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The Alternative Sigma Factor RpoN Is Required for *hrp* Activity in *Pseudomonas syringae* pv. Maculicola and Acts at the Level of *hrpL* Transcription

ERIK L. HENDRICKSON,† PABLO GUEVERA, AND FREDERICK M. AUSUBEL*

Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114

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β-Glucuronidase (*uidA*) reporter gene fusions were constructed for the *hrpZ*, *hrpL*, and *hrpS* genes from the phytopathogen *Pseudomonas syringae* pv. maculicola strain ES4326. These reporters, as well as an *avrRpt2-uidA* fusion, were used to measure transcriptional activity in ES4326 and a ES4326 *rpoN* mutant. *rpoN* was required for the expression of *avrRpt2*, *hrpZ*, and *hrpL* in vitro in minimal media and in vivo when infiltrated into *Arabidopsis thaliana* leaves. In contrast, the expression of *hrpS* was essentially the same in wild-type and *rpoN* mutant strains. Constitutive expression of *hrpL* in an *rpoN* mutant restored *hrpZ* transcription to wild-type levels, restored the hypersensitive response when infiltrated into tobacco (*Nicotiana tobacum*), and partially restored the elicitation of virulence-related symptoms but not growth when infiltrated into *Arabidopsis* leaves. These data indicate that *rpoN*-mediated control of *hrp* gene expression acts at the level of *hrpL* and that in planta growth of *P. syringae* is not required for the elicitation of disease symptoms.

In gram-negative bacteria, transcriptional activation in response to external stimuli often involves the alternative sigma factor σ^{54} (1, 50). σ^{54}, which is encoded by *rpoN*, works in conjunction with members of the NtrC superfamily of transcriptional activators (1, 50). Among the different enzymatic pathways under σ^{54} control are those responsible for nitrogen utilization, dicarboxylate transport, xylene degradation, and hydrogen utilization (8, 39, 47, 79).

In the case of some phytopathogenic bacterial species, *rpoN* has been implicated indirectly as a regulator of a large cluster of pathogenicity-related genes known as the *hrp* gene cluster (17, 27). For example, *Pseudomonas syringae* pv. syringae strain 61 contains a 25-kb *hrp* cluster consisting of 27 genes arranged as follows: *hrpKL*, *hrpJ*, *hrcV*, *hrpQ*, *hrcN*, *hrpOP*, *hrcQRSTU*, *hrpVT*, *hrcC*, *hrpGF*, *hrpED*, *hrcJ*, *hrpBZA*, and *hrpSR* (11, 33). The acronym *hrp* stands for hypersensitive response and pathogenicity. The genes within the *hrp* cluster are required not only for pathogenicity but also for elicitation of the plant resistance reaction known as the hypersensitive response (HR) (44, 45). The HR involves rapid, localized plant cell death, which is triggered by a gene-for-gene interaction between a particular avirulence (*avr*) gene in the pathogen and a corresponding resistance gene in the host (21, 56). The widely conserved members of the *hrp* cluster, designated *hrc*, show significant homology to members of the *Yersinia* type III secretory pathway and in the new simplified nomenclature bear the letter designation of the corresponding *Yersinia* gene (9). (The exception is *hrcV*, which is homologous to the *Yersinia lcrD* genes.)

Sequence analysis of the *hrp* cluster in *P. syringae* pv. phaseolicola suggested that σ^{54} would be required for *hrp* gene expression in conjunction with the *hrpRS* genes, which are required for expression of the remaining *hrp* genes in the cluster (17, 27). *hrpR* and *hrpS* encode proteins that contain the domain conserved among transcriptional activators such as NtrC, DctD, and NifA that work in concert with σ^{54} (29, 30; reviewed in references 1 and 50). Because of the HrpS-NtrC homology, it seemed likely that a *P. syringae* homologue of *rpoN* would be required for activation of the *hrp* gene cluster. Circumstantial evidence in support of this conclusion was provided by our findings presented in the accompanying study that the *rpoN* gene of *P. syringae* is required for pathogenesis and the HR (32).

The *P. syringae hrpL* gene product is related to the alternate sigma factor AlgU, which regulates genes involved in the biosynthesis and regulation of the exopolysaccharide alginate in *P. aeruginosa* (49, 80). The circuitry of *hrp* regulation in *P. syringae* appears to involve a transcriptional activation cascade in which HrpR activates *hrpS*, HrpS activates *hrpL*, and HrpL activates transcription of the remaining *hrp* genes (17, 26, 81), as well as *avr* genes that are responsible for eliciting the HR. One of these remaining *hrp* genes, *hrpV*, appears to negatively regulate *hrp* transcription and, while there is some evidence that *hrpV* functions upstream of *hrpRS*, the factor(s) involved in the regulation of *hrpRS* remain obscure (62).

Most of the remaining genes in the *hrp* cluster encode structural proteins that are involved in the synthesis or export of pathogenicity factors (78). As mentioned above, the nine *hrc* genes are homologous to members of the *Yersinia* type III secretory pathway that is responsible for translocating Yop proteins into host cells (18, 24, 53, 73). Similarly, *hrp* mutations block the export of PopA1 from *P. solanacearum* and HrpZ (harpin) and HrpW from *P. syringae*, of which the latter two elicit an HR-like response on incompatible and nonhost plants (2, 10, 11, 31). Given the role of the *hrp* cluster in protein secretion, the most likely cause for nonpathogenic and non-HR-eliciting phenotypes of *hrp* mutants is the inability to secrete a variety of virulence factors or elicitors. Recent studies suggest that several Avr proteins function directly inside of host plant cells in analogy with the type III-mediated transfer of virulence factor by *Y. enterocolitica* (23, 71, 75, 76).

* Corresponding author. Mailing address: Massachusetts General Hospital, Department of Molecular Biology, Wellman 10, Boston, MA 02114. Phone: (617) 726-5969. Fax: (617) 726-5949. E-mail: ausubel@frodo.mgh.harvard.edu.

† Present address: Department of Microbiology, University of Washington, Seattle, WA 98195.

TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant genotype, phenotype, or role in this study	Source or reference
Strains		
E. coli		
DH5a	F$^-$ *lacZ*ΔM15 *endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1* λ$^-$	Bethesda Research Laboratories (28)
MM294	Host of pRK2013	G. Walker (4)
HB101	Host of pHoGus and pSshe	48
MT609	Recipient for cosmids with Tn*3-uidA* insertions	T. Finan (20)
P. syringae pv. phaseolicola LRG94	*hrpS*::Tn*3-spice*	L. Rahme (64)
P. syringae pv. maculicola		
ES4326	Wild type	K. Davis (14)
ES4326 *hrpZ*::*uidA*	Contains chromosomal *uidA* translational fusion in *hrpZ*	This study
ES4326 *rpoN*::Km^r	Contains Kan^r cassette inserted into *rpoN* gene	This study
P. syringae pv. tomato		
DC3000	Wild type	D. Cuppels (57)
DC3661	Coronatine biosynthetic mutant	D. Cuppels (57)
MM1065	Origin of *avrRpt2*	M. Mindrinos (16)
Plasmids		
pRK2013	Kan^r Tra$^+$, mating helper	G. Ditta (15)
pLAFR3	Tc^r cosmid cloning vector	B. Staskawicz (72)
pBluescript SK(+)	Ap^r, cloning vector	Stratagene
pUC18	Ap^r, plasmid cloning vector	Boehringer Mannheim
pRR54cos	Ap^r, cosmid cloning vector	L. Rahme (64)
pPL6	Source of *P. syringae* pv. phaseolicola *hrpL* gene	L. Rahme (64)
pNN56	Source of *P. syringae* pv. phaseolicola *hrpS* gene	L. Rahme (64)
pHoGus	Ap^r Km^r, source of Tn*3-uidA*	J. Glazebrook (22)
pSshe	Cm^r, provides transposase *in trans*	J. Glazebrook (22)
pRAJ235	Source of *E. coli uidA* gene	R. A. Jefferson (37)
pRSR0	1.4-kb *Sal*I fragment containing *avrRpt2* in pUC119	R. Innes (35)
pLH12	1.4-kb *Sal*I fragment containing *avrRpt2* in pLAFR3	R. Innes (77)
pAVR12	*avrRpt2-uidA* translational fusion in pLAFR3	This study
pAVRC	*lacZ-avrRpt2* transcriptional fusion in pLAFR3	This study
pEHS	ES4326 *hrpS* and *hrpR* in pBluescript SK(+)	This study
pEHSpuc	2.0 HB *Sal*I-*Apa*I fragment containing *hrpS* in pUC18	This study
pEH10	ES4326 *hrp* clone containing *hrpS* in pLAFR3	This study
pEHL	ES4326 *hrp* clone containing *hrpL* in pRR54cos	This study
pEHL3	5.8-kb *Eco*RI fragment containing *hrpL* in pLAFR3	This study
pHRPLC	*lacZ-hrpL* transcriptional fusion in pLAFR3	This study
pHRPLgus	*hrpL-uidA* translational fusion in pLAFR3	This study
pHRPZgus	*hrpZ-uidA* translational fusion in pLAFR3	This study
pHRPSgus	*hrpS-uidA* translational fusion in pLAFR3	This study
pXIVB4	ES4326 *hrp* clone containing *hrpL* in pRR54cos	This study

avr genes are often coordinately regulated with *hrp* gene expression. A conserved sequence, GGAACCNA-N14-CCAC NNA, which appears to be responsible for *hrpL*-dependent transcription, has been identified upstream of a variety of *avr* genes (81). In several cases, including *avrRpt2* and *avrRpm1*, *hrpL* dependence has been confirmed experimentally (34, 35, 46, 63, 66, 70, 81). Thus, the loss of *hrp* by mutation of a putative *hrp* regulator such as *rpoN* should affect HR induction by blocking *avr* gene transcription and type III-mediated protein delivery.

As described above, the presence of the NtrC-like regulators HrpR and HrpS in *P. syringae* suggests a direct role for σ^{54} in *hrp* gene regulation. However, *rpoN* is often found to control multiple functions, including flagellar synthesis and nitrogen utilization (41, 50, 60). Moreover, *P. syringae* pathovars produce a number of toxins, including syringomycins, tabtoxin, phaseolotoxin, tagetitoxin, and coronatine (6), and we have found that *rpoN* is required for the expression of coronatine (32). Therefore, *rpoN* might be involved in the regulation of a number of virulence factors in addition to the *hrp* cluster, and our finding that *rpoN* mutants are nonpathogenic is not definitive evidence that *rpoN* regulates *hrp* gene expression.

To dissect the plant defense response using a molecular-genetic approach, our laboratory (13, 14, 16, 82) has established a model pathogenicity system that involves the infection of *Arabidopsis thaliana* with *P. syringae* pv. maculicola strain ES4326. Strain ES4326 proliferates extensively in *Arabidopsis* ecotype Columbia leaves and causes the development of water-soaked disease lesions (14, 16). In contrast, ES4326 carrying the avirulence gene *avrRpt2* elicits a visible HR about 16 h after infiltration, and its multiplication in planta is 50- to 100-fold less than ES4326 (16). In the accompanying publication, we describe the isolation and characterization of an ES4326 *rpoN* mutant (32). We describe here experiments examining the effect of the *rpoN* mutant on *hrp* and *avr* gene regulation in ES4326. Our results indicate that neither *hrp* nor *avr* genes are transcribed in an *rpoN* mutant in vitro or during pathogenesis, that synthesis of the *avrRpt2* gene product in the absence of *hrp* gene expression is insufficient to elicit an HR, that the coordinate *rpoN* regulation of *hrp* and *avr* genes acts through *hrpL*, that additional *rpoN*-regulated factors may be important for pathogenesis, and that the growth of ES4326 in *Arabidopsis* leaves is not required for the elicitation of disease symptoms.

MATERIALS AND METHODS

Strains and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. ES4326 and its derivatives were grown at 28°C in L broth (48), minimal M9 medium (48) or King's B (40) medium. *Escherichia coli* strains were grown at 37°C in L broth. Antibiotic concentrations for *E. coli* and *P. syringae* strains were as follows: streptomycin, 150 μg/ml; kanamycin, 25 μg/ml;

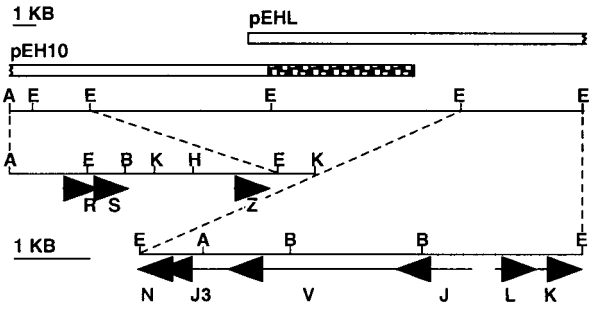

FIG. 1. Physical and genetic map of the *P. syringae* pathovar maculicola *hrp* cluster. Plasmid pEH10 was assayed for complementation of the *P. syringae* pv. phaseolicola *hrpS* mutant LRG94 as described in Materials and Methods. The regions of the *hrp* cluster contained in pEHL and pEH10 are shown. The fragment used to probe for overlap between the clones is hatched. The arrows represent the regions of the genes that have been sequenced and indicate the direction of transcription. Restriction enzymes: A, *Apa*I, B, *Bam*HI, E, *Eco*RI, H, *Hin*dIII, K, *Kpn*I, S, *Sal*I.

tetracycline, 12 μg/ml; gentamicin, 20 μg/ml; spectinomycin, 20 μg/ml; and ampicillin, 100 μg/ml. Marker exchange of *hrpZ*::*uidA* was carried out by serially culturing ES4326 containing pHRPZgus without antibiotic selection, plating on medium containing kanamycin to select for the insertion, and screening on plates containing kanamycin and tetracycline for strains that had lost the vector (68).

