Nucleotide Sequence and Properties of the *hrm*A Locus Associated with the *Pseudomonas syringae* pv. *syringae* 61 *hrp* Gene Cluster

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The *hrm*A locus, isolated from *Pseudomonas syringae* pv. *syringae* 61, is essential for phenotypic expression of the *P. s.* pv. *syringae* 61 *hrp* cluster in *Escherichia coli* strains and enables bacteria carrying the *hrp/hrm* gene cluster to elicit the hypersensitive response (HR) associated with plant disease resistance. The phenotype of *P. s.* pv *syringae* 61 *hrm*A mutants (pathogenicity$^+$, delayed HR) was distinct from that of *hrp* mutants. The locus was localized to a 3.6-kb *Bam*H1-*Eco*R1 fragment whose nucleotide sequence was determined. A single open reading frame was identified that encodes for a 41,457-Da protein of unknown biochemical function. Production of the deduced protein product was confirmed by using T7 RNA polymerase-directed expression of the locus and N-terminal sequence analysis of the isolated HrmA. The deduced protein product did not exhibit homology with any of the characterized *avr* genes or the *hrp*N product of *Erwinia amylovora*. Transcription was shown to initiate 37 nucleotides upstream of the translational start from an apparent σ^{70} promoter. Two *hrp* genes were shown to act as positive transcriptional factors for *hrm*A expression. Expression of *hrm*A in P. *syringae* pv. *glycinea* race 4 did not exhibit the phenotypic properties of an *avr* gene or HrpN, but suggested that this locus may serve a regulatory function. A homolog to *hrm*A was present in strains of only three of the 23 *P. syringae* pathovars tested.

Additional keywords: gene regulation, host range, virulence.

Pseudomonas syringae strains frequently elicit localized defense responses during incompatible interactions with nonhost plant species and resistant cultivars of susceptible plant species (Dixon and Lamb 1990; Slusarenko *et al.* 1991). These responses appear to limit further colonization of plant tissue and the establishment of disease. A rapid necrotic reaction, known as the hypersensitive response (HR), is thought to be a macroscopic manifestation of this process (Klement 1982). Huang *et al.* (1988) reported the isolation of a 30-kb DNA fragment from *P. syringae* pv. *syringae* 61 that enables nonpathogenic bacteria, such as *P. fluorescens* and *Escherichia coli* strains, to elicit an HR in tobacco. A 25-kb *hrp* gene cluster has been identified within this fragment (Huang *et al.* 1991). The *hrp* gene cluster consists of at least 13 apparent translational units organized as seven apparent transcriptional units (Hutcheson *et al.* 1993; Xiao *et al.* 1992; Huang *et al.* 1991). Associated with the *hrp* cluster is a phenotypically distinct locus that is required for the production of HR-eliciting signal in *E. coli*, but not in *P. syringae*. *P. s.* pv. *syringae* 61 mutants at this locus exhibit a delayed HR phenotype (36–48 hr) when inoculated into tobacco and appear to retain the weak pathogenicity in bean typical of the parent strain (Huang *et al.* 1991). This locus has been designated as *hrm*A for HR modulation.

The mechanism by which the *P. s.* pv. *syringae hrp/hrm* gene cluster produces an HR-eliciting signal has not been established. Wei *et al.* (1992) has reported that the *hrp*N locus associated with the *Erwinia amylovora hrp* cluster produces an exported protein, harpin, that elicits the HR. A homolog to the *E. amylovora hrp*N locus does not appear to be associated with the *P. s.* pv. *syringae* 61 *hrp/hrm* gene cluster or be present in the genome (Beer *et al.* 1993), although homologies between other regions of the two *hrp* clusters have been identified (Laby and Beer 1992). In several *P. syringae* strains, *avr* genes have been predicted to produce the HR-eliciting signal via an unknown mechanism (see Keen 1990). *Avr* genes appear to control race-cultivar interactions among *P. syringae* strains of the same pathovar and may function to limit the host range of *P. syringae* strains at the plant species level (Dong *et al.* 1991; Kobayashi *et al.* 1989). Current genetic models describing incompatible interactions frequently invoke *avr* genes to produce the postulated recognition signal (e.g., Fenselau *et al.* 1992; Keen 1990).

Because of its unique phenotype, the function and properties of *hrm*A are of interest. To further investigate the properties of *hrm*A, we have obtained the nucleotide sequence for the locus and characterized the physical and phenotypic properties of the gene product. Here we report: 1) the nucleotide sequence for *hrm*A; 2) the confirmation of the expected gene product and translational initiation by using a T7 RNA polymerase

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expression system; 3) the identification of the transcriptional initiation and termination sites for *hrm*A; 4) the regulation of *hrm*A expression by two *hrp* genes; 5) the distribution of *hrm*A among *P. syringae* strains; and 6) the analysis of the phenotypic properties of *hrm*A. A preliminary report of this work has been presented (Heu and Hutcheson 1991).

RESULTS

Nucleotide sequence of the *hrm*A locus.

The 3.6-kb *Bam*H1-*Eco*R1 fragment from pHIR11 to which *hrm*A mutations map was subcloned into pLAFR3 (Table 1) and found to be capable of complementing *P. s.* pv. *syringae* 61-2070. Transconjugants elicited a rapid HR in tobacco equivalent to that induced by *P. s.* pv. *syringae* 61 (data not shown). This fragment was then cloned into pBluescriptII SK$^+$ to create pSG1B, and the nucleotide sequence of the entire fragment was determined. A complete open reading frame (ORF1) beginning at nucleotide 705 and ending at base 1830 was identified within the fragment (Fig. 1). This ORF is consistent with the mapped insertion sites for *hrm*A::Tn*pho*A mutations 2070 and 2071 (Huang *et al.* 1991). A second apparent ORF, beginning near nucleotide 2080 and extending past the *Eco*R1 site (ORF2), was also identified. No substantial open reading frames were identified on the opposite strand.

To confirm that ORF1 is *hrm*A, pBSG1, a derivative of pSG1B in which nucleotides 2115–3662 were deleted, was tested for its ability to confer the capacity to elicit an HR upon *E. coli* G1045 (pHIR12). During other experiments, we had observed that G1045 carrying pHIR12 caused transient water-soaking symptoms in tobacco. Water-soaking symptoms were typically observed 12 hr after inoculation, and they dissipated after 30–48 hr. In contrast, G1045 (pHIR11) elicited an HR similar to that reported for *E. coli* MC4100 (pHIR11) (Li *et al.* 1992). G1045(pHIR12)(pBSG1), like G1045 (pHIR11), was able to elicit a rapid HR in tobacco. Little, if any, multiplication of G1045 (pHIR12) was detected *in planta*, irrespective of the presence of pBSG1 (data not shown).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Escherichia coli		
DH5α	*end*A1 *hsd*R17 (r_K^- m_K^-) *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 Δ(*arg*F-*lac*ZYA)U169 φ80d*lac*ZDM15	Bethesda Research Laboratory
MC4100	F′ *ara*D139 Δ(*arg*F-*lac*)U169 *rps*L159 *rel*A1 *flb*-5301 *pts*F25 *deo*C1	Casadaban 1976
G1045	F′ *his rps*L *rel*A *crp*::Cm^r *cya*854	A. Sankar, NCI-NIH
BL21 (DE3)	B strain F$^-$ *omp*T $r_b^-m_b^-$ *hsd*S *gal* (λDE3 c*Its*857 *int*l *Sam*7 *nin*5 *lac*UV5-T7 gene1)	Studier *et al.* 1990
Pseudomonas syringae		
pv. *syringae* 61	Wild type; Nx^r [a]	Huang *et al.* 1988
pv. *syringae* 61-2XXX[b]	61 marker exchange mutants carrying *hrp*/*hrm*::Tn*pho*A	Huang *et al.* 1991
pv. *glycinea* race 4	Wild type; Rf^r Am^r	Keen and Buzzell 1991
Plasmids		
pHIR11	Tc^r; *P. syringae* pv. *syringae hrm*A *hrp*I-XIII cloned into pLAFR3	Huang *et al.* 1988
pHIR12	Tc^r; *P. syringae* pv. *syringae hrp*I-XIII cloned into pLAFR3	Huang *et al.* 1988
pBluescriptII SK+	ColE1, *bla*	Stratagene
pSG1B	3.6-kb *Bam*H1-*Eco*R1 fragment cloned into pBluescript II SK+	This report
pBSG1	Deletion derivative of pSG1B carrying a 2.1-kb insert	This report
pET3a	pBR322 derivative carrying φ10 promoter	Studier *et al.* 1990
pVEX11f+T8/24	Derivative of pET3a	A. Sankar
pSG2V	1.55-kb *Alw*N1 fragment cloned into pVEX11f+T8/24	This report
pMLB1034	pBR322 derivative carrying promoterless *lac*Z	Silhavy *et al.* 1984
pSGAMS1	0.8-kb *Bam*H1-*Hin*cII fragment cloned into pMLB1034	This report
pLysS	*lys*S in pACY184	Studier *et al.* 1990
pRG960SD	IncP, promoterless *gus*A Sm^r/Sp^r	Van der Eede *et al.* 1992
pRG970	IncP, promoterless *lacZ* and *gusA* opposite orientation	Van der Eede *et al.* 1992
pSGR6	0.8-kb *Bam*H1-*Hin*cII fragment cloned into pRG960SD to create *hrm*A′-*gus*A transcriptional fusion	This report
pSGR7	0.8-kb *Bam*H1-*Hin*cII fragment cloned into pRG970 to create *hrm*A′-*lacZ* transcriptional fusion	This report

