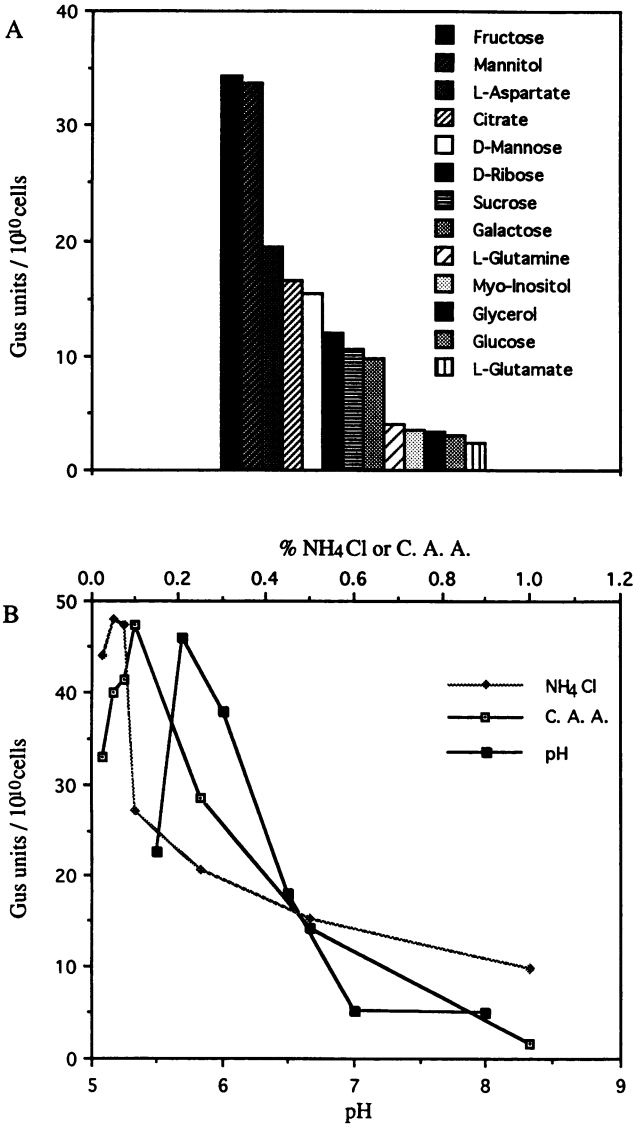

FIG. 3. Expression from the *avrD* promoter when PsgR4(HE-*gus*) cells were grown in different culture conditions. β-Glucuronidase activities were measured after 15 h of growth, and the means of three independent experiments are reported. (A) Different carbon sources (10 mM) were added to the medium composed of basal salts (pH 5.7), micronutrients, and 0.1% NH_4Cl. (B) Different concentrations of NH_4Cl or Casamino Acids (C. A. A.) were added to the medium composed of basal salts (pH 5.7), micronutrients, and 10 mM mannitol. For assays with media of different pH values, media composed of basal salts, micronutrients, 10 mM mannitol, 0.1% NH_4Cl, and 0.03% Casamino Acids were adjusted to the desired pH values with sodium phosphate buffer in basal salts medium.

from the translational start site was seen in all experiments with RNA prepared by two methods from bacteria grown in IM or plants. It is present in several *Pseudomonas* promoters and appropriately positioned with regard to a consensus sequence (GG-10 bp-GC) which interacts with the σ^{54} RNA polymerase holoenzyme (Fig. 5) (9, 32, 52).

Successive exonuclease III deletions were made from the 3′ end of the 150-bp *Hin*dIII-*Eco*RI promoter fragment, and the endpoints were mapped by DNA sequencing. Selected deletions were transcriptionally fused to the *gus* reporter

TABLE 2. Expression of the *avrD* promoter in different *P. syringae* pv. glycinea mutants

