

A Single Promoter Sequence Recognized by a Newly Identified Alternate Sigma Factor Directs Expression of Pathogenicity and Host Range Determinants in *Pseudomonas syringae*

YINGXIAN XIAO AND STEVEN W. HUTCHESON*

Department of Botany and Center for Agricultural Biotechnology of the University of Maryland
Biotechnology Institute, University of Maryland, College Park, Maryland 20742

Received 22 December 1993/Accepted 8 March 1994

A conserved sequence motif associated with transcription of *avr* genes was identified in the promoter regions of six *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* operons. A 34-bp fragment carrying this motif was cloned from the HrpZ promoter region and was shown to confer HrpL-dependent promoter activity. Expression of pathogenicity and host range determinants in *P. syringae* strains is thus directed by the apparent alternate sigma factor HrpL.

Hypersensitive response and pathogenicity (*hrp*) genes control the ability of *Pseudomonas syringae* and many other plant pathogenic bacteria to elicit a hypersensitive response associated with resistance in nonhost plants and pathogenesis in susceptible host plants (11, 24). In *P. syringae* pv. *syringae* Pss61, the *hrp* genes are clustered in a 25-kb region of the genome (8, 9) and are organized into eight transcriptional units (11, 15, 25) (Fig. 1). One of the transcriptional units, *hrpZ*, encodes harpin_{Pss}, a protein that appears to be directly responsible for the Hrp⁺ phenotype in plants (5). The deduced products of three transcriptional units (*hrpJ*, *hrpU*, and *hrpH*) appear to function in the secretion of harpin_{Pss} (7, 10, 15). The functions of *hrmA* and *hrpK* have not been established yet (6, 25).

The expression of most *hrp* genes is repressed in rich media and is affected by the carbon and nitrogen source, pH, osmotic conditions, and postulated plant factors (2, 18, 19, 26). A three-component regulatory cascade, consisting of HrpR and HrpS (unusual members of the NtrC family of transcriptional regulators) and HrpL (a putative alternate sigma factor), has been shown to mediate the environmental regulation of *hrp* genes in *P. syringae* pv. *syringae* Pss61 (6, 25). HrpR and HrpS positively regulate *hrpL* expression, and HrpL, which has properties of an alternate σ factor, activates the expression of *hrpJ*, *hrpZ*, and *hrmA* and, likely, *hrpK*, *hrpU*, and *hrpH* as well (25). Expression of *hrpL* alone was sufficient to induce transcription of *hrpJ*, *hrpZ*, and *hrmA* (25). This regulatory system functioned in both Pss61 and in *Escherichia coli* MC4100 (6, 25).

These observations prompted a search for a conserved promoter element(s) that is recognized by HrpL. A consensus harp box sequence, GN(A/C)(C/A)TG(A/C)AANCNNN(G/A), had been proposed to function in *hrp* regulation (2). However, a survey of the Pss61 *hrp* cluster found only two matching sequences within the 25,892-bp *hrp* region, and these motifs are located within the coding regions of two well-defined genes: *hrpJ2* (previously designated *hrpI*) and *hrpZ2* (previously designated *hrpZ*) (5, 10). A partially homologous harp box had been reported upstream of *hrpH2* (previously designated *hrpH*) (7), but subclones of this region failed to show promoter activity (10a).

Recently, a conserved sequence motif was identified in the upstream regions of 10 *avr* genes whose positions relative to

transcriptional initiation suggested that this motif could function as a promoter (13, 20, 21). A homolog to the *avr* conserved sequence motif was identified in the promoter-active regions of *hrmA*, *hrpJ*, *hrpZ* and in the deduced promoter regions of *hrpK*, *hrpU*, and *hrpH* (Fig. 1). Subsequent analysis similar to that of Xiao et al. (25) showed that the regions carrying the deduced *hrpK* and *hrpU* promoters exhibit HrpL-dependent promoter activity in *E. coli* MC4100 (15, 19a).

The *hrpZ* promoter had been cloned previously as an 868-bp *Bgl*III-*Hind*III fragment in pRG970 (23) to create a transcriptional fusion with *lacZ* (25). To determine whether the *hrp* conserved sequence is a necessary element of the *hrpZ* promoter, a 780-bp *Bsp*HI-*Hind*III fragment derived from the *hrpZ* promoter-active region but lacking the *hrp* conserved sequence motif was cloned into pRG970 to create pYXPZ2R. The promoter activity of the resulting construct in Pss61 was tested as described elsewhere (25). The resulting construct exhibited no significant promoter activity, irrespective of the medium (Fig. 2). In contrast, the 92-bp *Bgl*III-*Bsp*HI fragment containing the *hrp* conserved sequence motif cloned in a similar manner into pRG970 (pYXPZ3R) retained 46% of the original activity under these conditions.