[a] Nx, nalidixic acid; Tc, tetracycline; Am, ampicillin.
[b] 2XXX, reference number for TnphoA mutation.

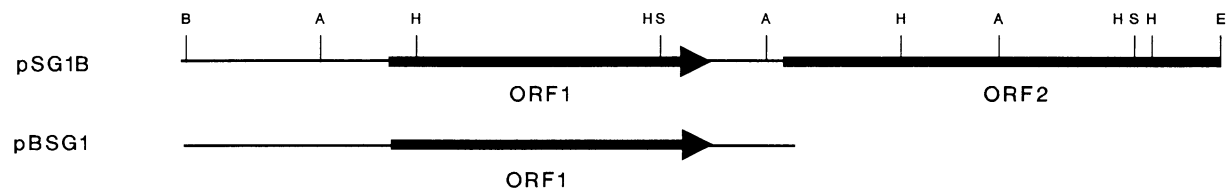

Fig. 1. Restriction map and organization of the *hrm*A region. Deduced open reading frames are indicated by filled arrows. The subclone pBSG1 was generated by exonuclease digestion of pSG1B as described in the text. **A,** *Alw*N1; **B,** *Bam*H1, **E,** *Eco*R1, **H,** *Hin*cII; **S,** *Sal*I.

The nucleotide sequence and the deduced protein product of *hrm*A are shown in Figure 2. A GUG codon is predicted to initiate translation. A potential ribosome binding site, consisting of AAGGAG (Miller 1992), is located six bases upstream of the apparent translational start codon. The deduced protein product contains 375 amino acid residues with an estimated molecular weight of 41,457. The ORF ends at a TGA codon. The deduced protein product lacks a signal sequence typical of proteins exported by *sec*-dependent mechanisms (Pugsley 1989). Hydropathy profiles of the predicted amino acid residues did not reveal hydrophobic domains typical of membrane proteins (Pugsley 1989). The MOTIFS algorithm employing PROSITE version 9.2 did not indicate any peptide domains of known function.

T7 RNA polymerase-directed expression of *hrm*A.

The HrmA product was visualized by using T7 RNA polymerase to direct the expression of the locus (Studier *et al.* 1990). The presence of a potential ribosome binding site with homology to the *E. coli* consensus sequence suggested that the expressed mRNA should be translated well

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                                                                AlwNI
421 TTGCTGATTGGTCTGCTTGAGCCACACGAGGATCTACCCTTGCAGCGCCTGTTGGCTGGC 480

481 GCTCTCAACCCCCTTGTGAATGCCGGCCCCGCATTGGCTGGGATGAGCAAAGCGGCCTGT 540

541 ACCACGCTTACCAAAGCATCCCGCGGGAAAAAGTCAGCGTGGAGATGCTGAAGCTCGAAA 600

601 TTTGCAGGATTCGGTCGAATGGATGAAGTGTTGGCGAGAAGCCGCACGTGACCGCGCTTT 660
                                  -35
                  **
661 CAATCTTCCGACGTATTTCAACTACTCTTGCTTAGCGTAAGGAGCTTTTAGTGAACCCTA 720
    -10                                  RBS
                                                    M  N  P  I
721 TCCATGCACGCTTCTCCAGCGTAGAAGCGCTCAGACATTCAAACGTTGATATTCAGGCAA 780
      H  A  R  F  S  S  V  E  A  L  R  H  S  N  V  D  I  Q  A  I
781 TCAAATCCGAGGGTCAGTTGGAAGTCAACGGCAAGCGTTACGAGATTCGTGCGGCCGCTG 840
      K  S  E  G  Q  L  E  V  N  G  K  R  Y  E  I  R  A  A  A  D
841 ACGGCTCAATCGCGGTCCTCAGACCCGATCAACAGTCCAAAGCAGACAAGTTCTTCAAAG 900
      G  S  I  A  V  L  R  P  D  Q  Q  S  K  A  D  K  F  F  K  G
901 GCGCAGCGCATCTTATTGGCGGACAAAGCCAGCGTGCCCAAATAGCCCAGGTACTCAACG 960
      A  A  H  L  I  G  G  Q  S  Q  R  A  Q  I  A  Q  V  L  N  E
961 AGAAAGCGGCGGCAGTTCCACGCCTGGACAGAATGTTGGGCAGACGCTTCGATCTGGAGA 1020
      K  A  A  A  V  P  R  L  D  R  M  L  G  R  R  F  D  L  E  K
1021 AGGGCGGAAGTAGCGCTGTGGGCGCCGCAATCAAGGCTGCCGACAGCCGACTGACATCAA 1080
      G  G  S  S  A  V  G  A  A  I  K  A  A  D  S  R  L  T  S  K
1081 AACAGACATTTGCCAGCTTCCAGCAATGGGCTGAAAAAGCTGAGGCGCTCGGGCGATACC 1140
      Q  T  F  A  S  F  Q  Q  W  A  E  K  A  E  A  L  G  R  Y  R
1141 GAAATCGGTATCTACATGATCTACAAGAGGGACACGCCAGACACAACGCCTATGAATGCG 1200
      N  R  Y  L  H  D  L  Q  E  G  H  A  R  H  N  A  Y  E  C  G
1201 GCAGAGTCAAGAACATTACCTGGAAACGCTACAGGCTCTCGATAACAAGAAAAACCTTAT 1260
      R  V  K  N  I  T  W  K  R  Y  R  L  S  I  T  R  K  T  L  S
1261 CATACGCCCCGCAGATCCATGATGATCGGGAAGAGGAAGAGCTTGATCTGGGCCGATACA 1320
      Y  A  P  Q  I  H  D  D  R  E  E  E  E  L  D  L  G  R  Y  I
1321 TCGCTGAAGACAGAAATGCCAGAACCGGCTTTTTTAGAATGGTTCCTAAAGACCAACGCG 1380
      A  E  D  R  N  A  R  T  G  F  F  R  M  V  P  K  D  Q  R  A
1381 CACCTGAGACAAACTCGGGACGACTTACCATTGGTGTAGAACCTAAATATGGAGCGCAGT 1440
      P  E  T  N  S  G  R  L  T  I  G  V  E  P  K  Y  G  A  Q  L
1441 TGGCCCTCGCAATGGCAACCCTGATGGACAAGCACAAATCTGTGACACAAGGTAAAGTCG 1500
      A  L  A  M  A  T  L  M  D  K  H  K  S  V  T  Q  G  K  V  V
1501 TCGGTCCGGCAAAATATGGCCAGCAAACTGACTCTGCCATTCTTTACATAAATGGTGATC 1560
      G  P  A  K  Y  G  Q  Q  T  D  S  A  I  L  Y  I  N  G  D  L
1561 TTGCAAAAGCAGTAAAACTGGGCGAAAAGCTGAAAAAGCTGAGCGGTATCCCTCCTGAAG 1620
      A  K  A  V  K  L  G  E  K  L  K  K  L  S  G  I  P  P  E  G
1621 GATTCGTCGAACATACACCGCTAAGCATGCAGTCGACGGGTCTCGGTCTTTCTTATGCCG 1680
      F  V  E  H  T  P  L  S  M  Q  S  T  G  L  G  L  S  Y  A  E
1681 AGTCGGTTGAAGGGCAGCCTTCCAGCCACGGACAGGCGAGAACACACGTTATCATGGATG 1740
      S  V  E  G  Q  P  S  S  H  G  Q  A  R  T  H  V  I  M  D  A
1741 CCTTGAAAGGCCAGGGCCCCATGGAGAACAGACTCAAAATGGCGCTGGCAGAAAGAGGCT 1800
      L  K  G  Q  G  P  M  E  N  R  L  K  M  A  L  A  E  R  G  Y
1801 ATGACCCGGAAAATCCGGCGCTCAGGGCGCGAAACTGAAATCGGGGCTAAATAGCTGACA 1860
      D  P  E  N  P  A  L  R  A  R  N  *
1861 CACTCACTGCCCTTATTACCTGTGTGGTCCTGAGATAGATTTATACCTGTTTCACACTTA 1920

