

## Enhancer-Binding Proteins HrpR and HrpS Interact To Regulate *hrp*-Encoded Type III Protein Secretion in *Pseudomonas syringae* Strains

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**In *Pseudomonas syringae* strains, the *hrp-hrc* pathogenicity island consists of an HrpL-dependent regulon that encodes a type III protein translocation complex and translocated effector proteins required for pathogenesis. HrpR and HrpS function as positive regulatory factors for the *hrpL* promoter, but their mechanism of action has not been established. Both HrpR and HrpS are structurally related to enhancer-binding proteins, but they lack receiver domains and do not appear to require a cognate protein kinase for activity. *hrpR* and *hrpS* were shown to be expressed as an operon: a promoter was identified 5' to *hrpR*, and reverse transcriptase PCR detected the presence of an *hrpRS* transcript. The *hrpR* promoter and coding sequence were conserved among *P. syringae* strains. The coding sequences for *hrpR* and *hrpS* were cloned into compatible expression vectors, and their activities were monitored in *Escherichia coli* transformants carrying an *hrpL'*-*lacZ* fusion. HrpS could function as a weak activator of the *hrpL* promoter, but the activity was only 2.5% of the activity detected when both HrpR and HrpS were expressed in the reporter strain. This finding is consistent with a requirement for both HrpR and HrpS in the activation of the *hrpL* promoter. By using a yeast two-hybrid assay, an interaction between HrpR and HrpS was detected, suggestive of the formation of a heteromeric complex. Physical interaction of HrpR and HrpS was confirmed by column-binding experiments. The results show that HrpR and HrpS physically interact to regulate the  $\sigma^{54}$ -dependent *hrpL* promoter in *P. syringae* strains.**

*Pseudomonas syringae* is a causal agent of leaf blights and related diseases in many plant species (19). When introduced into tissue of a susceptible plant, the bacterium colonizes the intercellular spaces of parenchymatous tissue, remaining external to plant cell walls. Colonizing bacteria produce extracellular polysaccharides, derivatized peptide toxins, and plant hormones that lead to altered ion fluxes across cellular membranes and slowly developing tissue necroses typical of leaf blights. Although *P. syringae* is capable of causing disease in most economically important plant species, a single strain usually causes disease only in a specific subset of plant species or in some cases specific genetic lines of a single plant species. When introduced into plants other than the susceptible host, a *P. syringae* strain elicits an active defense response that culminates in a rapid programmed cell death. This programmed cell death, also known as the hypersensitive response, and the associated defense responses prevent further colonization of the tissue and are thought to be major factors in the determination of a strain's host range (8, 23).

The colonization of plant tissue and elicitation of active defense responses by *P. syringae* strains have both been linked to a pathogenicity island (PAI) called the *hrp* gene cluster (for a recent review, see reference 8). The *P. syringae* *hrp* gene cluster encodes a type III protein export complex (PEC) sim-

ilar to those encoded by PAIs of mammalian pathogens (8, 22, 24), such as *Yersinia*, *Salmonella*, *Shigella*, enteropathogenic and enterohemorrhagic *Escherichia coli*, and *P. aeruginosa* strains and, more recently, *Chlamydia* spp. (11, 20) and *Bordetella pertussis* (66). The gene products conserved among all known type III PECs are present in the *hrp* gene cluster and are required for activity. Like other type III PECs, the *hrp*-encoded type III PEC functions in the translocation of effector proteins into the cytoplasm of host cells (13, 39, 43), most likely through the HrpA pilus (57).

Effector proteins translocated by the *hrp*-encoded type III PEC are postulated to function as pathogenicity determinants to allow colonization of susceptible plant hosts (5, 26, 46). Some of these translocated effector proteins, identified as products of *avr* (avirulence) genes, can also elicit the aforementioned active defense responses in resistant plants through a host protein-mediated recognition process (see references in references 23, 32, and 39). In addition, differential regulation of the *hrp* regulon in *P. syringae* strains may also affect host range by altering when pathogenicity and host range factors are secreted during pathogenesis.