Bacterial genetics. pLAFR3 derivatives were introduced into *Pseudomonas* strains via triparental matings with pRK2013 as the mobilizing plasmid (15).

Cloning of ES4326 *hrpR*, *hrpS*, *hrpZ*, and *hrpL* genes. *hrpS* was cloned from a ES4326 genomic library constructed by *Sau*3A partial digestion of ES4326 genomic DNA (3). The partial digest was size selected for 5- to 10-kb fragments on a sucrose gradient (48) and ligated into the *Bam*HI site of pBluescript. A plasmid carrying a 5.0-kb fragment containing *hrpS*, pEHS, was identified by colony hybridization using a randomly labeled probe (3) derived from cosmid pNN56 which contains *hrpS* from *P. syringae* pv. phaseolicola. Sequencing 500 bp of pEHS confirmed the presence of *hrpR* and a portion of *hrpS* on pEHS. A larger 24-kb clone, pEH10, containing the entire *hrpS* gene, was obtained by colony hybridization of an ES4326 genomic library constructed in pLAFR3 using probe containing a portion of *hrpS* generated from the 1.1-kb *Pvu*II fragment of pEHS (Fig. 1). The presence of a functioning *hrpS* gene on pEH10 was confirmed by complementing the *P. syringae* pv. phaseolicola *hrpS* mutant LRG94 for disease symptoms on bean. Southern blot analysis delimited *hrpS* to a 2.8-kb *Kpn*I fragment in pEH10. Sequencing the junction of a Tn*3*-*uidA* transposon insertion in pEH10 (see below) identified a homolog of the *P. syringae* pv. phaseolicola *hrpZ* gene on an adjacent 2.1-kb *Kpn*I fragment.

A 36-kb clone, pEHL, containing *hrpL* was isolated from an ES4326 pRR54cos library by colony hybridization. This library was constructed similarly to the others except that 30- to 40-kb fragments were isolated. A *hrpL* probe was generated from a 2.4-kb *Eco*RI-*Sal*I fragment from plasmid pPL6 containing *hrpL* from *P. syringae* pv. phaseolicola. A 5.8-kb *Eco*RI fragment identified as containing *hrpL* by Southern hybridization was subcloned into pLAFR3 to construct pEHL3 and sequenced.

RNA analysis. RNA preparation and Northern blot analysis was carried out using standard techniques (3). An *avrRpt2* probe was made by the Klenow random priming reaction using an internal fragment of *avrRpt2* as the template (3). *hrpL* and *hrpZ* probes were made by PCR labeling using primers with the sequences 5′-CATACCCCCATTCAGGC-3′ and 5′-GTCTCAGTCTTAACAG C-3′, respectively (25, 67).

Construction of translational fusions. A translational gene fusion that contains the amino-terminal half of *avrRpt2* fused in frame to the entire *E. coli uidA* gene, including its ATG translation initiation codon, was constructed as follows. Plasmid pRAJ275, which contains the *uidA* gene, was digested with *Nco*I; the resulting 5′ overhangs were filled using Klenow polymerase (3), and the *Nco*I-cleaved DNA was digested with *Sal*I. A 575-bp *Sal*I-*Bal*I fragment containing the promoter and the first 133 amino acids of *avrRpt2* was purified from plasmid pRSR0 (35) and ligated to the *Nco*I-*Sal*I-digested pRAJ275 DNA. The resulting plasmid containing the *avrRpt2-uidA* fusion was digested with *Hin*dIII and *Eco*RI, which cut within the polylinker and the 3′ end of the *uidA* gene, respectively. This 2.25-kb *Hin*dIII-*Eco*RI fragment was then cloned into pLAFR3 that had been linearized using the same two enzymes, resulting in the construction of pAVR12. ES4326 transconjugants carrying pAVR12 formed white colonies with blue centers on King's B or Luria-Bertani (LB) agar and dark blue colonies on minimal M9 medium containing indolyl-4-methylumbelliferyl-β-D-glucuronide (X-Gluc; Biosynth AG).

Translational gene fusions were made to *hrpZ* (pHRPZgus) and *hrpL* (pHRPLgus) by transposon mutagenesis using Tn*3*::*uidA* (22). For *hrpZ*, pEH10 was introduced by triparental mating into HB101 (pHoGus pSshe) which contains Tn*3*::*uidA* on pHoGus and the transposase gene on pSshe. The resulting strain was incubated overnight, mated with MT609, and then spread on LB agar containing tetracycline and kanamycin 6 h later. Because pHoGus is unable to replicate in MT609, Tc^r Km^r exconjugants should only arise by a transposition event. Presumptive pEH10 Tn*3*::*uidA* isolates were analyzed by restriction mapping to locate the position of the *uidA* insert. A primer internal to the *uidA* gene (5′-GCAATTGCCCGGCTTTC-3′) was used to sequence the Tn*3*::*uidA* junctions. For *hrpL*, the 5.8-kb *Eco*RI *hrpL* subclone pEHL3 was mated into HB101 (pHoGus pSshe) and analyzed as described above for pEH10. An *hrpS* translational fusion to *uidA* was constructed by first subcloning a 2.0-kb *Sal*I-*Kpn*I fragment from pEH10 containing *hrpS* into pUC18 to produce pEHSpuc. Primers containing *Bsp*EI sites corresponding to the amino-terminal (5′-AGTTCTC CGGAGCATGCCTGCAGGTCG-3′) and carboxyl-terminal (5′-AGTTCTCC GGAGAGGCTGTAGCCGACG-3′) sequences of the *uidA* gene were used to generate PCR fragments containing *uidA* which were digested with *Bsp*EI and ligated into the *Bsp*EI site of pEHSpuc. The resulting fusion was digested with *Kpn*I and *Hin*dIII which cut in the polylinker, the overhangs were filled using Klenow polymerase, and the fragment was ligated into the *Hin*dIII site of pLAFR3 (also filled using Klenow polymerase) to give pHRPSgus.

Construction of P_{lacZ}-*avrRpt2*. The 1.22-kb *Xmn*I-*Pst*I fragment of pRSR0 containing the *P. syringae avrRpt2* gene and its putative Shine-Dalgarno sequence was cloned into the *Eco*RI (filled in with Klenow polymerase) and *Pst*I sites of plasmid pLAFR3 to form pAVRC. Transcription originating at the *lacZ* promoter present in pLAFR3 (proximal to the *Eco*RI site) reads through into *avrRpt2*.

Construction of P_{lacZ}-*hrpL*. A PCR-based strategy using primers with engineered *Eco*RI sites was used to construct a translational fusion between *hrpL* and the *lacZ* promoter of cosmid pLAFR3. One primer (5′-AGTCAGAATTCCCA GAACCTTGTGATC-3′) corresponded to the amino-terminal end of *hrpL* starting with the third codon, and the other primer (5′-AGTTCGAATTCCTGTGT GGTTTCGGGC-3′) corresponded to *hrpL* sequence 27 bp downstream of the stop codon. The PCR product was digested with *Eco*RI and ligated to the *Eco*RI site of pLAFR3 to generate pHRPLC. The pHRPLC fusion was verified by sequencing the *lacZ-hrpL* junction.