1921 AAACGCTCGATGCACCGCATCGGGTTTTCTATTTCAGGGGTGTTTAGCAGTTCATTAAAA 1980
                                                    AlwNI
1981 GCCGGAACAGTACTGAGCGCCGCAGCCTGAATGCTGAATCAGCCTCTGATCGGTTTTTTG 2040
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Fig. 2. Nucleotide sequence of the 3,662 bp region of the *P. syringae* pv. syringae 61 *hrp/hrm* gene cluster containing *hrm*A. The deduced amino acid sequence is shown by single letter codes below. A potential ribosome binding site is indicated by the underlined bold characters. The transcriptional start site is shown by the asterisks. A potential σ^{70} promoter is shown in overlined bold characters. A deduced terminator is shown by the inverted arrows.

in *E. coli*. The *hrm*A locus was subcloned as a 1.55-kb *Alw*N1 fragment into pVEX11f + T8/24, which had been cut with *Xba*I beforehand to remove the vector's ribosome binding site, to create pSG2V and expression of the *hrm*A locus driven by the vector's T7 promoter after transformation into *E. coli* BL21 (DE3)(pLysS). Lysates of IPTG-induced cells contained a novel 40 + 1-kDa protein that was associated with expression of the *hrm*A locus (Fig. 3). This protein was readily detectable in polyacrylamide gels stained with Coomassie blue and appeared to be resolved from other proteins. Most of the apparent HrmA protein was found in the precipitate fraction after centrifugation, suggesting that the protein forms inclusion bodies when expressed at this level.

To confirm the identification of HrmA and its deduced translation start codon, the N-terminal sequence of the 40-kDa protein was obtained. The N-terminal sequence (MNPIHAR) agreed with the deduced N-terminal sequence for ORF1 as shown in Figure 2 (data not shown).

Homology of HrmA with other proteins.

The deduced protein sequence of HrmA was compared with that of previously characterized *avr* genes and the *E. amylovora hrp*N locus. No significant homology was found with *avr*A (Napoli and Staskawicz 1987), *avr*B and *avr*C (Tamaki *et al.* 1988), *avr*Bs1 (Ronald and Staskawicz 1988), *avr*Bs2 (Kearney and Staskawicz 1990), *avr*Bs3 (Bonas *et al.* 1989), and *avr*Rpt2 (R. Innes, personal communication) or *hrp*N (Wei *et al.* 1992). FASTA and TFASTA searches of SwissProt release 24, GenBank release 73.2, EMBL release 34 databases did not reveal any substantial homology with proteins of known biochemical function.

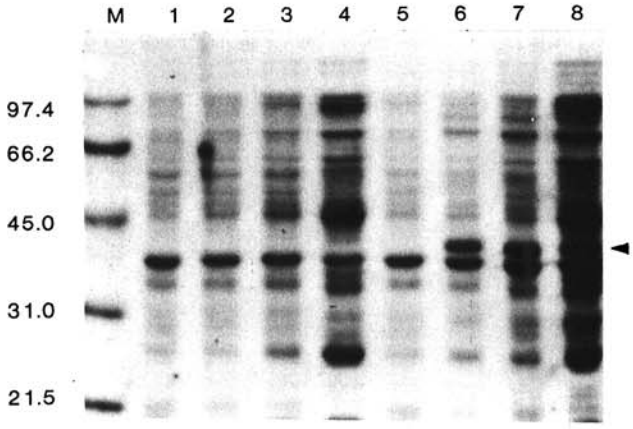

Fig. 3. T7 polymerase-directed expression of *hrm*A. *E. coli* BL21 (DE3) (pLysS) was transformed with the vector pVEX11f+T8/24 alone (lanes 1–4) or with the *hrm*A locus cloned as a 1.55-kb AlwN1 fragment in pVEX11f+T8/24 (lanes 5–8). Bacteria were harvested after induction of T7 RNA polymerase with 1 mM IPTG for 0 (lanes 1, 5), 1.5 (lanes 2, 6), and 3 (lanes 3,4,7,8) hr and lysed by freeze-thaw/sonication. The whole lysate (lanes 4,8) or the precipitate collected by centrifugation for 20 min at 2,000 (lanes 2–3, 5–7) were fractionated by SDS-polyacrylamide gel electrophoresis and proteins stained with Coomassie Blue. A protein unique to strains expressing the *hrm*A locus is indicated by the arrow.

Transcription initiation and termination.

Promoter activity of the upstream region from *hrm*A was demonstrated by ligating the *Bam*H1–*Hin*cII fragment carrying 705 nt upstream of *hrm*A and the sequence encoding the N-terminal 33 amino acids of *Hrm*A into pMLB1034 to create a translational fusion between ORF1 and the truncated *lac*Z gene of the vector. *E. coli* MC4100 (pSGAMS1) produced intensely blue colonies when cultured on M63M agar containing X-Gal.

The transcriptional start site was mapped by using primer extension. A synthetic oligonucleotide, corresponding to the opposite strand from nucleotides 713 to 729 (5′ GTGCATGGATAGGGTTC), was used to initiate synthesis of a cDNA from isolated RNA. Transcription was shown to initiate 37 nt upstream of the GUG translational start codon in *P. s.* pv. *syringae* 61 (pHIR11) and in *E. coli* MC4100 (pBSG1)(pHIR12) (Fig. 4). In wild-type *P. s.* pv. *syringae* 61 and MC4100(pBSG1), transcript levels were below the sensitivity of the assay irrespective of culture conditions. It was, therefore, necessary to use strains carrying plasmid-borne *hrm*A constructs. Similar

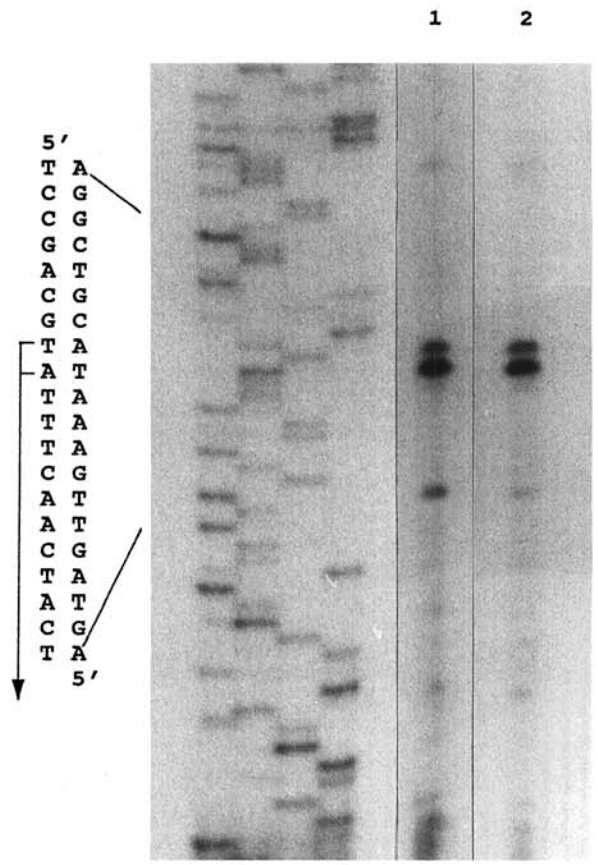

Fig. 4. Determination of the transcriptional start site of *hrmA* by primer extension. Total RNA was isolated from *P. syringae* pv. syringae 61(pHIR11) (lane 1) or *E. coli* MC4100 (pHIR12) (pBSG1) (lane 2) as described in the text. A ^{32}P-end-labeled synthetic oligonucleotide primer, corresponding to the stand opposite base positions 718-735, was annealed to the RNA at 42° C and a cDNA produced by using aAMV reverse transcriptase. The cDNA product was electrophoresced in parallel with G/A/T/C sequence reactions generated by using the same primer.

results were obtained irrespective of the host strain or the plasmid construct. In both pBSG1 and pHIR11, *hrm*A is separated from vector sequences by 670 bp which contains two intervening, apparent, *rho*-independent transcription terminators. The plasmid pHIR12 was included in the experiments employing MC4100 because other work had shown *hrm*A expression to be dependent on the activity of other *hrp* genes (see below).