Like many other type III PECs, expression of most *P. syringae* *hrp* and *hrc* genes is environmentally regulated. *hrp*, *hrc*, and *avr* expression is low during growth in most rich media containing broad-spectrum amino acid sources and is induced during pathogenesis or by culture in an acidic minimal salts medium (45, 56, 65). The acidic minimal salts medium is thought to mimic conditions found in planta. It is unclear at present whether *hrp* and *avr* genes are regulated by host cell contact similar to that postulated to occur during pathogenesis by *Yersinia* spp. (45, 48) or whether they are regulated via

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nutritional or physiological signals related to the growth conditions.

Several transcriptional factors that mediate the environmental regulation of the *P. syringae* *hrp*, *hrc*, and *avr* genes have been identified. The primary transcriptional factor controlling expression of most *hrp*, *hrc*, and *avr* genes is the alternative sigma factor HrpL (63), a member of the extracytoplasmic function (ECF) family of sigma factors (34). An HrpL-dependent promoter consensus sequence that is present in all known HrpL-dependent promoters (24) was identified (64) and is a required *cis*-acting element associated with transcription initiation (25, 49, 51). The operons carrying the *hrp* and *hrc* genes, which encode structural components of the type III PEC, as well as the genes for secreted effector proteins, such as the *avr* and *hop* genes, form the *hrp* regulon, which is dependent upon HrpL for expression (24). Related sigma factors controlling the type III PEC have been identified in *Erwinia* strains carrying closely related group I *hrp* clusters (HrpL) (59) and in *Bordetella* (Trs) (66).

Because HrpL is the primary transcription factor controlling expression of *hrp* regulon genes, regulation of *hrpL* transcription may in part control the environmental regulation of the *hrp* regulon. HrpR and HrpS have been reported to be positively acting regulators of *hrpL* expression (63). Both HrpR and HrpS are unusual members of the enhancer-binding family of proteins (9, 15, 16, 63) that normally function as response regulators of two-component regulatory systems (41). Most enhancer-binding proteins are typically modular, consisting of a large regulatory receiver (AB) domain, a central domain (C) involved in the interaction with  $\sigma^{54}$ , and an enhancer- or upstream activating sequence-binding domain (D) (37, 41). Similar to other enhancer-binding proteins, HrpR and HrpS retain the  $\sigma^{54}$  interaction (C) and DNA binding domains (D) (37, 40). HrpR and HrpS differ from most enhancer-binding factors that function in two-component regulatory systems by the apparent absence of a receiver domain that functions in phosphorylation-dependent modulation of response regulator activity (see reference 52). Thus, HrpR and HrpS are similar to the stress response regulator PspF, which also lacks these domains (27, 28).

The mechanism by which HrpR and HrpS regulate *hrpL* promoter activity has not been established. Xiao et al. (63) reported that *hrpL* promoter activity in *Escherichia coli* transformants was dependent upon the expression of both *hrpR* and *hrpS* and suggested that an interaction between the two proteins may be required to activate expression of the  $\sigma^{54}$ -dependent *hrpL* promoter. Grimm et al. (15) reported that *hrpS* expressed from a plasmid-borne construct could rescue the ability of an *hrpR::Tn5* mutant of *P. syringae* NPS3121 to elicit the hypersensitive response in tobacco leaves. An apparent *hrpS* transcript was detected that appeared to initiate near a minimal  $\sigma^{54}$  promoter consensus sequence internal to the *hrpR* coding sequence. HrpS was thus proposed to function independently of HrpR to activate expression of the *hrp* regulon in *P. syringae* strains (15). Other bacteria carrying closely related group I *hrp* PAIs found in *Erwinia* strains carry an apparent HrpS homolog (30, 58) but not an HrpR homolog. Since other aspects of type III secretion in *Erwinia* strains appear to be similar to those of *P. syringae*, the role of *hrpR* in the regulation of group I *hrp* PAIs is unclear (22, 58).