The *hrp* conserved sequence motif alone was shown to be sufficient to generate the observed promoter activity by subcloning from the *hrpZ* promoter a 34-bp *Bgl*III-*Bfa*I fragment containing the complete motif. The promoter activity for the 34-bp fragment in Pss61 is equivalent to that observed for the 868-bp *hrpZ* promoter-active fragment (Fig. 2). Since pYXPZAR carries a transcriptional fusion between the 34-bp fragment and a promoterless *lacZ* construct, the only explanation for the observed β -galactosidase activity is that the *hrp* conserved sequence motif forms all or part of the *hrpZ* promoter.

Further analysis with *E. coli* MC4100 showed that the promoter activity of the *hrp* promoter motif is *hrp* dependent. None of the constructs exhibited significant promoter activity in the absence of the Pss61 *hrp/hrmA* cluster (Table 1). When pHIR11-2096, which carries the entire Pss61 *hrp/hrmA* gene cluster, was introduced into the MC4100 derivatives, an approximately 50-fold increase in promoter activity from all three clones containing the *hrp* promoter motif was observed (Table 1).

To determine whether the promoter activity of the *hrp* promoter motif is dependent on HrpL (σ^L), the promoter constructs were transformed into MC4100(pYXL2SP) which carries an inducible *hrpL* construct (25). In the absence of

* Corresponding author. Phone: (301) 405-1606. Fax: (301) 314-9082. Electronic mail address: sh53@umail.umd.edu.

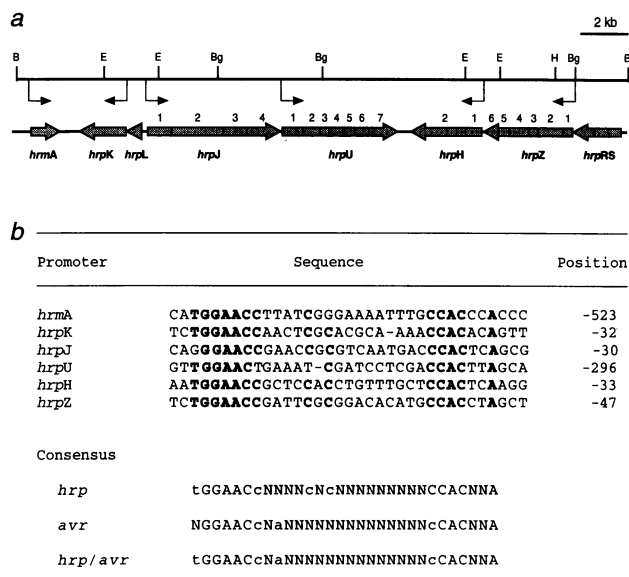


FIG. 1. (a) Transcriptional organization of Pss61 *hrp* gene cluster. Shaded arrows indicate apparent transcriptional units and their orientations, which were deduced from sequence and complementation analyses (5, 6, 8, 10, 15, 26). Numbers indicate deduced translational units of polycistronic transcriptional units. Bent arrows show the locations and orientations of the *hrp* conserved sequence motif. B, *Bam*HI; E, *Eco*RI; Bg, *Bgl*II; H, *Hind*III. (b) Conserved sequence motifs upstream of Pss61 *hrp* transcriptional units. Conserved nucleotides are shown in boldface type. In the consensus sequences, nucleotides denoted by uppercase letters exhibit 100% conservation, whereas nucleotides denoted by lowercase letters retain at least 75% conservation among the surveyed *hrp* and *avr* promoters. The *avr* conserved sequence motif was derived from the promoter regions of 10 *avr* genes (13). The position of the conserved sequence motif relative to that of the initial codon of the first apparent open reading frame of the transcriptional unit is indicated.

isopropyl- β -D-thiogalactopyranoside (IPTG), little promoter activity was observed from any of the five constructs that were tested (Table 2). Induction of *hrpL* expression by the addition of 1 mM IPTG resulted in at least a 25-fold increase in the promoter activity detected in the strains carrying the *hrp* promoter motif.