Sequences that exhibit partial homology with the −10 and −35 regions associated with σ^{70}-type promoters (see Fig. 2; Directic *et al.* 1989) are located upstream of the apparent transcriptional start site. No properly positioned σ^{54}-like promoter sequences were apparent. Consistent with this observation, the *hrm*A′-*lac*Z fusion carried by pSGAMS1 was expressed in an *E. coli rpo*N mutant at levels equivalent to the wild-type strain (data not shown). No sequences homologous to the "*hrp* box" described by Fellay *et al.* (1989) were associated with this region. Approximately 523 nucleotides upstream of the transcriptional start site is an apparent "*avr* box" (KGGAACC (N)16 CCAC; Innes *et al.* 1993). Seventy seven nucleotides downstream of the apparent termination codon is a set of inverted repeats followed by a T-rich region typical of a *rho*-independent terminator (Miller 1992).

Induction of *hrm*A′–*uid*A expression *in planta*.

Several *P. s.* pv. *syringae* 61 *hrp* genes associated with *hrm*A have been shown to be environmentally regulated by nutritional conditions (Xiao *et al.* 1992). To study the environmental regulation of *hrm*A, the 804-bp promoter-active *Bam*H1-*Hin*cII fragment upstream of the *hrm*A ORF was cloned into pRG960SD to create a transcriptional fusion with *gus*A and the resulting plasmid, pSGR6, transformed into *P. s.* pv. *syringae* 61. *P. s.* pv. *syringae* 61 (pSGR6) exhibited low level expression from the construct during growth in KB medium. Upon inoculation into tobacco leaves, expression increased sixfold during the initial 3 hr of the interaction (Fig. 5). Previous studies had established that the induction stage for the HR elicited by this strain in tobacco was 2 hr (Xiao *et al.* 1992).

Nutritional regulation of *hrm*A expression.

As with the *hrp* genes previously studied in this strain (Xiao *et al.* 1992), the observed induction appeared to result from a shift in the nutritional conditions. *hrm*A′-driven expression of *gus*A increased within 1 hr after

Table 2. Effect of carbon source on the expression of plasmid-borne *hrmA′-lacZ* fusion

Medium	β-Galactosidase activity[a] (units)
King's B	3 ± 1
M9	
Fructose[b]	460 ± 49
Glucose[c]	39 ± 4
Sucrose	10 ± 1
Fructose + glucose	42 ± 3
Fructose + peptone[d]	2 ± 1

[a] The indicated medium (pH 7.0) was inoculated with *P. syringae* pv. *syringae* 61 (pSG7) at 5×10^7 cells/ml and incubated for 9 hr. Cells were harvested and β-galactosidase activity determined as described in the text. No significant differences in basal β-galactosidase activity was observed in the various media when *P. syringae* pv. *syringae* 61 (pRG970) was employed.

[b] Sugar concentration was 20 mM.

[c] In a parallel experiment, the β-galactosidase activity levels detected when the following carbon sources were substituted for glucose were (Miller units): mannitol (104), myo-inositol (34), glycerol (10), pyruvate (10), oxalacetic acid (10), and succinate (44).

[d] Peptone concentration was 1%. Similar results were obtained when Casamino Acids were substituted.

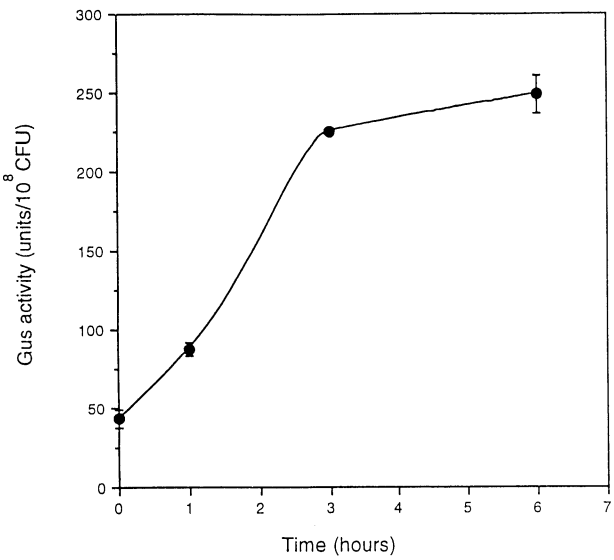

Fig. 5. Induction of *hrm*A expression following inoculation of tobacco leaves. A suspension of *P. syringae* pv. syringae 61 (pSGR6) (10^9 cells/ml) was inoculated into tobacco leaves and GUS activities and bacterial populations measured at the indicated time as described in the text. The values reported represent the means of three replicates. Error bars indicate standard deviations.

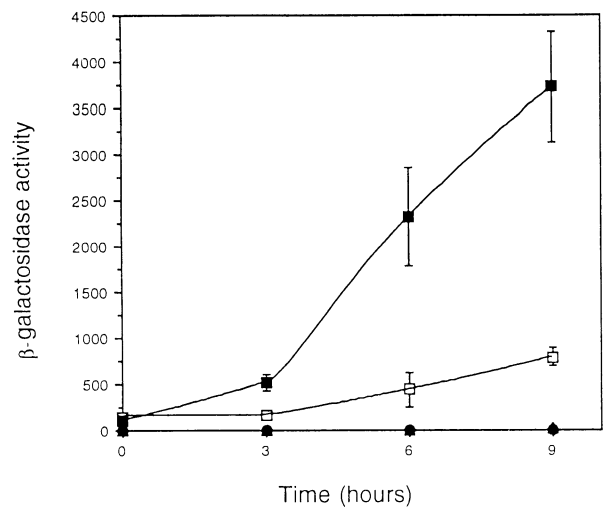

Fig. 6. Effect of *hrp* genes on the nutritional regulation of *hrm*A in *E. coli* MC4100. KB (open symbols) or M63M (closed symbols) media were inoculated with washed MC4100 (pSGSMS1) (triangles) or MC4100 (pSGMS1) (pHIR12) (Boxes) to 5×10^7 cells per milliliter. At the indicated times, cells were harvested and β-galactosidase activity determined as described in the text. The values reported represent the mean of three replicates.

transfer of *P. s.* pv. *syringae* 61 (pSGR6) to a minimal salts medium and after 3 hr was fivefold higher than when the strain was maintained in KB medium (data not shown). Similar results were obtained when *P. s.* pv. *syringae* 61 (pSGR7) was tested for *hrm*A′-driven expression of *lac*Z (see Table 2).

Induction of the *hrm*A′–*lac*Z expression in *P. s.* pv. *syringae* 61 (pSGR7) was dependent on the carbon source. Maximal expression was observed in an M9 medium containing fructose as the sole carbon source (Table 2). Significantly less expression was observed in media containing other carbon sources or complete amino acid sources, such as peptone or Casamino Acids. Substitution of fructose for the glycerol in KB medium did not enhance the expression of the construct in this medium. Growth on sucrose produced only moderate levels of *hrm*A′–*lac*Z expression. To determine if *hrm*A expression is subject to a form of catabolite repression, the influence of glucose and fructose on *hrm*A′-driven expression of *lac*Z was investigated. The addition of glucose to M9 medium containing fructose suppressed expression of the construct to levels equivalent to that observed in media containing glucose alone (Table 2).