The purpose of our experiments was to elucidate the role of HrpR and HrpS in the regulation of the *P. syringae* *hrp* regulon. Here we report that *hrpR* and *hrpS* are expressed as a single operon and that the gene products function together as a positive transcriptional factor for the *hrpL* promoter. For maximal activity of the *hrpL* promoter, both HrpR and HrpS were required. Physical interaction of HrpR and HrpS was detected by yeast two-hybrid analysis and confirmed biochemically in column-binding experiments. The results indicate that HrpR and HrpS form a stable heteromeric complex to regulate the  $\sigma^{54}$ -dependent *hrpL* promoter in *P. syringae* strains.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** Bacterial strains, plasmids, and primers used in this work are listed in Table 1. *E. coli* strains were grown at 37°C in King's B broth (1) unless otherwise noted. *P. syringae* strains were grown in King's B broth or M63 minimal salts medium supplemented with glucose, fructose, and/or 1% Casamino Acids as indicated in the text. Yeast (*Saccharomyces cerevisiae*) strains were maintained on defined media (7). The following antibiotics were included where indicated below at the indicated concentrations (in micrograms per milliliter): ampicillin, 200; kanamycin, 50; spectinomycin, 100; tetracycline, 25; and nalidixic acid, 50.

**General DNA manipulations.** Restriction enzymes were purchased from Invitrogen BRL (Bethesda, Md.), and T4 DNA ligase was purchased from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's recommendations. Basic manipulations were done using standard procedures (50). PCRs were performed using a Hybaid PCRSprint thermal cycler with 50- $\mu$ l reaction volumes. Unless indicated otherwise, *Pwo* polymerase (Boehringer Mannheim) was used for amplifying fragments for cloning.

**Construction of *hrpR'*-*lacZ* and *hrpS'*-*lacZ* promoter fusions.** The *hrpR* promoter region was isolated from pYXRS1B as a 690-bp *Bst*YI fragment (Fig. 1). This fragment was cloned into *Bam*HI-digested pRG970 to create pDRR1R. The fusion of the *hrpR* promoter to the vector's promoterless *lacZYA* cassette was confirmed by sequence analysis. The potential *hrpS* promoter was amplified from pHIR11 using the tailed primers P24607 and P25677R. The resulting 1,087-bp fragment encompassed the region extending from 44 bp 5' of the *hrpR* coding sequence to 34 bp inside the *hrpS* coding sequence. The *Bgl*II- and *Xma*I-digested fragment was ligated into *Bam*HI- and *Xma*I-digested pRG970 to construct pTSR4R. Inserts were confirmed by sequence analysis. To construct the equivalent constructs using DC3000 sequences, the primers DC715 and DC1412 (R6 fragment) and DC985 and DC2045 (R7 fragment) were used to amplify fragments from DC3000 genomic DNA that were subsequently ligated into *Sma*I- and *Bam*HI-digested pRG970 as *Eco*RV-*Bam*HI fragments to create pJBR6R carrying the *hrpR* promoter and pJBR7R carrying the putative *hrpS* promoter.

**RNA extraction from *P. syringae* cells.** RNA was isolated from cells grown for 3 h in M63 medium (pH 5.5) containing fructose as the carbon source using hot Trizol (Gibco BRL) extraction as recommended by the manufacturer.

**RT-PCR.** Extracted RNA was DNase I treated and used for cDNA synthesis with Superscript II reverse transcriptase (RT; Gibco BRL) and with the P24200 primer by using the manufacturer's protocol for DNA synthesis from high-GC-content RNA templates. A control reaction lacking RT (-RT) was run in parallel with cDNA synthesis. The cDNA preparation and the -RT control were treated with RNase H and used as templates for PCR. The PCR amplification, using *Taq* polymerase (Gibco BRL) and the primers indicated below, involved 35 cycles of 94°C for 10 s, 50°C for 10 s, and a 1.5-min extension at 72°C followed by a 4-min fill-in reaction at 72°C. Primers used to amplify Pss61 sequences were P24200, P24634, P24901, and P25591 (Fig. 1). For DC3000, the corresponding primer sequences were DC24200, DC24901, and DC25619.