Bacterial strain[a]	Mutant locus	Gus U/10^{10} cells grown in[b]: IM	Plants
P. syringae pv. glycinea			
Race 0 (pLAFR6-*gus*)	Wild type	0.1	0.5
Race 0 (pHE-*gus*)	Wild type	30.5	52.7
E10-6 (pHE-*gus*)	*hrpAB*::Tn*5*	28.3	48.2
E10-3 (pHE-*gus*)	*hrpC*::Tn*5*	30.6	38.8
E10-20 (pHE-*gus*)	*hrpD*::Tn*5*	23.8	61.3
E64 (pHE-*gus*)	*hrpE*::Tn*5*	32.9	46.3
E67 (pHE-*gus*)	*hrpF*::Tn*5*	27.1	32.5
E43 (pHE-*gus*)	*hrpL*::Tn*5*	1.0	1.3
E10-18 (pHE-*gus*)	*hrpS*::Tn*5*	0.9	0.8
P. syringae pv. phaseolicola			
NSP3121 (pHE-*gus*)	Wild type	35.2	ND[c]
ME50 (pHE-*gus*)	*ntrA*::Tn*3-spice*	0.8	ND[c]

[a] Strains are described in Table 1.
[b] pLAFR6(*gus*) fusions were introduced into the noted strains and *gus* activity was measured after 15 h; data reported are the means of three independent experiments.
[c] ND, not determined because the *ntrA* mutant was unable to grow in plants.

gene in pLAFR6(*gus*), and these were conjugated into PsgR4 and assayed for β-glucuronidase activity in both IM and in plants. As shown in Fig. 6, promoter activity was retained in the +25 and −1 deletions located downstream from the σ^{54} promoter consensus sequence. Deletions within the promoter consensus sequence at position −23 and further 5′ at −37 completely abolished promoter activity, however. These data therefore suggest that the *avrD* gene is transcribed from the GG-10 bp-GC promoter.

P. syringae pv. phaseolicola is a bacterium related to *P. syringae* pv. glycinea and *P. syringae* pv. tomato. An *ntrA* (encoding σ^{54}) mutant of *P. syringae* pv. phaseolicola ME50 was constructed in the laboratory of N. Panopoulos at the University of California, Berkeley. The HE-*gus* fusion was introduced into ME50 as well as the wild-type strain, NPS3121. Growth of strain ME50 required the addition of glutamine to the IM, as expected (13), and the strain did not grow sufficiently well in plant leaves to permit measurement of promoter activity. Little expression was observed from the HE-*gus* fusion in mutant ME50 grown in IM, whereas expression in the wild-type strain, NPS3121, was comparable to that observed in PsgR4 and PstPT23 (Table 2). These results indicate that transcription of the *avrD* gene requires the σ^{54} cofactor.

Transcription of *avrD* requires an upstream *cis*-acting element. Transcription from σ^{54} promoters is positively controlled, and these promoters generally possess an upstream activator sequence which is required for expression (32, 52). The region upstream of the *avrD* GG-10 bp-GC promoter was therefore analyzed by 5′ deletions and base substitution mutations. As shown in Fig. 6, deletions 5′ to position −85 did not affect promoter activity. A deletion at position −64 resulted in a reduced expression level (ca. 20% of the wild type) in plants and no expression in IM. A sequence motif was identified in the region between the −85 and −64 positions that is similar to the consensus harp box found in the promoter regions of several *P. syringae hrp* genes (11) (Fig. 6). Deletions at the −51 position and further 3′ completely eliminated promoter activity. A sequence motif (GCC ACACA) located between positions −61 and −53 of the

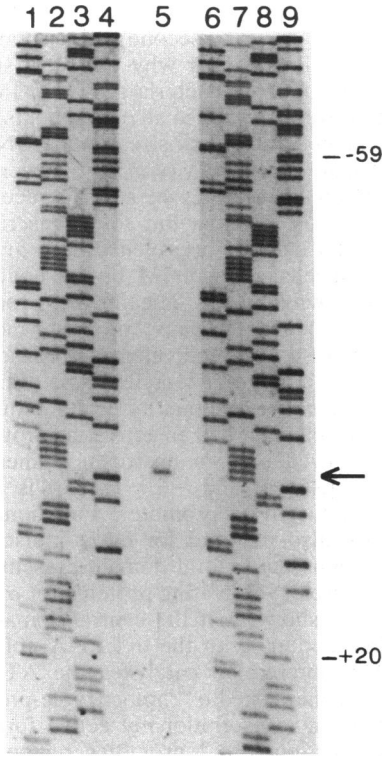

FIG. 4. Primer extension product of the *avrD* gene transcript. The extension product shown was produced from RNA isolated from *P. syringae* pv. glycinea cells grown in IM. The DNA sequence shown is that of the sense strand and corresponds to that given in Fig. 8. 5'-CAAAGAAACGATCTTTAGCGGGTCCC-3' was used as primer in the extension and DNA sequencing reactions. Lanes: 1 to 4 and 6 to 9, DNA sequencing lanes for G, A, T, and C, respectively; 5, primer extension reaction, with the single detected product designated by the arrow.