Phenolic compounds have been shown to control the expression of *vir* genes in *Agrobacterium* (Peters and Verma 1990), *nod* genes in rhizobial bacteria (Horvath *et al.* 1987), and *syr* genes in some *P. syringae* strains (Mo and Gross 1991). Compounds representative of the phenolic compounds functioning in the regulation of the aforementioned genes (e.g., arbutin, esculin, salicin, flavone, 3HO-flavone, narigenin, narigin, quercitin, rutin, acetosyringone, catechin, and chlorogenic acid) were screened for their effect on *hrm*A′–*lac*Z expression. Addition of up to 100 μM esculin, salicin, or chlorogenic acid to the medium only caused 30–35% increases in expression of the fusion, irrespective of whether glucose or fructose was used as the carbon source (data not shown; c.f., Mo and Gross 1991). The other compounds had little, if any, effect on *hrm*A promoter activity. Since the magnitude of these effects was small relative to the responses induced by the shift to a minimal medium containing fructose or to the effect of these compounds in other bacteria, it is unlikely that the observed effects of these phenolic compounds are significant.

Table 3. Induction of *hrmA′-lacZ* expression in *E. coli* MC4100 by *P. syringae* pv. *syringae* 61 genes

Plasmids[a]	Mutation	β-Galactosidase activity[b]
None	None	70 ± 17
pHIR12	None	2,945 ± 300
pHIR11-2070	*hrm*A::Tn*pho*A	3,511 ± 230
pHIR11-2074	*hrp*II::Tn*pho*A	37 ± 13
pHIR11-2075	*hrp*III::Tn*pho*A	4,058 ± 537
pHIR11-2077	*hrp*IV::Tn*pho*A	2,891 ± 736
pHIR11-2081	*hrp*V::Tn*pho*A	3,417 ± 771
pHIR11-2084	*hrp*VI::Tn*pho*A	4,817 ± 189
pHIR11-2086	*hrp*VII::Tn*pho*A	3,753 ± 970
pHIR11-2087	*hrp*VIII::Tn*pho*A	3,620 ± 537
pHIR11-2088	*hrp*IX::Tn*pho*A	3,620 ± 537
pHIR11-2089	*hrp*X::Tn*pho*A	3,759 ± 884
pHIR11-2091	*hrp*XI::Tn*pho*A	5,103 ± 261
pHIR11-2092	*hrp*XII::Tn*pho*A	3,224 ± 713
pHIR11-2094	*hrp*XIII::Tn*pho*A	32 ± 12

[a] MC4100 (pSGAMS1) carrying the indicated plasmid.
[b] M63M medium was inoculated with each bacterium to 5×10^7 cells per millimeter. After 18 hr, cells were harvested and β-galactosidase activity determined as described in the text.

Role of *hrp* genes in the nutritional regulation of *hrp* genes.

E. coli MC4100 (pSGAMS1) failed to exhibit any effect of culture medium on *hrm*A′–*lac*Z expression. Transformation of MC4100 (pSGAMS1) with pHIR12 caused a 60-fold increase in the basal expression observed from the *hrm*A′–*lac*Z fusion in KB medium and restored the induction observed in M9 medium. The *hrm*A′–*lac*Z expression increased 35-fold upon transfer of MC4100 (pSGAMS1)(pHIR12) to M9 medium (Fig. 6).

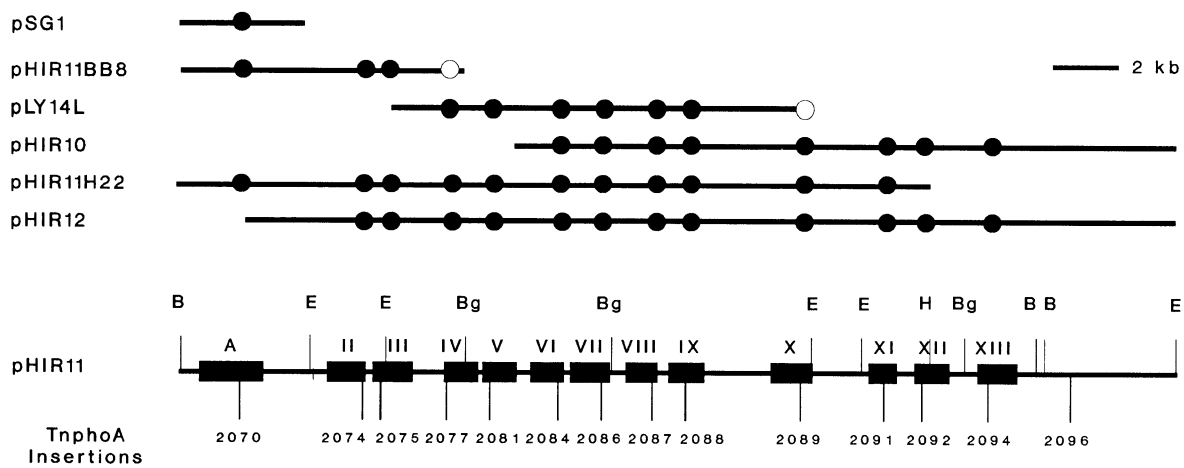

Fig. 7. Subclones and pHIR11::Tn*phoA* derivatives tested for their effect on *P. syringae* pv. *glycinea* race 4 virulence. All subclones use pLAFR3 as the vector. Filled circles indicate that the subclone complements the corresponding *P. s.* pv. *syringae* 61::Tn*phoA* mutant (2070-2094) described by Huang et al. (1991). The subclones or pHIR11::Tn*phoA* derivatives carrying the indicated insertions were transferred into *P. s.* pv. *glycinea* race 4 by triparental matings and transconjugants screened for their virulence in soybean cultivars Linderin, Harosoy, Flambeau, Merit, Norchief, Chippewa, Acme, and Centennial. All transconjugants produced water soaking and flecked necrosis symptoms typical of disease after 72 hr except the control strain *P. s.* pv. glycinea race 4 (pHIR11-2096), which produced the HR in inoculated regions.

To identify which *hrp* genes within the cluster are mediating the nutritional regulation of *hrm*A in MC4100 (PSGAMS1)(pHIR12), pHIR11::Tn*pho*A derivatives were transformed into MC4100 (pSGAMS1) and expression of the *hrm*A′-*lac*Z construct monitored in M9 medium. Insertional inactivation of complementation groups II and XIII (sensu Xiao *et al.* 1992) reduced β-galactosidase levels to that observed in the absence of *hrp* genes (Table 3).

Identification of *hrp* "XIII" as a homolog of *P. s.* pv. *phaseolicola hrp*RS.

The *P. s.* pv. *phaseolicola hrp*RS region has previously been shown to produce a positive-acting transcriptional factor (Grimm and Panopoulos 1989). To determine if either *hrp*II or *hrp*XIII are homologous to the *hrp*RS region, a 0.95-kb *Nde*I-*Hin*dIII fragment carrying *hrp*R (a gift of N. Panopoulos) was used to probe a set of subclones of the *hrp/hrm* cluster. Only subclones carrying *hrp* "XIII" hybridized to the *hrp*R probe (data not shown).

Phenotypic properties of *hrm*A.