**$\beta$ -Galactosidase assays.**  $\beta$ -Galactosidase activity in bacterial cells was estimated by the procedures of Miller (36).

**Yeast two-hybrid analysis.** *hrpR* (codons 2 to 314) was amplified by PCR using the tailed primers RN and RC from pYXRS1D. Similarly, *hrpS* (codons 2 to 302) was amplified using the tailed primers SN and SC. The resulting fragments were cloned as *Eco*RI-*Bam*HI fragments into pBTM116 to create pDWR3BTM (LexA'-HrpR fusion) and pDWS4BTM (LexA'-HrpS fusion) or into pGAD424 to generate pDWR3GAD (GAL4'-HrpR fusion) and pDWS4GAD (GAL4'-HrpS fusion). Translational fusions to the LexA' binding domain (BD) of pBTM116 or the GAL4' activating domain (AD) of pGAD424 were confirmed by sequence analysis. Truncated derivatives of *hrpR* were constructed using the

Strain, plasmid, or primer	Relevant characteristic(s)	Reference or source
<b>Strains</b>		
<i>P. syringae</i>		
DC3000	Tomato and <i>Arabidopsis</i> pathogen	60
Pss61	Weak bean pathogen	2
<i>E. coli</i>		
DH5 $\alpha$	( $r_K^- m_K^-$ ) <i>recA1 relA1</i> $\Delta$ ( <i>argF-lacZYA</i> ) <i>U169</i> $\phi$ 80 <i>dlacZ</i> DM15	Invitrogen BRL
MC4100	F' $\Delta$ ( <i>argF-lacZYA</i> ) <i>U169</i>	4
<i>S. cerevisiae</i>		
L40	<i>MATa his3D200 trp1-901 leu2-3, 112 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ gal80</i>	S. Hollenberg
<b>Plasmids</b>		
pBTM116	<i>ori<sub>ColE1</sub> ori2<math>\mu</math>m TRP1 P<sub>adh</sub>::LexA'-BD::mcs</i>	3
pBTM-lamin	Lamin gene cloned into pBTM116 to create a LexA BD-lamin fusion	3
pDRR1R	690-bp <i>Bst</i> YI fragment cloned into pRG970 to create P <sub>hrpR</sub> - <i>lacZ</i>	This report
pDSK519	incQ Kn <sup>r</sup>	29
pDSK600	incQ Sp <sup>r</sup> , triple <i>lacUV5</i> promoter, <i>mcs</i>	38
pDWR3BTM	0.9-kb PCR product cloned into pBTM116, LexA'-HrpR	This report
pDWR3GAD	0.9-kb PCR product cloned into pGAD424, GAL4'-HrpR	This report
pDWS4BTM	0.9-kb PCR product cloned into pBTM116, LexA'-HrpS	This report
pDWS4GAD	0.9-kb PCR product cloned into pGAD424, GAL4'-HrpS	This report
pFLAG-CTC	C-terminal FLAG epitope expression vector	Sigma
pGAD424	<i>ori<sub>ColE1</sub> ori2<math>\mu</math>-m LEU2 P<sub>adh</sub>::GAL4'-AD::mcs</i>	3
pHIR11	<i>hrp</i> PAI from Pss61 cloned into pLAFR3	21
pJBR6R	697-bp PCR product amplified from DC3000 and cloned into pRG970 to create P <sub>hrpR</sub> - <i>lacZ</i>	This report
pJBR7R	1,087-bp PCR product amplified from DC3000 and cloned into pRG970 to create P <sub>hrpS</sub> - <i>lacZ</i>	This report
pLAFR3	incP-1 Tc <sup>r</sup> cosmid vector	54
pMLB1034	pBR322 derivative carrying ' <i>lacZ</i> ' for constructing translational fusions	53