avrD promoter also occurs in the promoter regions of the *avrA* and *avrB* genes (38, 51) and all of five characterized *avrD* alleles (31, 58). This sequence was therefore a candidate for an upstream *cis*-acting element. A mutant with base substitutions in the −53 to −61 region of the *avrD* promoter, OMD1, had no promoter activity (Fig. 6), confirming its importance.

The data presented above identified two DNA regions that were important for *avrD* promoter activity (GGAACC-N15/16-CCAC). Sequence comparison revealed that these regions are conserved in the promoters of nine different

avrD	AATTGGTAGCTGTATAGCTTCCGCAGTCGAAAA
PAK	ATTTGGCATGGTAAGTGCTTGGTAGGGTTA
xylCAB	CAATGGCATGGCGGTTGCTAGCTATACGAGA
CPG2	CAGTGGCACTCGAATTGCTATAAGAACCATGG
xylS	GCTTGGCGTTATTTTTGCTTGGAAAAGTGG
consensus	TGGCA TTGCTT

FIG. 5. Comparison of the *avrD* promoter with other σ^{54} promoters from *Pseudomonas* spp. The canonical sequences (GG-10 bp-GC) recognized by the σ^{54} RNA polymerase holoenzyme are boxed. Sequence data for the various genes can be found in the following references: *avrD*, 30; PAK from *P. aeruginosa*, 21; the *xylCAB* and *xylS* genes from *P. putida*, 17 and 18, respectively; and CPG2 from *Pseudomonas* sp. strain RS16, 37.

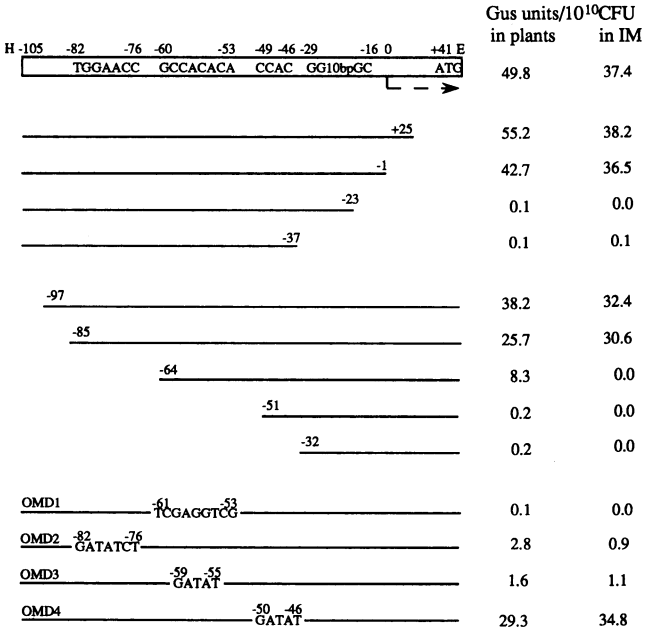

FIG. 6. Deletion and base substitution analysis of the *avrD* promoter. Deletions and mutant sequences were transcriptionally fused to the *gus* reporter gene of pLAFR6(*gus*) and transformed into *P. syringae* pv. glycinea race 4. *gus* activities are the means of three independent experiments and are expressed as β-glucuronidase units/10^{10} CFU.

avirulence genes from *P. syringae* pathovars (Fig. 7). In addition to the *avrD* gene of *P. syringae* pv. tomato, this sequence motif also occurs in the promoter regions of four *avrD* alleles from other *P. syringae* pathovars (31, 58) as well as in three transcriptional units closely linked to the *P. syringae* pv. tomato *hrp* cluster. To further test their importance, additional base substitutions were introduced into these two regions through site-directed oligomutagenesis. The mutant promoters OMD2 and OMD3 gave little expression, as predicted, when bacteria were grown in either IM or soybean leaves (Fig. 6). However, mutation of a second CCAC sequence motif occurring between positions −46 and