These results demonstrate that the 30-bp *hrp* conserved sequence motif functions as an HrpL-dependent promoter. The bipartite motif is highly conserved among the 16 *hrp* and *avr* promoters that were characterized. Nine positions are perfectly conserved, and greater than 75% conservation is observed at four other positions. An apparent HrpL-dependent promoter consensus sequence is shown in Fig. 1. Although transcription initiation was not investigated in this study, equivalent analyses performed on selected *P. syringae* *avr* genes predict that transcription begins 6 to 8 bp downstream of the HrpL-dependent promoter consensus sequence (13, 20). Shen and King (21) demonstrated that substitution for either region of the promoter consensus sequence suppressed the activity of the *avrD* promoter; however, their studies failed to identify the nature of this *cis*-acting element. Partial deletion of the upstream region of the HrpL-dependent promoter consensus sequence has been reported to reduce the activity of the *avrPto* promoter (20). The HrpL-dependent promoter consensus sequence, therefore, is found in all HrpL-dependent promoters that have characterized thus far, functions as a *cis*-acting element for promoter activity, is associated with transcription initiation, and is required for HrpL-dependent promoter activity.

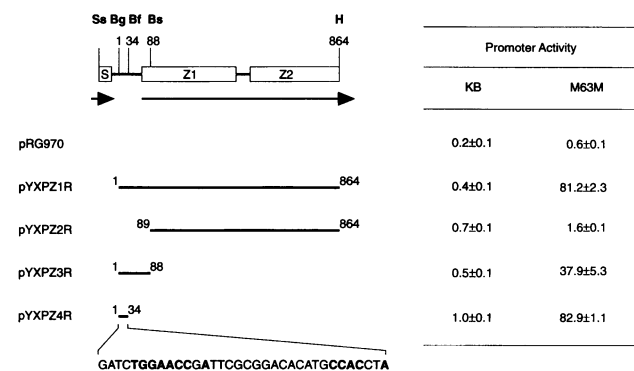


FIG. 2. Role of the conserved sequence motif in *hrpZ* promoter activity. The *hrpZ* promoter region and cloned restriction fragments are indicated on the left. The arrows indicate transcriptional orientations of the *hrpRS* and *hrpZ* transcriptional units. Ss, *Ssr*I; Bg, *Bgl*II; Bf, *Bfa*I; Bs, *Bsp*HI; H, *Hind*III. The numbers indicate positions relative to the *Bgl*II site. The sequence of the 34-bp fragment is given at the bottom left, and the conserved promoter motif is indicated by the boldface letters. The promoter activities of the cloned fragments in the indicated medium are listed on the right in Miller's units of β -galactosidase activity. KB, King's medium B (14), a rich medium containing proteose peptone; M63M, M63 minimal salts medium (22) with 0.2% mannitol as the carbon source.

HrpL is most closely related to AlgU, a putative alternate sigma factor controlling extracellular polysaccharide biosynthesis in *P. aeruginosa* (25). The near identity between the deduced DNA binding motifs (16) in the two proteins had predicted that these proteins recognize similar promoter motifs. Interestingly, there are two motifs in the AlgU-dependent *algD* promoter (17) that are similar to the *hrp/avr* promoter consensus sequence. One, cGGAACtccctcgagagaaaaCatCctA, is located at the -6 position relative to the transcription initiation site, and the other, cGGAACtgcacatcttttcaCgCccAgcCCACagA, is located at the -337 position, a region also essential for *algD* promoter activity (17). In light of the similarities among several *P. syringae* *hrp* products and key pathogenicity factors of mammalian pathogens, such as *Yersinia* spp., *Shigella* spp., and *Salmonella* species (3, 4, 7, 10), alternate sigma factors may be a common mechanism to control the production and deployment of pathogenicity factors.