To determine if *hrm*A exhibits the phenotypic traits of an *avr* gene, plasmids carrying *hrm*A alone or in combination with one or more *hrp* genes were transformed into *P. s.* pv. *glycinea* race 4 and the reaction of soybean cultivars screened. *P. s.* pv. *glycinea*–soybean interactions have been used previously to detect *avr* genes from heterologous *P. syringae* strains (e.g., Keen and Buzzell 1991; Kobayashi *et al.* 1989). As observed previously with other *P. syringae* strains (Huang *et al.* 1988; Hutcheson *et al.* 1993), the avirulence activity associated with pHIR11 was phenotypically expressed in *P. s.* pv. *glycinea* race 4. Confluent tissue collapse typical of the HR was observed after 16 hr when *P. s.* pv. *glycinea* race 4 (pHIR11) was inoculated into any of the soybean cultivars at inocula levels larger than 10^7 cells per milliliter. Only limited multiplication (100-fold) of *P. s.* pv. *glycinea* (pHIR11) was observed in soybean leaves during the initial 36 hr of the interaction. In comparison, wild-type populations of *P. s.* pv. *glycinea* race 4 increased 10^6-fold during this period (data not shown). There was no cultivar specificity to the soybean reaction produced by *P. s.* pv. *glycinea* race 4 (pHIR11) (c.f. Kobayashi *et al.* 1989). A comprehensive set of subclones carrying *hrm*A alone or in combination with adjacent *hrp* genes was then tested for an effect on the virulence of *P. s.* pv. *glycinea* race 4. Each of the subclones was capable of complementing at least one of the known *hrp* or *hrm* genes in *P. s.* pv. *syringae* 61 and all known *hrp* and *hrm* genes were collectively represented in the set (Fig. 7). *P. s.* pv. *glycinea* race 4 transconjugants carrying any of the pHIR11-derived subclones retained virulence and caused typical disease symptoms after 3 days. To confirm the expression of *hrm*A in *P. s.* pv. *glycinea* race 4, *hrm*A′-directed expression of *gus*A was monitored in *P. s.* pv. *syringae* 61 and *P. s.* pv. *glycinea* race 4 transformed with pSGR6. Similar levels of expression were detected in both strains (data not shown).

Because of the possibility that the avirulence activity produced by pHIR11 in *P. s.* pv. *glycinea* race 4 may depend on one or more of the *P. s.* pv. *syringae* 61 *hrp* genes in addition to *hrm*A, pHIR11::Tn*pho*A derivatives were introduced into *P. s.* pv. *glycinea* race 4 by triparental mating, and the response of the soybean cultivars to the transconjugants was screened. *P. s.* pv. *glycinea* race 4 transconjugants carrying pHIR11::Tn*pho*A derivatives in

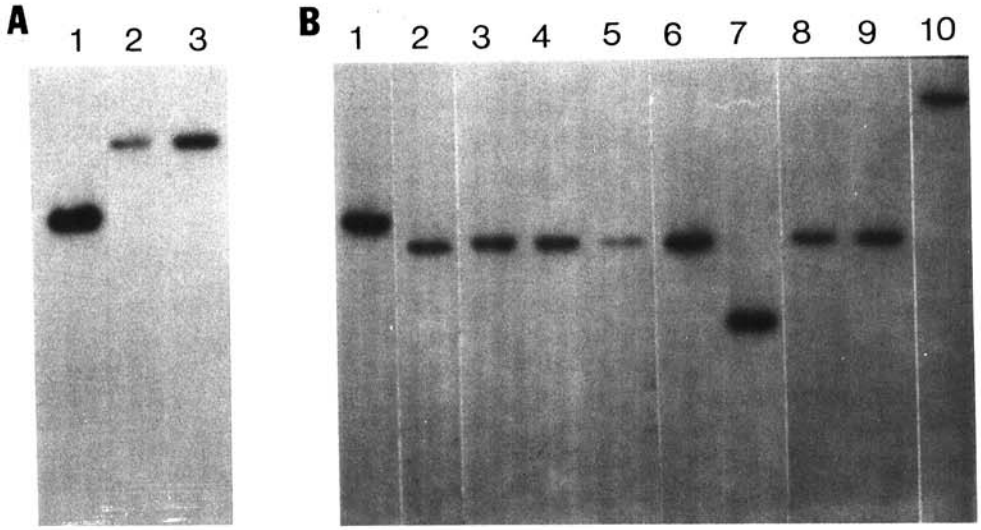

Fig. 8. Distribution of *hrm*A among *P. syringae* strains. Genomic DNA from 142 *P. syringae* strains were digested with *Eco*R1, fractionated by agarose gel electrophoresis and transferred to a blotting membrane (a gift of T. Denny). Hybridizations, employing the ^{32}P-labeled 1.55-kb *Alw*N1 fragment carrying the *hrm*A locus, were performed as described in the text. Selected hybridizing strains are shown. **A.** lane 1) *P.s.* pv. *syringae* 61; 2) *P.s. papulans*; 3) *P.s. lachrymans*. **B.** *P.s.* pv. *syringae* strains. Lane 1) strain 61; 2) sieva bean isolate; 3-6) tomato isolates; 7) okra isolate; 8,9) soybean isolates; 10) pepper isolate. Strains classified into the following pathovars did not hybridize to the *hrm*A probe: *antirrhini, atrofaciens, atropurpurea, berberidis, coronafaciens, delphinii, glycinea, hibisci, maculicola, mori, morsprunorum, passiflorae, persicae, phaseolicola, pisi, savastanoi, striavaciens, tabaci, tagetis,* and *tomato*. A list of strains not hybridizing is available upon request.

which each of the known *hrp* or *hrm* genes were individually inactivated by transposon insertion (Fig. 7) retained virulence in the soybean cultivars tested and produce disease symptoms after 3 days. In *P. s.* pv. *glycinea* race 4, pHIR11 was stably maintained *in planta* for at least 72 hr.

Presence of *hrm*A in other *P. syringae* strains.

Total genomic DNA's isolated from 142 strains of *P. syringae* representing 23 pathovars and 120 unique isolates of *P. s.* pv. *syringae* were probed by Southern blot analysis with the 1.55-kb *Alw*N1 fragment carrying *hrm*A. Of the strains screened, those representing only three pathovars hybridized with the probe: *P. s.* pv. *syringae*, *P. s.* pv. *papulans*, and *P. s.* pv. *lachrymans* (Fig. 8). A homolog to the *hrm*A locus was detected in only 28 of the *P. syringae* pv. *syringae* strains tested under the experimental conditions. A majority of these strains were collected in the southern United States and were isolated from a variety of citrus or vegetable crops (T. Denny, personal communication). All strains tested exhibited homology to the remaining *P. s.* pv *syringae* 61 *hrp* region (T. Denny, personal communication). No linkage to host range could be established, but many of the strains carrying a *hrm*A homolog were isolated as epiphytes. The strains of *P. s.* pv. *phaseolicola*, *P. s.* pv. *glycinea,* and *P. s.* pv. *tomato* tested did not carry a homolog to *hrm*A. *Hrm*A did not cross hybridize with *avr*Psp2 gene that is associated with the *P. s.* pv. *phaseolicola* race 2 *hrp* gene cluster (a gift of J. Mansfield).

DISCUSSION

The *P. s.* pv. *syringae* 61 *hrp/hrm* gene cluster forms a minimum genetic unit sufficient to enable nonpathogenic bacteria to elicit the HR. The properties and function of the genes contributing to production of the HR^+ phenotype are, therefore, of interest. The *hrm*A locus has been shown to be essential for the production of the HR^+ phenotype in nonpathogenic bacteria, such as *P. fluorescens* (Huang *et al.* 1989). Tn*pho*A mutations with the *hrm*A phenotype had been localized to a 3.6-kb *Bam*H1–*Eco*R1 fragment bordering the *P. s.* pv. *syringae* 61 *hrp* cluster (Huang *et al.* 1991). The nucleotide sequence of this fragment and directed expression of the locus indicates that *hrm*A encodes a single 41,456-Da polypeptide. The deduced amino acid sequence of *hrm*A, however, was devoid of features indicative of function or compartmentation. The deduced protein product of *hrm*A lacks a signal sequence and hydrophobic domains typical of Sec-exported or membrane proteins. A survey of the current databases and direct comparison to the known *avr* genes and *E. amylovora hrp*N did not reveal other proteins of known biochemical function with significant sequence homology.

Prior characterization of the *hrp/hrm* gene cluster had failed to define the *hrm*A transcriptional unit (Huang *et al.* 1991; Xiao *et al.* 1992). By using primer extension, transcription was shown to initiate 37 nt upstream of the initiation codon for the *hrm*A ORF and appears to terminate approximately 90 nt downstream of the stop codon at a *rho*-independent terminator. Identical transcriptional initiation sites were identified in both the *E. coli* and *P. syringae* strains tested, irrespective of the resident plasmid construction. Two apparent *rho*-independent terminators were identified in the region separating *hrm*A from vector sequences that could inhibit vector-directed expression of the locus in these plasmids. The phenotypic expression of the cluster in *E. coli* to produce the HR^+ phenotype and the retention of the nutritional regulation in *E. coli* suggests that the deduced transcriptional signals are functional. The *hrm*A locus thus appears to be monocistronic.