pNTRS3D	2-kb PCR product amplified from Pss61 genomic DNA using <i>Pwo</i> polymerase and ligated as an <i>Xba</i> I- <i>Hind</i> III fragment in to pDSK519	This work
pQE30	N-terminal six-His expression vector	Qiagen
pREP4	Supplies LacI, Kn <sup>r</sup>	Qiagen
pRG970	incP Sp <sup>r</sup> , promoterless ' <i>lacZYA</i> ' for constructing transcriptional fusions	55
pSGL4MS	340-bp fragment from pYXL1R cloned into pMLB1034 as a <i>Bam</i> HI- <i>Eco</i> RI fragment, P <sub>hrpL</sub> - <i>lacZ</i>	This report
pSHS23Q30	0.9-kb PCR product cloned into pQE30, P <sub>lac</sub> -six-His- <i>hrpS</i>	This report
pSJR2L	PCR-amplified 1-kb fragment carrying <i>hrpR</i> cloned into pLAFR3, P <sub>lac</sub> - <i>hrpR</i>	This report
pSJS3DS	PCR-amplified 1-kb fragment carrying <i>hrpS</i> cloned into pDSK600 as an <i>Xba</i> I- <i>Bam</i> HI fragment, P <sub>lacUV5</sub> - <i>hrpS</i>	This report
pSJR2S3L	<i>hrpS</i> fragment from pSJS3DS cloned into pSJR2L as a <i>Bam</i> HI- <i>Hind</i> III fragment, P <sub>lac</sub> - <i>hrpR</i> $\Delta$ 30bp <i>hrpS</i>	This report
pTSR4GAD	899-bp PCR product cloned into pGAD424, GAL4'-HrpR $\Delta$ N	This report
pTSR4R	1,087-bp PCR product amplified from Pss61 cloned into pRG970 to create P <sub>hrpS</sub> - <i>lacZ</i>	This report
pTSR5GAD	730-bp PCR product cloned into pGAD424, GAL4'-HrpR $\Delta$ C'	This report
pTSR6GAD	691-bp PCR product cloned into pGAD424, GAL4'-HrpR $\Delta$ N $\Delta$ C'	This report
pTSR8CTC	0.9-kb PCR product cloned into pFLAG-CTC, P <sub>lac</sub> - <i>hrpR</i> -FLAG fusion	This report
pYXL1R	340-bp PCR product cloned into pRG970, P <sub>hrpL</sub> - <i>lacZ</i>	63
pYXRS1B	<i>Bam</i> HI- <i>Bgl</i> II fragment carrying <i>hrpRS</i> cloned into pBluescript SK(+)	63
pYXRS1D	<i>Bam</i> HI- <i>Bgl</i> II fragment carrying <i>hrpRS</i> cloned into pDSK519	63
<b>Primers</b>		
DC715	CGGATATCGACCGCTTTGCCAGTATCC	
DC985	CGGATATCTCCGCTTGCCACCCACCA	
DC1412	CGGGATCCTGATGACCCGCTGATAATGC	
DC2045	CGGGATCCCTCGTCCAGATCATCTCTCAA	
DC24200	GGAGGCGATCACGCAGATA	
DC24901	CAGCAAGGGGAAGCCGAG	
DC25619	GATAAGGACGTCCGAGAGTG	
P23740	GCGCAAGCTTTCAGATGCTCAATTCCTTGATGCGA	
P24200	AGCCGACGCAATCACACAG	
P24607	GCAGATCTCATCCAGGTCATCGTCAAAC	
P24634	GATGATGAGTTTGACCATGA	
P24640	GCGGATCCAATCTCGATGATGAGTTTGACGA	
P24692	GCGAATTCGGACTCGGCTGCCAGGTC	
P24901	TCACAGCAACGGCAATCCGAGG	
P25591	GTAAGTGCATTATCGGCTGG	
P25630R	CCTCAAGCTTAGCACAGACATTGATAACGA	
P25677R	TGTCCCGGGCGTGGTATTAATATTCGCCCT	
RC	GCGGATCCTCAGGACTCGGCTGCCAGGTCGA	
RN	GCGAATTCAGCACAGACATTGATAACGACG	
R $\Delta$ C	GCGGATCCCAGCAACGGCAATCCGAGGAC	
R $\Delta$ N	GCGAATTCGTAATCGATTATCGGCTGGT	
SC	GCGGATCCTCAGATGCTCAATTCCTTGAT	
SN	GCGAATTCATCTCGATGATGAGTTTGAC	