Screen for *hrp* regulators. ES4326 *hrpZ*::*uidA* was mutagenized using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NG) (52) by subculturing an overnight culture of ES4326 *hrpZ*::*uidA* in KB media, growing it to an optical density at 600 nm (OD_{600}) of 1.0, resuspending four 5-ml samples in 4 ml of 0.1 M sodium citrate (pH 5.5), adding 0.2 ml of a 1-mg/ml solution of NG in 0.1 M sodium citrate (pH 5.5) to three of the cultures, and incubating the mixture in a 37°C water bath for 5, 15, and 30 min, respectively. Each sample was then pelleted, washed in 0.1 M phosphate buffer (pH 7.0), resuspended in 10 ml of KB, plated to determine the extent of killing, and grown overnight. The mutants were screened by plating 100 to 500 CFU on M9 plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide X-Gluc per ml.

β-Glucuronidase assays. *avr* and *hrp* gene induction following transfer from rich medium to minimal medium was measured by growing cultures in King's B sucrose, in which *hrp* expression is suppressed, to late exponential phase (OD_{600} = 0.6 to 1.0), washing with M9 media, and resuspending the bacteria at an OD_{600} of 0.02 in M9 medium incubated at 28°C. At the indicated times, aliquots were pelleted in a microcentrifuge and resuspended in 10 mM $MgSO_4$. Duplicate serial dilutions were then plated on LB media containing appropriate antibiotics, and the bacterial concentration was determined by CFU counting. Then, 100 μl of each sample was added to an equal volume of 2× extraction buffer (36). β-Glucuronidase assays were performed by adding 50 μl of sample to 500 μl of assay buffer containing 6.25 mM methylumbelliferyl-β-D-glucuronide as substrate (37). Fluorometric measurements were performed using a Hoefer Scientific Instruments fluorimeter with an excitation wavelength of 365 nm and an emission wavelength of 460 nm. Standard curves were obtained with 4-methylumbelliferone (Sigma). Enzymatic activity was calculated as rate of accumulation of 4-methylumbelliferone (nanomoles/minute) per bacterial cell and represents the average of two experiments with three replicates per experiment.

The accumulation of β-glucuronidase produced by bacteria in *Arabidopsis* leaves was determined after infiltrating the leaves with cultures grown in King's B to late exponential phase (OD_{600} = 0.6 to 1.0) that had been washed with 10 mM $MgSO_4$ and resuspended in 10 mM $MgSO_4$ at an OD_{600} of 0.2. At the indicated times, leaves were harvested, and six leaf punches obtained with a no. 2 cork borer were ground in 200 μl of 10 mM $MgSO_4$ and then handled as described above. Values presented for glucuronidase activity represent the average of two or three experiments with three replicates per experiment.

Plant pathogenicity assays. *Arabidopsis* ecotype Columbia was germinated, grown, and infiltrated with ES4326 strains at a titer of either 1×10^4 or 5×10^7 CFU/cm^2 of leaf area as described earlier (74). Growth of *P. syringae* strains in leaves was measured by individually grinding four to six 0.2-cm^2 leaf punches (excised with a no. 2 cork borer) in 10 mM $MgSO_4$, plating appropriate dilutions on King's B medium (ES4326) containing appropriate antibiotics, and counting the CFU. *Nicotiana tabacum* (tobacco) cv. Xanthi was grown under greenhouse conditions, inoculated with ES4326 strains, and assayed for HR as previously described (72). *Phaseolus vulgaris* cv. Red Kidney (bean) was grown under greenhouse conditions and vacuum inoculated as described earlier (45).

RESULTS

Cloning of ES4326 *hrpZ*, *hrpL*, *hrpS*, and *hrpR*. We cloned several genes that belong to the ES4326 *hrp* cluster which function at different levels in the *hrp* regulatory cascade. The ES4326 *hrpRS* genes were identified by colony hybridization using a heterologous *hrpS* probe from *P. syringae* pv. phaseolicola as described in Materials and Methods. Comparing the ES4326 sequences to *hrpR* and *hrpS* of *P. syringae* pv. syringae 61 revealed 83 and 85% DNA sequence identity, respectively. pEH10, a cosmid clone carrying ES4326 *hrpS* on a 24-kb insert complemented the inability of the *P. syringae* pv. phaseolicola *hrpS* mutant LRG94 to elicit disease symptoms on bean leaves, indicating that the ES4326 *hrpS* functions similarly to its counterpart in *P. syringae* pv. phaseolicola. As described in Materials and Methods, pEH10 was also shown to contain a presumptive *hrpZ* gene; a 90-bp sequence showed 82% identity to the *P. syringae* pv. syringae strain 61 *hrpZ* gene.

The ES426 *hrpL* gene was also identified by colony hybridization using a *P. syringae* pv. phaseolicola probe as described in Materials and Methods. A 5.8-kb *Eco*RI fragment containing *hrpL* was subcloned and sequenced, revealing a series of open reading frames corresponding to the *P. syringae* pv. syringae strain 61 *hrpK*, *hrpL*, *hrpJ*, *hrcV*, *hrpJ3*, and *hrcN* genes. The ES4326 and *P. syringae* pv. syringae strain 61 *hrp* genes are colinear and shared 73 to 89% DNA sequence identity, with *hrpL* being the most highly conserved.

Based on the structure of the *hrp* clusters in *P. syringae* pathovars phaseolicola and syringae, we expected that cosmid pEHL, which contains a 35-kb insert containing *hrpL* should overlap cosmid pEH10, which contains *hrpS*. Indeed, a 6-kb *Eco*RI fragment from pEH10 (that extends from the polylinker *Eco*RI site to the first *Eco*RI site; see hatched fragment in Fig. 1) hybridized to the 8-kb *Eco*RI fragment of pEHL. This allowed us to construct the restriction map of the ES4326 *hrp* cluster shown in Fig. 1.

Construction of *uidA* translational fusions. Translational reporter fusions were constructed as described in Materials and Methods between the *E. coli uidA* structural gene for β-glucuronidase and the ES4326 *hrpZ*, *hrpL*, and *hrpS* genes, as well as the avirulence gene *avrRpt2* from *P. syringae* pv. tomato. Plasmid pAVR12 contains 133 codons and 171 bp of upstream nontranslated sequence of *avrRpt2* fused to the entire *uidA* gene, including the ATG translational initiation codon. Plasmid pHRPSgus contains 68 codons of *hrpS* and 1.7 kb of upstream sequence fused to the entire *uidA* gene. Plasmids pHRPZgus and pHRPLgus carry *hrpZ-uidA* and *hrpL-uidA* fusions at codons 135 and 25 of *hrpZ* and *hrpL*, respectively.