Expression of *hrm*A in *P. syringae* appears to be directed by an atypical σ^{70}-type promoter. The deduced –35 and –10 regions were separated by 22 nucleotides instead of 17 + 1 spacing most frequently observed in *E. coli* (Miller 1992) and *Pseudomonas* σ^{70}-type promoters (Directic *et al.* 1989). The larger spacing between these regions may indicate a dependence on other trans-acting transcriptional factors for expression (Gottesman 1984). Consistent with this interpretation, we have observed that *hrm*A expression is dependent on two *hrp* genes. The *hrm*A transcript could only be detected in *E. coli* MC4100 when the *hrp* cluster is present and inactivation of *hrp* regions II and XIII blocked the *hrp*-dependent expression of a *hrmA′–lacZ* fusion. The *hrm*A promoter appears to be distinct from the σ^{54}-dependent promoters deduced to control the expression of several *P. s.* pv. *phaseolicola hrp* genes (Fellay *et al.* 1989; Grimm and Panopoulos 1989) and the *P. s.* pv. *syringae* 61 *hrp*H locus (Huang *et al.* 1992).

The environmental regulation of *hrm*A appears similar to that of the *P. s.* pv. *syringae* 61 *hrp*J locus. Enhanced expression can be detected approximately 1 hr after inoculation of tobacco leaves for both loci (Xiao *et al.* 1992). Enhanced expression of both loci could also be observed upon transfer of the strain to a minimal salts medium which was similar in magnitude to that observed *in planta*. Expression was suppressed by broad spectrum amino acid sources, such as Casamino Acids or peptone. No evidence for the involvement of a plant factor in the regulation of *hrm*A was detected.

The environmental regulation of *hrm*A in *P. s.* pv. *syringae* 61 was linked to the carbon source. Growth in a medium containing fructose supported the highest level of *hrm*A expression; lowest expression was observed in media containing peptone or Casamino Acids. Media containing sucrose or glucose produced intermediate levels of expression. Typical of the classical catabolite repression observed in enteric bacteria (Magasanik and Neidhardt 1987), the apparent stimulation of *hrm*A promoter activity by fructose could be reversed by the addition of glucose to the medium. HrmA expression thus appears to be regulated at two levels: repression by broad spectrum amino acid sources and activation linked to carbon source. Uptake of fructose by *P. aeruginosa* is mediated by a PEP:fructose 1-phosphotransferase system and is the only sugar imported by this pathway in this bacterium (Lessie

and Phibbs 1984). Since *P. syringae* strains are closely related to *P. aeruginosa* (Pallaroni 1984), it is likely that a similar pathway functions in *P. s.* pv. *syringae* 61. It is unlikely that this apparent catabolite repression is mediated by cAMP, as in enterobacteria (Lessie and Phibbs 1984; MacGregor *et al.* 1991). Fructose was not tested in the previous studies of Xiao *et al.* (1992) but subsequent analyses indicate that *hrp*J expression is maximal in a fructose-based minimal salts medium (Y. Xiao and S. W. Hutcheson, unpublished results). In contrast to the results reported here, *P. s.* pv. *phaseolicola* NPS3121 *hrp*::*ina*Z fusions were expressed at similar levels in minimal media containing fructose or sucrose (Rahme *et al.* 1992). It may be that the environmental regulation of *hrp* genes differs among the host range variants of *P. syringae* or there is a difference in the sensitivity of the reporter gene assays used.

Carbon source has been reported to affect the expression of *avr*B in *P. s.* pv. *glycinea* race 0 (Huynh *et al.* 1989) similarly to that reported here. Highest expression of a plasmid-borne *avr*B′–*lac*Z fusion was observed in media containing fructose, sucrose, and mannitol. Glucose, however, did not repress expression of the fusion when added to these other carbon sources. Instead, TCA cycle intermediates, such as succinate, repressed the *avr*B expression observed in a mannitol-containing medium to the level equivalent to that observed in the presence of peptone. Succinate has also been reported to repress expression of *hrp* genes in *P. s.* pv. *phaseolicola* NPS3121 (Rahme *et al.* 1992). Expression of the *hrm*A′–*lac*Z fusion in media containing succinate as the sole carbon source, in contrast, was equivalent to that observed in a glucose-containing medium.

The nutritional regulation of *hrm*A appears to be mediated by *hrp* regions II and XIII (sensu Xiao *et al.* 1992). This appears similar to the regulation of *avr*B expression in *P. s.* pv. *glycinea* race 0 (Huynh et al 1989). Two undefined *hrp* genes located near the borders of the *P. s.* pv. *glycinea* race 0 *hrp* region have been reported to control the expression of *avr*B. The *P. s.* pv. *phaseolicola* *hrp* cluster has also been reported to contain two positive-acting regulatory elements, *hrp*L and *hrp*RS (Grimm and Panopoulos 1989; Fellay *et al.* 1989; Rahme *et al.* 1991;1992). This organization appears to be conserved among the *P. syringae* strains. The *P. s.* pv. *syringae* 61 *hrp* region XIII was shown to carry a homolog to the *P. s.* pv. *phaseolicola* *hrp*R locus. Positional criteria suggest that region II is equivalent to *hrp*L. The activity of this regulatory system in *E. coli* MC4100 is a novel observation and suggests that either *hrp*L and *hrp*R(S) form an independent regulatory system or other components of the regulatory system are highly conserved among gram-negative bacteria.

A homolog to *hrm*A was detected in strains representing only three of the pathovars tested. The inability to detect a homolog in other strains may indicate the absence of this locus or considerable sequence variation. All of the strains tested exhibited homology with the *P. s.* pv. *syringae* 61 *hrp* cluster under these conditions. This confirms that *Hrm*A is not essential for the elicitation of the HR by many *P. syringae* strains. *P. syringae* strains lacking a homolog are capable of eliciting an HR in tobacco. *P. s.* pv. *syringae* 61 *hrm*A mutants can still produce a necrotic reaction in tobacco leaves that differs from a typical HR only by its timing (Huang *et al.* 1991). These results predict that the gene products of one or more of the *hrp* genes initiate the HR. Consistent with this prediction is the observation that *E. coli* G1045 (pHIR12) can elicit water-soaking symptoms in tobacco. Since this plasmid only carries the *hrp* cluster, any plant response is likely be a product of *hrp* gene activity. The water-soaking symptoms could result from reduced expression of the postulated plant response elicitor in the absence of *hrm*A, modification of the postulated elicitor via the HrmA product, or the possible secretion of an *E. coli* product by Hrp products (Huang *et al.* 1992, 1993; Hutcheson *et al.* 1993).

Several observations had suggested that *hrm*A may be a negative host-range determinant similar to an *avr* gene. The initial characterization of pHIR11 indicated that one or more host range determinants may be associated with the *P. s.* pv. *syringae* 61 *hrp* cluster (Huang *et al.* 1988) and *P. s.* pv. *glycinea* race 4 (pHIR11) was avirulent on its normal host. The phenotype of *P. s.* pv. *syringae* 61 *hrm*A mutants was similar to that reported for a *X. campestris* pv. *vesicatoria* *avr* mutant (Whalen *et al.* 1988). As reported here, the environmental regulation of *hrm*A is similar to that of the *P. s.* pv. *glycinea* race 0 *avr*B gene. The effect of *hrm*A expression on the phenotype of *E. coli* G1045(pHIR12) and the limited distribution of *hrm*A among *P. syringae* strains are consistent with a potential role of *hrm*A in determining host range.