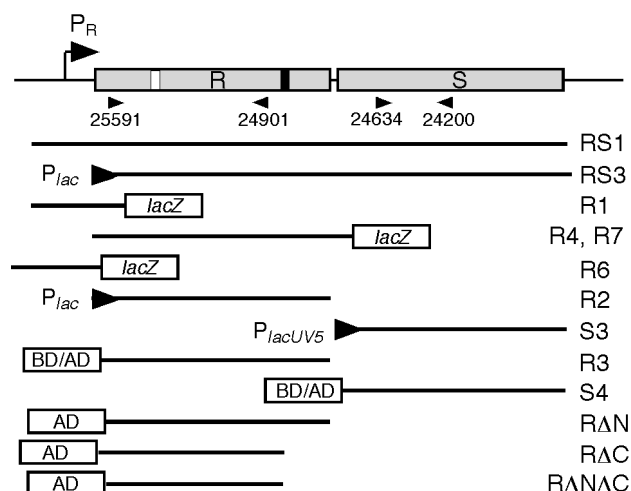


FIG. 1. Features of the *hprRS* region and primers and constructs used in experiments. A map of the *hprRS* region is shown at the top. Shaded boxes represent deduced coding sequences for *hprR* (R) and *hprS* (S) (9, 63). The location of the *hprR* promoter ( $P_R$ ) is shown by the bent arrow. The HrpR box (white bar) and *hprS* promoter (black bar) are positioned as proposed by Grimm et al. (15). The locations and orientations of the primers used in the RT-PCR experiments are indicated by the labeled arrowheads immediately below the map. Fragments used in other experiments are indicated by the labeled lines that represent the portion of the mapped region carried by the fragment. Promoter fusions are shown by the arrowheads, with the left side representing the left end of the fragment. Transcriptional fusions to *lacZ* are indicated by the labeled box. Translational fusions constructed to the LexA' BD or GAL4' AD are also shown. Not shown is the S23 fragment that is equivalent to the S4 fragment except that it has tails with different restriction sites; this fragment was used to construct a His-tagged derivative as described in Materials and Methods. The R8 fragment is equivalent to the R3 fragment but lacks the C-terminal stop codon and was used to make a FLAG-tagged derivative. Designations for plasmids carrying the above-described fragments represent the constructor's initials, the fragment designation shown above, and a suffix indicating the host vector. Vector codes are as follows: B, pBluescript SK+; BTM, pBTM116; CTC, pFLAG-CTC; D, pDSK519; DS, pDSK600; GAD, pGAD424; L, pLAFR3; MS, pMLB1034; Q30, pQE30; R, pRG970.

primers R $\Delta$ N and R $\Delta$ C and corresponding RN or RC primers. The resulting constructs were transformed into *S. cerevisiae* L40 singly or in combination with pBTM-lamin, pGAD424, and the corresponding pBTM116 or pGAD424 construct by using the Li acetate-polyethylene glycol one-step transformation protocol (12). Transformants were selected on defined media by complementation of Trp and/or Leu auxotrophy. The resulting transformants were initially screened for  $\beta$ -galactosidase activity by filter lift assay employing liquid  $N_2$ -lysed cells floated on X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside)-containing phosphate buffer (7).  $\beta$ -Galactosidase activity in liquid  $N_2$ -lysed cells was quantitatively estimated by the procedures of Clark et al. (7).

**Construction of epitope-tagged HrpR and HrpS.** To construct an N-terminal His-tagged HrpS, a fragment carrying *hprS* (codons 2 to 302) was amplified using the primers P24640 and P23740 and *Pwo* polymerase. The resulting fragment was cloned into *Bam*HI- and *Hind*III-digested pQE30 to create pSHS23Q30. To create a C-terminal FLAG-tagged HrpR, *hrpR'* (codons 1 to 313) was amplified using the primers P25630R and P24692 and *Pwo* polymerase. The *hrpR* fragment was ligated as a *Hind*III-*Eco*RI fragment into pFLAG-CTC to generate pTSR8CTC.