***hrp* and *avrRpt2* transcription is *rpoN* dependent in vitro.** To test whether transcription of *hrp* and *avr* genes in ES4326 is *rpoN* dependent, we assayed expression of the *hrpS-uidA*, *hrpL-uidA*, *hrpZ-uidA*, and *avrRpt2-uidA* gene fusions in ES4326 *rpoN*::Km^r. Strains were grown overnight in KB media which suppresses *hrp* gene expression. The bacteria were then pelleted, washed, and resuspended in minimal M9 medium, a medium that derepresses *hrp* and *avr* gene expression (65). At various times, samples were taken and β-glucuronidase activity was determined, with zero time points taken in KB media before resuspension. The results, summarized in Fig. 2, show that the expression levels of *hrpL-uidA*, *hrpZ-uidA*, and *avrRpt2-uidA* fusions were at least 2 orders of magnitude lower in ES4326 *rpoN*::Km^r than in the wild type, whereas the *hrpS-uidA* fusion displayed the same low level of expression in both the wild type and in the *rpoN*::Km^r mutant. Interestingly, the timing of induction of *hrpS-uidA* was delayed in ES4326 *rpoN*::Km^r compared to the wild type, possibly due to the slower rate of growth of the *rpoN* mutant. It should be noted that we do not know what affect the Hrp and Avr protein regions in the fusion proteins have on the relative activity and stability of β-glucuronidase, and so expression data can only be compared for each fusion and not between different fusions.

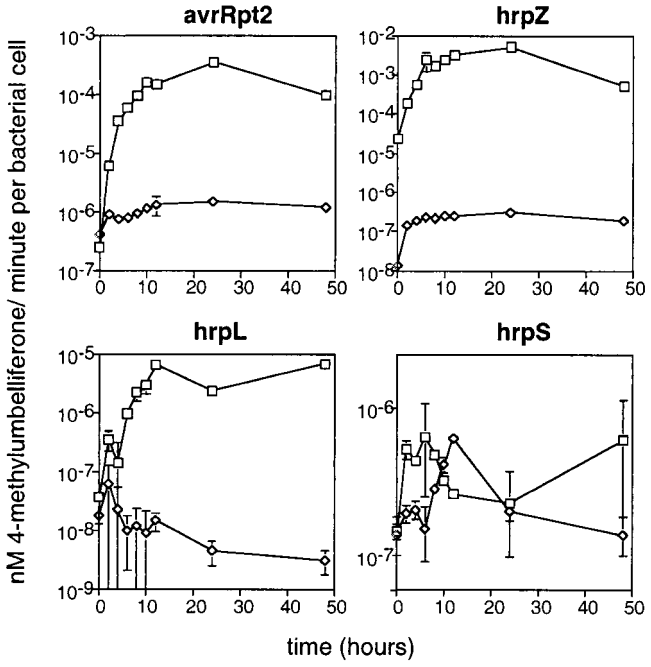

FIG. 2. *hrpS*, *hrpZ*, *hrpL*, and *avrRpt2* transcriptional activity in ES4326 and ES4326 *rpoN*::Km^r following transfer from rich medium to minimal medium. ES4326 (□) and ES4326 *rpoN*::Km^r (◇) carrying fusions were shifted from rich (KB) to minimal (M9; pH 5.5) media at time zero, and the β-glucuronidase activity was measured at the indicated times as described in Materials and Methods. Glucuronidase activity is expressed as nanomoles per minute per cell. Time is given in hours after transfer to minimal medium. Reporter fusions: avrRpt2, *avrRpt2-uidA*; hrpZ, *hrpZ-uidA*; hrpL, *hrpL-uidA*; hrpS, *hrpS-uidA*.

To confirm the results obtained with the gene fusions, RNA blots containing total RNA isolated from ES4326, ES4326 *rpoN*::Km^r, ES4326 expressing *avrRpt2* on plasmid pLH12, and ES4326 *rpoN*::km^r (pLH12) grown in M9 medium were probed with radiolabeled fragments containing *avrRpt2*. As shown in Fig. 3, the *avrRpt2* transcript was detected only in the case of the wild-type strain carrying the *avrRpt2* cosmid. The *hrpL-uidA* and *hrpZ-uidA* results were also confirmed by RNA blot analysis. Total RNA was isolated from ES4326 and ES4326 *rpoN*::Km^r grown in KB medium or after transfer to M9 minimal medium for 6 or 12 h. *hrpL* and *hrpZ* transcripts accumulated after transfer to minimal medium in ES4326 but not in ES4326 *rpoN*::Km^r (data not shown).

***rpoN* is required for in planta induction of *hrp* and *avr* genes.** To determine whether *hrp* and *avr* gene regulation are substantially different in planta and in vitro, we monitored the expression of the *hrpS-uidA*, *hrpL-uidA*, *hrpZ-uidA*, and *avrRpt2-uidA* gene fusions in *Arabidopsis* leaves. As with the induction experiments in vitro, strains were grown overnight in KB medium but were then washed and resuspended in 10 mM $MgSO_4$ before infiltration into *Arabidopsis* leaves. Figure 4, which presents β-glucuronidase activity data normalized to CFU, shows that *avrRpt2-uidA*, *hrpZ-uidA*, and *hrpL-uidA* were activated to significantly higher levels in wild-type ES4326 than in the ES4326 *rpoN*::Km^r mutant. In contrast to the in vitro experiment, some induction of the *avrRpt2-uidA*, *hrpZ-uidA*, and *hrpL-uidA* fusions appears to occur in the *rpoN*

FIG. 3. RNA blot analysis of *avrRpt2* expression in *P. syringae* strains 6 h after transfer to minimal medium. Growth of bacteria and the RNA preparation was performed as described in Materials and Methods. A radiolabeled fragment encoding an internal portion of the *avrRpt2* gene was used as a probe. Lanes: 1, ES4326(pLAFR3); 2, ES4326 *rpoN* (pLAFR3); 3, ES4326(pLH12); 4, ES4326 *rpoN* (pLH12).

mutant, although the standard deviations in these strains were quite high and the β-glucuronidase activities were near the limits of detection. Another notable difference between the in vitro and in planta experiment was that *hrpZ-uidA* was induced to at least 10-fold-higher levels per cell in vitro than in planta, while *hrpS-uidA* was induced 10-fold higher in planta.

As was the case in vitro, *hrpS-uidA* activity in the strain ES4326 *rpoN*::Km^r mutant was comparable to wild-type levels at 6 h. Unexpectedly, however, as shown in Fig. 4, *hrpS-uidA* activity dropped sharply in the wild-type strain after 24 h, whereas activity dropped only slightly in ES4326 *rpoN*::Km^r. After 24 h, expression in the wild-type strain actually fell below the level in ES4326 *rpoN*::Km^r.