The expression of *avr* and *hrp* genes in *P. syringae* strains has been shown previously to be affected by similar environmental conditions (6, 12, 19, 26) and to be controlled by a single

TABLE 1. Activity of *hrpZ* promoter constructs in *E. coli* MC4100

Plasmid	Promoter activity ^a	
	- <i>hrp</i>	+ <i>hrp</i>
pRG970	0.9 ± 0.1	1.0 ± 0.1
pYXPZ1R	2.2 ± 0.3	102.2 ± 1.5
pYXPZ2R	3.3 ± 0.1	3.0 ± 0.3
pYXPZ3R	1.6 ± 0.1	74.6 ± 4.2
pYXPZ4R	4.6 ± 0.2	217.0 ± 2.6

^a Promoter activities are given in Miller's units of β -galactosidase activity. The indicated plasmids, which are diagrammed in Fig. 2, were transformed into *E. coli* MC4100 (Δ *argF-lacZYA* [1]) (-*hrp*) or MC4100(pHIR11-2096) (+*hrp*) by electroporation. The plasmid pHIR11-2096 carries the entire Pss61 *hrp* gene cluster (8). The bacteria were cultured in M63M medium, and β -galactosidase activities were measured as described in the legend to Fig. 2. The data presented are the means of three replicates \pm the standard errors. This experiment was repeated twice, with similar results.

TABLE 2. Effect of *hrpL* expression on the activity of the *hrpZ* promoter constructs in *E. coli* MC4100(pYXL2SP)

Plasmid	Promoter activity ^a	
	Uninduced	Induced ^b
pRG970	0.1 ± 0.1	0.1 ± 0.1
pYXPZ1R	1.7 ± 0.1	45.5 ± 0.9
pYXPZ2R	0.3 ± 0.1	0.4 ± 0.1
pYXPZ3R	1.2 ± 0.1	30.4 ± 0.6
pYXPZ4R	2.8 ± 0.1	72.8 ± 2.0

^a Promoter activities are given in Miller's units of β-galactosidase activity. The *hrpZ* promoter constructs were electroporated into MC4100(pYXL2SP) (25). pYXL2SP is a pSPORT 1 (*lacI*⁺; GIBCO BRL-Life Technologies, Inc., Gaithersburg, Md.) derivative carrying *hrpL* cloned downstream of the vector's *lac* promoter as a 1-kb *SspI-HincII* fragment (25). The bacteria were cultured and the promoter activities were measured as described in footnote a Table 1.

^b Expression of *hrpL* was induced by the addition of 1 mM IPTG.

regulatory system, apparently consisting of HrpL and HrpR-S (6, 12, 13, 20, 21, 25). The results presented here clarify the mechanism for this linkage by demonstrating that a single conserved HrpL-dependent promoter controls the expression of both *hrp* and *avr* genes. HrpL thus controls the production of (i) harpin_{PSS} (25), (ii) the AvrD-linked low-molecular-weight plant response elicitor (13, 20, 21), (iii) a secretion system for harpins (15, 25), and (iv) the *avr* products that affect host range (13, 20, 21). The coregulation of *hrp* genes and *avr* genes at the same level of the regulatory hierarchy strongly implies that the final effectors of *hrp* genes and *avr* genes may act together, either directly or indirectly, to determine the outcome of the plant-bacterium interaction.

This research was supported by funds provided by NSF grant MCB9221670. Yingxian Xiao was supported by a fellowship from the Center for Agricultural Biotechnology of the University of Maryland Biotechnology Institute.