Four sets of observations, however, distinguish *hrm*A from previously characterized *avr* genes: 1) the absence of homology of *hrm*A with any of the known *avr* genes; 2) neither a subclone constructed to express the *hrm*A locus nor a comprehensive set of subclones carrying other regions of the *P. s.* pv. *syringae* 61 *hrp/hrm* gene cluster had an effect on *P. s.* pv. *glycinea* race 4 virulence (c.f. Kobayashi *et al.* 1989); 3) a set of pHIR11::Tn*phoA* derivatives in which individual *hrp* genes had been insertionally inactivated (including those that did not affect the expression of *hrm*A) did not exhibit phenotypic properties analogous to known *avr* gene; and 4) there is no specificity to the avirulence activity produced by the cluster in the variety of bacteria and host plants tested thus far (Hutcheson *et al.* 1993). We therefore conclude that no single gene or subset of genes associated with *P. s.* pv. *syringae* 61 *hrp/hrm* cluster have the qualities of an *avr* gene that could explain the avirulence activity of pHIR11 in *P. s.* pv. *glycinea* race 4. Either all bacteria carry a cryptic avirulence determinant whose phenotypic expression is dependent on the entire *hrp/hrm* gene cluster or one or more of the *hrp* genes produces the plant response elicitor.

The inability of the *P. s.* pv. *glycinea* race 4 *hrp* genes to complement the *P. s.* pv. *syringae* 61 *hrp*::Tn*phoA* mutations and the absence of specificity to the avirulence

activity may be due to the elevated expression of the *hrp/hrm* genes observed when the entire *hrp/hrm* cluster is plasmid-borne (Xiao *et al.* 1992; Hutcheson *et al.* 1993). Several pHIR11::Tn*5-gus*A1 derivatives were expressed greater than 50-fold higher than the equivalent chromosomal mutation. Other work has suggested that *hrp* genes are conserved among *P. syringae* strains (Lindgren *et al.* 1988). If this hypothesis is valid, it predicts that differential expression of one or more of the *hrp* genes during compatible and incompatible interactions controls the host range of *P. syringae* strains (Hutcheson *et al.* 1993).

The most likely explanation for the *hrm*A phenotype in *P. s.* pv. *syringae* (delayed HR/pathogenicity$^+$) and the effect of *hrm*A on G1045 (pHIR12) phenotype (water-soaking to HR$^+$), then, is that *hrm*A is a positive regulatory determinant for one or more *hrp* genes. Consistent with this hypothesis, recent work has shown that *hrp*J promoter activity is reduced in a *P. s.* pv. *syringae* 61 *hrm*A mutant (Y. Xiao, S. Heu, Y. Lu, J. Yi, and S. W. Hutcheson, unpublished). If *hrm*A is a regulatory determinant, the absence of sequence homology to known regulatory factors suggests that HrmA is a novel regulatory determinant or acts through other regulatory factors. An alternative hypothesis for the function of *hrm*A could be that HrmA enzymically modifies one or more *hrp* gene products. Experiments are currently in progress to test these hypotheses.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions.

Bacterial strains and plasmids are described in Table 1. *Pseudomonas* strains were grown on King's B (KB) broth or agar (King *et al.* 1954) at 28° C. *E. coli* strains were cultured on LM medium (Hanahan 1983), M9 (Sambrook *et al.* 1989), or M63M (Xiao *et al.* 1992) media at 37° C and pH 7.0. Media were supplemented with antibiotics when indicated, at the following concentrations (μg/ml): kanamycin, 50; nalidixic acid, 20; tetracycline, 20; and ampicillin, 50.

General DNA manipulations.

Plasmid DNA was isolated and manipulated by using standard techniques (Sambrook *et al.* 1989). Restriction enzymes and related reagents were purchased from Bethesda Research Laboratories (Gaithersburg, MD) and were used according to the manufacturer's instructions. Restriction fragments were purified following agarose gel electrophoresis by using Geneclean (Bio 101, Vista, CA). Electroporation-mediated transformation was performed as described in Li *et al.* (1992).

Triparental matings were accomplished as described previously (Huang *et al.* 1991). Transconjugants were purified by single colony transfer prior to use. Marker exchange mutagenesis was performed as described by Huang *et al.* (1991). Southern hybridizations were accomplished essentially as described in Huang *et al.* (1988). Total genomic DNA was isolated from the indicated strains and digested with *Eco*R1. After electrophoresis in 0.7% agarose gels, DNA was transferred onto Zetabind (Bio-Rad, Richmond, CA) membranes by capillary flow (blots were a gift of T. Denny, University Georgia, Athens). The *Alw*N1 fragment carrying the *hrm*A ORF was purified by agarose gel electrophoresis, and the isolated fragment was labeled by nick translation with α-^{32}P-dATP. Hybridizations were performed in 50% formamide at 42° C according to the membrane manufacturer's instructions. Blots were washed at room temperature prior to autoradiography.

Nucleotide sequence.

The 3.6-kb *Bam*H1–*Eco*R1 fragment from pHIR11 was cloned into pBluescriptII SK$^+$ (Stratagene, LaJolla, CA) and nested deletion derivatives created by using exonuclease III after *Kpn*I/*Xho*I digestion of the isolated plasmid. Following ligation and transformation into *E. coli* DH5α, plasmids from randomly selected colonies were isolated by using acid phenol (Gibco Focus 16:5-6, Gaithersburg, MD). The nucleotide sequence was obtained by using Sequenase 2.0 (U.S. Biochemicals, Cleveland, OH), double-stranded templates, and vector-priming sites. Gaps apparent in the sequence of either strand after compilation of the nested deletion data were filled by using synthetic oligonucleotide primers. Compressions were resolved by using dITP in the reaction mixtures. Sequence data was analyzed by using the algorithms of the University of Wisconsin Genetics Computer Group Package 7.1 (Devereux *et al.* 1984). The nucleotide sequence has been deposited in GenBank under accession number L14926.

T7 RNA polymerase-directed expression of *hrm*A.

The 1.55-kb *Alw*N1 fragment carrying the *hrm*A ORF was ligated into *Xba*I-digested pVEX11f+T8/24, a derivative of pET3a (Studier *et al.* 1990) that carries a multicloning site (a gift of A. Sankar, NCI-NIH, Bethesda, MD), to create pSG2V. The resulting construct was transformed into BL21(DE3)(pLysS). After induction of T7 RNA polymerase by IPTG treatment for 60, 90, and 120 min, cells were lysed into 0.05× culture volume of 50 mM Tris-Cl 2 mM EDTA (pH 8.0) by repeated freeze-thaw and sonication. The lysate was fractionated by centrifugation at 2,000 *g* for 20 min. The precipitate and supernatant fractions were solubilized in 2% SDS and proteins resolved by electrophoresis in 10% SDS-polyacrylamide gels (Laemmli 1970). Proteins were visualized by Coomassie blue staining.

N-terminal sequence of HrmA.

The protein band associated with the T7 RNA polymerase-directed expression of *hrm*A was electrophoretically transferred to Immobilon-P (Millipore Corp., Bedford, MA) matrix according to the manufacturer's instructions. The bound protein was subjected to Edman degradation by using a Applied Biosystems 977A protein sequencer.

Transcriptional start site.

Total RNA was isolated by using hot phenol from *E. coli* MC4100 (pBSG1)(pHIR12) and *P. s.* pv. *syringae* 61

(pHIR11), which had been cultured in M63M medium for 3 hr. A synthetic oligonucleotide primer was labeled at the 5′ end with polynucleotide kinase and γ-^{32}P-ATP and annealed to the RNA at 42° C. A cDNA was generated by using AMV reverse transcriptase (Promega, Madison, WI) according to the supplier's instructions and electrophoresed in a 7% acrylamide-urea sequencing gel in parallel with a set of sequence reactions generated by using the same primer.

Enzyme assays.

β-Galactosidase activity was monitored as described by Miller (1971). β-Glucuronidase activity was determined by the procedures of Jefferson (1987) as modified by Xiao *et al.* (1992).

Plant reaction assays.

Glycine max (L.) Merr. cvs. Harosoy, Linderin, Flambeau, Merit, Norchief, Chippewa, Acme, and Centennial were grown from seed in greenhouses in commercial potting soil. The bacterial suspensions were infiltrated into the primary leaves of 10-day-old plants through abaxial epidermal wounds by using disposable plastic syringes until approximately 1 cm^2 was water-soaked as described by Napoli and Staskawicz (1987). *Nicotiana tabacum* L. 'Samsun' plants were inoculated and scored for reaction as described by Huang *et al.* (1988). Bacterial populations were monitored by the procedures of Bertoni and Mills (1987).

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