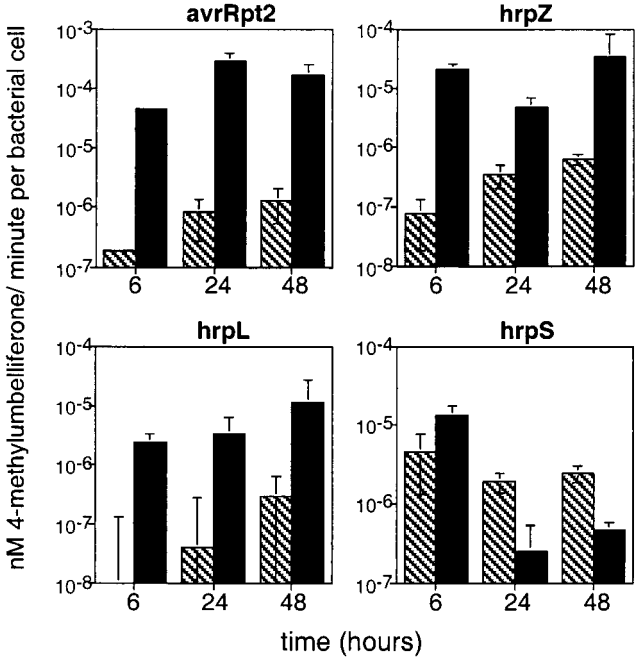

FIG. 4. Time course of *hrpS*, *hrpZ*, *hrpL*, and *avrRpt2* induction in ES4326 and ES4326 *rpoN*::Km^r following inoculation into *Arabidopsis* leaves. ES4326 (solid bars) and ES4326 *rpoN*::Km^r (cross-hatched bars) carrying *hrpS*, *hrpZ*, *hrpL*, and *avrRpt2* fusions to *uidA* were inoculated into *Arabidopsis* leaves, and the glucuronidase activity was measured as described in Materials and Methods. Glucuronidase activity is reported as nanomoles per minute per cell. Time is given in hours after inoculation.

FIG. 5. Constitutive expression of *hrpL* restores an HR when ES4326 *rpoN* is infiltrated into tobacco leaves. Tobacco leaves were infiltrated with bacterial suspensions (5×10^7 CFU/ml) as described in Materials and Methods, and symptoms were photographed 2 days postinoculation. Tobacco leaf sections: 1, ES4326 *rpoN*; 2, ES4326; 3, ES4326 *rpoN* (pHRPLC).

Constitutive expression of *hrpL* is sufficient to restore pathogenicity and the HR in *rpoN* mutants. Because HrpL is sufficient to activate transcription of *P. syringae* pv. syringae *hrp* genes in *E. coli* (80), it appears likely that *rpoN* regulates *hrp* expression by activating the transcription of *hrpL*. If this were the case, constitutive expression of *hrpL* would be expected to suppress the nonpathogenic and non-HR-inducing phenotypes of an *rpoN* mutant. A fusion between the promoter region of *lacZ* and *hrpL* was constructed by cloning an engineered *hrpL* gene into the *Eco*RI site of pLAFR3 as described in Materials and Methods. Constitutive expression of *hrpL* (from plasmid pHRPLC) was confirmed by RNA blot analysis (data not shown). *hrpZ* was expressed at very high levels in ES4326 *rpoN*::Km^r (pHRPLC) even in *hrp*-suppressing KB media (data not shown). However, pHRPLC had no effect on other *rpoN* phenotypes, such as nonmotility and the inability to utilize nitrate as a sole nitrogen source (data not shown). As shown in the accompanying study (32), constitutive *hrpL* expression also did not restore expression of the phytotoxin coronatine, suggesting that ES4326 *rpoN*::Km^r (pHRPLC) phenotypes are most likely due to the specific activation of *hrp* and *avrRpt2* genes. Interestingly, as shown in Fig. 5, ES4326 *rpoN*::Km^r carrying pHRPLC elicited a strong HR on tobacco leaves. ES4326 *rpoN*::Km^r (pHRPLC) also elicited water-soaked disease symptoms on *Arabidopsis* leaves, symptoms identical to the symptoms elicited by wild-type *P. syringae* (data not shown), when infiltrated at a titer of 5×10^7 CFU/ml. Lower doses of ES4326 *rpoN*::Km^r (pHRPLC) also elicited disease symptoms, though not to the same extent as the wild-type strain.

Despite the ability of strain ES4326 *rpoN*::Km^r (pHRPLC) to elicit disease symptoms, pHRPLC did not restore the ability of ES4326 *rpoN*::Km^r to grow in planta (Fig. 6). These data suggest that factors under *rpoN* control but not located in the *hrp* cluster may also be necessary for wild-type virulence in planta. However, there are two important caveats to this con-

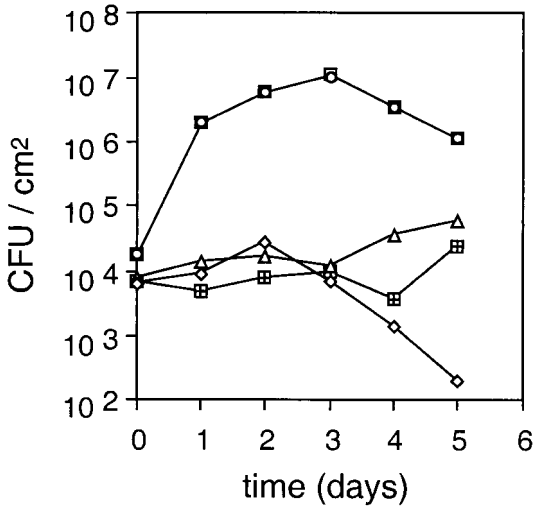

FIG. 6. Constitutive expression of *hrpL* does not restore growth to strain ES4326 *rpoN*::Km^r in *Arabidopsis* leaves. Six-week-old *Arabidopsis* (Columbia) leaves were inoculated with bacterial suspension at 10^4 CFU/cm^2, and bacterial titers were determined as described in Materials and Methods. Symbols: □, ES4326; ○, ES4326(pHRPLC) with no selection for pHRPLC; ◇, ES4326 (pHRPLC) with antibiotic selection for pHRPLC; △, ES4326 *rpoN*::Km^r; ⊞, ES4326 *rpoN*::Km^r (pHRPLC).

clusion. First, ES4326 *rpoN*::Km^r (pHRPLC) grows even more slowly than the *rpoN* mutant itself, implying that constitutive expression of *hrpL* is deleterious. Second, the effect of pHRPLC on wild-type *Psm* ES4326 in planta could not be determined because the plasmid was rapidly lost from the wild-type strain upon infiltration (Fig. 6).