Avr gene	*P. s.* pathovar	sequence in the promoter region
avrD	*tomato*	CATGGAACCAAATCCGTCCCAAAGGCCACACAGAG
avrA	*glycinea*	GATGAAACCGAAACGGCGTTGCTTGCCACACAGCA
avrB	*glycinea*	CGTGGAACCTAATTCAGGGTAAATGCCACACAGCT
avrC	*glycinea*	CTTGGAACCGTTCTGCAACTCGTG_CCACTAAGCT
avrPto	*tomato*	CTTGGAACCGATCCGCTCCCTATGACCACTCAAGT
avrRpt2	*tomato*	GTGGGAACCCATTCATTGTTTGGAACCACCAACGG
avrPpi1	*pisi*	CAGGGAACTCATTTTCTTTTAAAA_CCACACATGT
avrRpm1	*maculicola*	CAGGGAACTCATTTTCTTTTAAAA_CCACACATGT
avrPph3	*phaseolicola*	TTTGGAACCGAATGGGTCAGCTGG___ACACTTAG

FIG. 7. Comparison of the *cis*-acting element of the *avrD* gene with other *P. syringae* (*P. s.*) avirulence gene promoter regions. Sequences important for *avrD* gene expression that occur in other avirulence genes are underlined. Sequence data for the various genes can be found in the following references: *avrD*, 30; *avrA*, 39; *avrB* and *avrC*, 51; *avrPto*, 42; *avrRpt2*, 16; *avrPpi1* and *avrRpm1*, 8; *avrPph3*, 20.

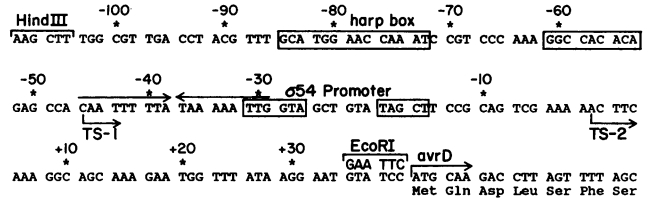

FIG. 8. Important elements identified in the 150-bp *Hin*dIII-*Eco*RI promoter sequence of the *avrD* gene from *P. syringae* pv. tomato. The *Eco*RI (GAATTC) site shown above the wild-type sequence was previously introduced by oligonucleotide site-directed mutagenesis (25) to facilitate manipulation of the promoter region. TS-1 and TS-2 denote the transcriptional start sites determined by primer extension experiments. Numbering is relative to TS-2. The function of the AT-rich palindromic sequence denoted by the arrows is unknown; however, the fully functional promoter region from the *avrD* allele of *P. syringae* pv. glycinea was previously shown to be similar to that of *P. syringae* pv. tomato, except for deletion of bases −52 to −43, which encompass part of the palindromic sequence (31).

−49 (OMD4) did not affect promoter activity. This result was also expected since the promoter of the *avrD* allele in *P. syringae* pv. glycinea lacks this sequence but is fully active (31, 45).

DISCUSSION

The interaction between plants and pathogens is a dynamic process involving signal exchanges between the two partners. For example, expression of the *avrD* gene in *P. syringae* or other gram-negative bacteria leads to the production of related elicitor molecules that trigger the HR in plants carrying the cognate *Rpg4* disease resistance gene (25, 35, 47). We have further shown in this paper that expression of the *avrD* gene only occurs in response to particular environmental conditions presumably occurring in plant leaves. Attempts to identify a specific plant inducer(s) of *avrD* gene expression, however, have thus far been unsuccessful. If there is a discrete plant signal molecule involved in induction, it must be unstable and/or present in trace amounts. Nonetheless, our results showed that the *avrD* promoter is expressed only when bacteria are grown in conditions presumably occurring in plants, such as low pH, low nitrogen, and containing only certain carbon sources. *hrp* genes in *P. syringae* pv. phaseolicola and *Xanthomonas campestris* pv. vesicatoria are also regulated by similar environmental cues and, possibly, by plant signal molecules (41, 44).