REFERENCES

- Casadaban, M. J. 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J. Mol. Biol.* **104**:541-555.
- Fellay, R., L. G. Rahme, M. N. Mindrinos, R. D. Frederick, A. Pisi, and N. J. Panopoulos. 1989. Genes and signals controlling the *Pseudomonas syringae* pv. phaseolicola-plant interaction, p. 45-52. In H. Hennecke and D. P. S. Verma (ed.), *Advances in molecular genetics of plant-microbe interactions*, vol. 1. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Fenselau, S., I. Balbo, and U. Bonas. 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. vesicatoria are related to proteins involved in secretion in bacterial pathogens of animals. *Mol. Plant-Microbe Interact.* **5**:390-396.
- Gough, C. L., S. Genin, C. Zischek, and C. A. Boucher. 1992. *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. *Mol. Plant-Microbe Interact.* **5**:384-389.
- He, S. Y., H. C. Huang, and A. Collmer. 1993. *Pseudomonas syringae* pv. *syringae* harpin_{PSS}: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell* **73**:1255-1266.
- Heu, S., and S. W. Hutcheson. 1993. Nucleotide sequence and properties of the *hrmA* locus associated with the *P. syringae* pv. *syringae* 61 *hrp* gene cluster. *Mol. Plant-Microbe Interact.* **6**:553-564.
- Huang, H.-C., S. Y. He, D. W. Bauer, and A. Collmer. 1992. The *Pseudomonas syringae* pv. *syringae* 61 *hrpH* product: an envelope protein required for elicitation of the hypersensitive response in plants. *J. Bacteriol.* **174**:6878-6885.
- Huang, H. C., S. W. Hutcheson, and A. Collmer. 1991. Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and *TnphoA* tagging of exported or membrane-spanning Hrp proteins. *Mol. Plant-Microbe Interact.* **4**:469-476.
- Huang, H. C., R. Schuurink, T. P. Denny, M. M. Atkinson, C. J. Baker, I. Yucel, S. W. Hutcheson, and A. Collmer. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco. *J. Bacteriol.* **170**:4748-4756.
- Huang, H. C., Y. Xiao, R.-H. Lin, Y. Lu, S. W. Hutcheson, and A. Collmer. 1993. Characterization of the *Pseudomonas syringae* pv. *syringae* 61 *hrpJ* and *hrpI* genes: homology of HrpI to a superfamily of proteins associated with protein translocation. *Mol. Plant-Microbe Interact.* **6**:515-520.
- Hutcheson, S. Unpublished data.
- Hutcheson, S. W., S. Heu, H. C. Huang, M. Lidell, and Y. Xiao. 1994. Organization, regulation and function of *Pseudomonas syringae* pv. *syringae* 61 *hrp* genes, p. 593-603. In J. Crosa and C. I. Kado (ed.), *Bacterial virulence mechanisms*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Huynh, T., D. Dahlbeck, and B. J. Staskawicz. 1989. Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. *Science* **245**:1374-1377.
- Innes, R. W., A. F. Bent, B. N. Kunkel, S. R. Bisgrove, and B. J. Staskawicz. 1993. Molecular analysis of avirulence gene *avrRpt2* and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. *J. Bacteriol.* **175**:4859-4869.
- King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* **44**:301-307.
- Lidell, M., and S. W. Hutcheson. Characterization of the *hrpJ* and U operons of *Pseudomonas syringae* pv. *syringae* Pss61: similarity with components of enteric bacteria involved in flagellar biogenesis and demonstration of their role in protein translocation. *Mol. Plant-Microbe Interact.*, in press.
- Lonetto, M., M. Gribskov, and C. A. Gross. 1992. The σ^{70} family: sequence conservation and evolutionary relationships. *J. Bacteriol.* **174**:3843-3849.
- Mohr, C. D., D. W. Martin, W. M. Konyecsni, J. R. W. Govan, S. Lory, and V. Deretic. 1990. Role of the far-upstream sites of the *algD* promoter and the *algR* and *rpoN* genes in environmental modulation of mucoidy in *Pseudomonas aeruginosa*. *J. Bacteriol.* **172**:6576-6580.
- Rahme, L. G., M. N. Mindrinos, and N. J. Panopoulos. 1991. Genetic and transcriptional organization of the *hrp* cluster of *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* **173**:575-586.
- Rahme, L. G., M. N. Mindrinos, and N. J. Panopoulos. 1992. Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* **174**:3499-3507.
- Rowley, D., and S. W. Hutcheson. Unpublished data.
- Salmeron, J. M., and B. J. Staskawicz. 1993. Molecular characterization and *hrp*-dependence of the avirulence gene *avrPto* from *Pseudomonas syringae* pv. *tomato*. *Mol. Gen. Genet.* **239**:6-10.
- Shen, H., and N. T. Keen. 1993. Characterization of the promoter of avirulence gene D from *Pseudomonas syringae* pv. *tomato*. *J. Bacteriol.* **175**:5916-5924.
- Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. *Experiments with gene fusions*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Van den Eede, G., R. Deblaere, K. Goethals, M. Van Montagu, and M. Holsters. 1992. Broad host range and promoter selection vectors for bacteria that interact with plants. *Mol. Plant-Microbe Interact.* **5**:228-234.
- Willis, K., J. J. Rich, and E. M. Hrabak. 1990. The *hrp* genes of phytopathogenic bacteria. *Mol. Plant-Microbe Interact.* **4**:132-138.
- Xiao, Y., S. Heu, J. Yi, Y. Lu, and S. W. Hutcheson. 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. *J. Bacteriol.* **176**:1025-1036.
- Xiao, Y., Y. Lu, S. Heu, and S. W. Hutcheson. 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. *J. Bacteriol.* **174**:1734-1741.