Constitutive expression of *avrRpt2* is not sufficient for the elicitation of the HR in an *rpoN* mutant. We report in the accompanying study that an ES4326 *rpoN* mutant carrying the avirulence gene *avrRpt2* failed to elicit an HR on *A. thaliana* (32). This is not surprising since *avrRpt2* is expressed at very low levels in a *rpoN* mutant. Moreover, it is likely that AvrRpt2 is delivered directly into plant cells via the type III secretory system encoded by the *hrp* genes (58, 59, 61, 76). Therefore, we reasoned that constitutive *avrRpt2* expression in ES4326 *rpoN*::Km^r would not be sufficient for elicitation of an HR. Indeed, ES4326 *rpoN*::Km^r containing plasmid pAVRC1 (which contains a *lacZ-avrRpt2* fusion) did not elicit a detectable HR on tobacco or *Arabidopsis* leaves (data not shown).

Interestingly, ES4326(pAVRC1) did not elicit an HR in *Arabidopsis* at lower inoculation doses than ES4326 carrying pLH12, which contains *avrRpt2* expressed from its own promoter. However, approximately 75% of the plants infiltrated with ES4326(pAVRC1) at a dose of 10^5 CFU/cm^2 developed an HR 22 h after infiltration compared to only 12% of the plants inoculated with ES4326(pLH12). These data suggested that constitutive expression of *avrRpt2* could potentially lead to enhanced resistance of the host. To investigate this latter point further, we determined the growth of ES4326(pLAFR3), ES4326(pLH12), and ES4326(pAVRC1) in *Arabidopsis* leaves. However, both pLH12 (*avrRpt2*) and pAVRC1 (*lacZ-avrRpt2*) resulted in the same decrease in growth compared to ES4326 (pLAFR3) (data not shown).

Screen to find additional *hrp* regulatory factors. The results described above show that *rpoN* gene is required for the transcriptional activation of the *hrpL*, *hrpZ*, and *avrRpt2* genes but that *hrpS* transcription is *rpoN* independent. Assuming that *rpoN* itself is not transcriptionally regulated under *hrp*-inducing conditions in vitro or in planta and that HrpS plus RpoN activate *hrpL*, these results suggest that HrpS plus RpoN activity is regulated by environmental factors. In turn, this line of reasoning suggests that there could be previously unknown genes outside of the *hrp* cluster that play a role in *hrp* gene regulation. In this section we describe a series of experiments that were undertaken to isolate such mutants.

The *hrpZ-uidA* fusion described above was transferred from pHRPZgus to the ES4326 chromosome to produce ES4326 *hrpZ*::*uidA* by marker exchange. ES4326 *hrpZ*::*uidA* was then mutagenized using nitrosoguanidine and plated onto M9 medium containing X-Gluc. Among approximately 14,000 colonies screened, 297 exhibited reduced or no blue color and were tested further.

Among the 297 putative mutants, we were particularly interested in identifying mutations that mapped outside of the *hrp* gene cluster. We first screened the 297 mutants for putative *hrpR*, *hrpS*, or *hrpZ*::*uidA* mutants by complementation with pEH10 or pHRPZgus. All but five of the mutants were complemented by one of these two plasmids. The five remaining mutants were each mated to a strain ES4326 chromosomal library in pRR54cos, and the exconjugants were plated on M9 X-Gluc plates to isolate plasmids that restored β-glucuronidase activity. Two such plasmids, pEHL and pXIVB4, restored activity to all five of the mutants. These clones were isolated and shown by DNA blot analysis to contain *hrpL*. The simplest interpretation of these data is that no *hrp* regulators outside of the *hrp* cluster were identified in the screen.

DISCUSSION

Circuitry of *hrp* regulation. We have shown in the accompanying study that ES4326 *rpoN* is nonpathogenic and that ES4326 *rpoN* carrying *avrRpt2* fails to elicit an HR on *Arabidopsis* (32). In the experiments reported here we sought to determine whether the alternative sigma factor σ^{54} encoded by *rpoN* is required for the expression of *hrp* and *avr* genes, which would explain the nonpathogenic and non-HR-inducing phenotypes of the ES4326 *rpoN* mutant. The rational for investigating whether *rpoN* regulates *hrp* expression is that *hrpS* and *hrpR* encode proteins that are homologous to the NtrC group of transcriptional activators that work in concert with σ^{54} to activate target gene expression (26, 27, 54).

In the case of *P. syringae* pv. phaseolicola, it was shown that HrpR is required for *hrpS* transcription and that *hrpS* mutants fail to activate transcription of the remaining *hrp* and *hrc* genes in the cluster (26, 27). While *hrpRS* transcription has been shown to be strongly affected by plant signals (65), it is not clear from previous work whether *rpoN* is regulated at the transcriptional level or whether the activity of the HrpR or HrpS is environmentally regulated.

Expression studies, conducted both in vitro and in planta, determined that *hrpL*, *hrpZ*, and *avrRpt2* expression is significantly higher in wild-type ES4326 than in an *rpoN* mutant. Curiously, the *hrpZ-uidA* fusion was activated at least 10-fold higher in vitro than in planta. In contrast to the other *hrp* genes, the activation of *hrpS* transcription does not appear to be *rpoN*-dependent. A *hrpS-uidA* fusion was expressed at the same levels in vitro in both wild-type and *rpoN* mutant cells. In planta, *hrpS-uidA* expression per cell was about 10-fold higher than in vitro, a finding similar to what has been reported previously for *P. syringae* pv. phaseolicola (65). Despite some inconsistencies, these data shown that the loss of pathogenicity and HR phenotypes of the *rpoN* mutant is most easily explained by the failure to activate *hrp* and *avr* transcription in the *rpoN* mutant.

In general, our data are consistent with previously proposed models of *hrp* regulation in *P. syringae* in which HrpS plus RpoN (σ^{54}) activate *hrpL* and in which HrpL activates *avr* genes and the remaining genes in the *hrp* cluster (63, 80, 81). To further test this model, we placed *hrpL* expression under control of the *E. coli lacZ* promoter. Constitutive expression of *hrpL* not only restored *hrpZ* transcription to ES4326 *rpoN*::Km^r but also restored the elicitation of an HR on tobacco and disease symptoms in *Arabidopsis*. These data suggest that *rpoN* does not play a significant role in *hrp* gene expression downstream of HrpL. Moreover, constitutive expression of *hrpL* did not result in complementation of other *rpoN* phenotypes, including lack of motility, slow growth, and the inability to utilize nitrate as a sole nitrogen source. The results suggest that the role of *rpoN* in eliciting disease symptoms and the HR, at least in *Arabidopsis* and tobacco plants, respectively, is primarily limited to the activation of *hrpL*. On the other hand, as discussed further below, we report in the accompanying study that *rpoN* is also required for the *hrp*-independent synthesis of the phytotoxin coronatine, and it is possible that the failure to activate the coronatine pathway contributes to the loss of pathogenicity phenotype of the *rpoN* mutant (32).