Several lines of evidence presented in this study demonstrate that *avrD* utilizes a promoter transcribed by the σ^{54} RNA polymerase holoenzyme (32, 52). This class of bacterial promoters with the consensus sequence (GG-10 bp-GC) was first recognized in genes involved in nitrogen assimilation but was subsequently shown to occur in other genes involved in specialized metabolic functions and environmental adaptation. A transcriptional start site (TS-2, Fig. 8) observed in all experiments with RNA prepared from bacteria grown in plants or IM is appropriately positioned relative to a near-consensus GG-10 bp-GC σ^{54} promoter sequence. The role of this putative promoter in *avrD* expression was confirmed by the loss of function observed in 3′ deletions which removed part of the element (Fig. 6). This indicates that the σ^{54} promoter is essential for *avrD* expression when bacteria are grown both in plants and in IM.

In some but not all experiments with RNA from Psg cells grown in IM, we observed a second transcriptional start site (TS-1, Fig. 8). It is not clear why this start site was only detected with RNA from bacteria grown in IM but not in plants or why it was not seen in all experiments. Of interest, TS-1 occurs immediately downstream of a DNA sequence conserved in many different *P. syringae* avirulence genes, as will be discussed later. Innes et al. (16) recently demonstrated that there is a similar but sole transcriptional start site similar to TS-1 for the avirulence gene *avrRpt2*. They utilized bacterial cells grown on IM, but did not extract RNA from bacteria grown in plants. The *avrRpt2* promoter region does not contain an identifiable σ^{54} promoter motif, explaining the absence of a TS-2 equivalent and raising the possibility that this gene may utilize a new type of promoter.

Expression of σ^{54} promoters typically requires an upstream *cis*-acting element and an activating protein (32). Our experiments showed that an upstream element occurring between positions −53 and −61 (Fig. 8) is required for transcription of the *avrD* promoter. Functional *hrpL* and *hrpS* genes are also required for *avrD* transcription. The *hrpS* gene contains the central domain present in the *ntrC* and *nifA* subclasses of activating proteins for σ^{54} promoters (12). It has been shown that the central domain of *nifA* is critical for interactions with the σ^{54} RNA polymerase holoenzyme. This interaction catalyzes the ATP-dependent isomerization of the closed σ^{54} holoenzyme-promoter complex into an open, transcriptionally active form (13). One peculiarity of the *avrD* σ^{54} promoter region is that the upstream element is located relatively close to the promoter. Generally, these elements occur 80 to 140 bp upstream of the promoter, presumably to permit DNA looping (32). However, the upstream element that we identified between positions −53 and −61 (Fig. 8) is presumably too close to the promoter to permit looping. This unusual feature therefore necessitates further work. However, while it has not been demonstrated that the *hrpS* gene product directly interacts with the *avrD* promoter and functions as an activator, preliminary results of gel retardation assays with extracts from wild-type *P. syringae* pv. glycinea cells showed two DNA-protein complexes specific to DNA carrying the *avrD* promoter that were absent when protein extracts from an *hrpS* mutant strain were used (45a). Possible interaction of the *hrpS* gene product with the *cis*-acting *avrD* promoter element identified in this work therefore merits further investigation.

The consensus *cis*-acting element required for *avrD* gene expression occurs in several different *P. syringae* avirulence genes (16 [Fig. 7]). Significantly, this element has recently been observed in the promoter regions of three transcriptional units occurring immediately adjacent to *hrpR/S* of the *P. syringae* pv. tomato *hrp* gene cluster (33a). As discussed earlier, this motif may also function as a promoter, per se, with certain *hrp*-regulated genes. The wide distribution of the upstream element raises the possibility that all of these avirulence genes may be specifically expressed in plants and may collectively contribute to bacterial fitness during the course of infection and colonization of plants. The consensus sequence for σ^{54} promoters (GG-10 bp-GC) is present in the promoters of some but not all of the *P. syringae* avirulence genes studied. Whether other avirulence genes also utilize a σ^{54} promoter therefore requires further analysis. σ^{54} promoters occur in genes involved with specialized metabolism or environmental adaptation (32, 52). It is therefore appealing to speculate that some avirulence genes may be involved with nutrient metabolism or other adaptations to

environmental conditions encountered during bacterial growth on or in plants.

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