***hrpS* expression and the role of HrpS versus HrpR in the regulation of the *hrp* cluster.** In vitro, the final induction level of the *hrpS-uidA* fusion was similar in the wild type and the *rpoN* mutant; however, *hrpS-uidA* induction was delayed in ES4326 *rpoN*::Km^r, possibly due to the relatively slow growth of the *rpoN* mutant. In planta, *hrpS-uidA* induction was initially the same in the wild-type and *rpoN* mutant. Interestingly, *hrpS-uidA* activity dropped dramatically in the wild-type strain 24 h after infection but only declined moderately in the mutant. One explanation for these data is that the lack of growth of ES4326 *rpoN*::Km^r in planta and its failure to induce host defense responses (32) might mimic the early stages of infection at which *hrpS-uidA* activity is normally high. Preston et al. have reported that *hrpV* acts as a negative regulator of *hrp* transcription, possibly upstream of *hrpS* (62). Thus, *hrpS* downregulation could be the result of feedback inhibition by *hrpV*, an effect similar to the feedback regulation of the type III secretion system of *Yersinia pestis* (43). This seems unlikely though, as we would not then have expected to see the downregulation of the *hrpS-uidA* fusion in the *rpoN* mutant in vitro (see Fig. 2).

Another aspect of *hrp* gene regulation that needs further study is the relationship between HrpR and HrpS. The *hrpS* gene is downstream of *hrpR*, but they do not appear to constitute an operon (26). In both *P. syringae* pv. phaseolicola and ES4326, *hrpS* transcription must initiate within the *hrpR* gene and in *P. syringae* pv. phaseolicola, *hrpR* is required for *hrpS* transcription (26). However, because *hrpS* expression is not significantly reduced in *rpoN* mutants, either HrpR activity is *rpoN* independent or *hrpS* transcription is not dependent on HrpR in *P. syringae* pv. maculicola.

It is conceivable that in ES4326 *hrpR* is transcriptionally regulated by upstream factors and that HrpR plus RpoN activate *hrpL*. We have not specifically tested ES4326 *hrpS* or *hrpR* mutants to determine if they have the same phenotype as *P. syringae* pv. phaseolicola *hrpS* mutants.

***rpoN*- and *hrpL*-regulated virulence factors.** One unexpected aspect of our studies with constitutive *hrpL* expression is that *hrpL* restored both disease and HR symptoms to ES4326 *rpoN*::Km^r but did not restore growth in planta. This is a complicated result, and the failure of ES4326 *rpoN*::Km^r (pHRPLC) to grow in planta needs to be discussed separately from the fact that it elicited host symptoms. One simple explanation for the failure to grow relates to the highly pleiotropic nature of the *rpoN* mutation. For example, the *rpoN* mutant might be unable to metabolize a major nutrient source in the plant, irrespective of *hrpL* expression. Another explanation for the failure to grow in planta could be related to the fact that HrpL is an alternative sigma factor (80, 81) and that constitutive expression could interfere with a variety bacterial functions not related to pathogenicity. Indeed, ES4326 strains expressing *hrpL* constitutively grew more slowly in vitro, whereas in planta the plasmid carrying the *hrpL* construct was rapidly lost from a wild-type strain.

A more interesting possibility for the failure of ES4326 *rpoN*::Km^r (pHRPLC) to grow in planta is that important virulence factors required for in planta growth may lie outside of the *hrp* regulation pathway but under *rpoN* control. We show in the accompanying study that the phytotoxin coronatine is one such factor, being absent in *rpoN* mutants and not restored by constitutive *hrpL* expression (32). Coronatine is a non-host-specific toxin that primarily elicits chlorosis but is also known to induce hypertrophy and inhibit root elongation (7, 19, 69). Studies of coronatine-defective mutants of *P. syringae* pv. tomato have shown that the toxin normally contributes to lesion expansion, chlorosis, and bacterial multiplication in *Arabidopsis* (55). While the absence of coronatine alone seems insufficient to explain the failure of ES4326 *rpoN*::Km^r (pHRPLC) to grow in planta, coronatine may be one of several virulence factors under *rpoN* control but not *hrpL* control that contribute to pathogenicity.

In any case, our *hrpL* overexpression studies have shown that there is not a necessary linkage between symptom development in Arabidopsis and growth of *P. syringae* pv. maculicola in planta but that *hrpL*-dependent factors are required for the elicitation of these symptoms. This is consistent with the result reported in the accompanying study (32) that ES4326 *rpoN*::Km^r (pHRPLC) elicits defense gene induction, whereas ES4326 *rpoN*::Km^r does not when infiltrated into *Arabidopsis* leaves. An important future experiment is to determine whether the symptoms elicited by ES4326 *rpoN*::Km^r (pHRPLC) involve necrosis or programmed cell death (PCD) or both. One intriguing possibility is that *hrpL*-dependent factors are involved in eliciting PCD in virulent interactions that cause disease as well as in avirulent interactions that result in an HR.

Failure to find upstream regulatory mutants. Considering the relatively large number of mutants screened and because putative mutations were obtained in each of the known *hrp* regulatory loci, *hrpR*, *hrpS*, and *hrpL*, it seems likely that the screen for *hrp* regulatory mutants was saturated. However, there is a potential problem with the screen design that relates to the *trans*-complementation method used to identify, and thus remove for further consideration, *hrpR*, *hrpS*, and *hrpZ*::*uidA* mutants. It is conceivable that overexpression of *hrpR* and *hrpS* from pEH10 or pHRPZgus compensated for mutations in upstream regulatory factors or titrated out a factor that interacts directly with HrpS or HrpR to downregulate its activity.

It is possible that *hrp* gene regulation is mediated by a factor that interacts with HrpR and/or HrpS and/or RpoN. Another possibility is that *rpoN* expression itself is transcriptionally regulated. Regulation of *rpoN* transcription has been reported in *Bradyrhizobium japonicum* (42) and *Rhodobacter capsulatus* (12). In *P. aeruginosa* (38) and *Klebsiella pneumonia* (51), genes found downstream of *rpoN* appear to modify RpoN activity. Unfortunately, mutations that affect *rpoN* transcription or activity would probably have a slow-growth phenotype similar to *rpoN* mutants and would probably not have been identified in our screen.

Some regulatory genes such as the *P. aeruginosa fur* gene, which encodes an iron-responsive protein, are essential for

bacterial survival (5). However, mutagenesis using NG should produce mutants that retain partial function, resulting in an intermediate phenotype allowing identification of such essential genes (52). We did not identify such mutants, though the screen might not have been sufficiently sensitive to detect small changes in activity.

hrpV has been reported to be a negative regulator of *hrp* expression, and there is some evidence that *hrpV* functions upstream of *hrpRS* (62). It is not likely, however, that we would have detected mutations in negative regulators because the screen was designed specifically to identify positive regulators outside of the *hrp* cluster. The high level of *hrpZ-uidA* expression in wild-type cells on the minimal media used in the screen most likely obscured the recovery of mutants with enhanced levels of expression. In any case, it is likely that *hrpV* mutants would have been complemented by the plasmids that we used to screen against *hrpRS* and *hrpL* mutants. Taken together, our results suggest that upstream regulators of *hrp* are either negative regulators, essential genes, or regulators of σ^{54} activity or transcription.

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