**Column binding assay.** DH5 $\alpha$ (pREP4)(pSHS23Q30) or DH5 $\alpha$ (pTSR8CTC) cultures (optical density at 600 nm [OD<sub>600</sub>] = 0.5) were induced by the addition of 1 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) and grown for an additional 5 h. Cells from 50-ml cultures were harvested and stored at  $-20^\circ\text{C}$  until use. Frozen cells were thawed on ice in 5 ml of lysis buffer (50 mM NaHPO<sub>4</sub> [pH 8.0], 100 mM KCl, 10 mM imidazole), and 5 mg of lysozyme was added. After 30 min of incubation, cells were lysed by sonication and fractionated by centrifuga-

tion at 10,000  $\times g$  for 30 min. The supernatant was collected and clarified by centrifugation in a microcentrifuge for 10 min at  $4^\circ\text{C}$ .

The clarified lysate of DH5 $\alpha$ (pREP4)(pSHS23Q30) was mixed with 1.5 ml of Ni-nitrilotriacetic acid (NTA) slurry (Qiagen, Valencia, Calif.) and incubated for 2 h at  $4^\circ\text{C}$  with shaking. Resin was collected in a 6-ml polypropylene column and washed with 2 column volumes of lysis buffer, 2 column volumes of wash buffer (lysis buffer supplemented with 20 mM imidazole), and 1 column volume of lysis buffer. The clarified lysate of DH5 $\alpha$ (pTSR8CTC) was applied to the column, and the column was washed with 1 column volume of lysis buffer, two column volumes of wash buffer, and two column volumes of lysis buffer. Bound proteins were eluted in elution buffer (lysis buffer supplemented with 250 mM imidazole).

**Immunoblotting.** Proteins in samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% polyacrylamide gels. Proteins were electroblotted onto polyvinylidene difluoride membranes in Tris-glycine buffer (pH 8.3) containing 20% methanol. Membranes were then blocked with 5% dry milk in phosphate-buffered saline (PBS)-0.05% Tween 20 (PBST) and incubated with anti-His antibody (Bio-Rad, Hercules, Calif.) and/or anti-FLAG M2 antibody (Sigma) in 3% bovine serum albumin in PBST for 1 h at room temperature. Membranes were washed three times in PBST and incubated with a 1:3,000 dilution of anti-mouse immunoglobulin G-horseradish peroxidase conjugate (Bio-Rad) in 5% dry milk in PBST for 1 h at room temperature. Membranes were washed three times in PBST and once in PBS, and immunoreactive proteins were detected by using an enhanced-chemiluminescence detection kit (Amersham Pharmacia Biotech).

## RESULTS

***hprR* and *hprS* are expressed as an operon.** To determine whether promoters were associated with *hprR* and/or *hprS*, fragments 5' to the *hprR* and to the *hprS* coding sequences from *P. syringae* Pss61 (R1 and R4) (Fig. 1) or *P. syringae* DC3000 (R6 and R7) genomic DNA were cloned into the low-copy-number plasmid pRG970 (Table 1) as described in Materials and Methods to create transcriptional fusions to *lacZYA*. The resulting constructs were confirmed by sequence analysis and transformed into *P. syringae* Pss61 or *P. syringae* DC3000. Cells were assayed for  $\beta$ -galactosidase activity during mid-log-phase growth in the inductive M63 fructose medium. Promoter activity was detected from the R1 construct carrying 259 bp upstream of the *hprR* coding sequence, irrespective of the host bacterium (Table 2). Strains carrying the R1 construct exhibited >30-fold more  $\beta$ -galactosidase activity than the background. In contrast, little promoter activity was detected from the 1,070-bp R4 and R7 constructs, which include the predicted HrpR-dependent regulatory site (HrpR box) and potential *hprS* promoter (HrpS box) (15), the *hprR-hprS* intergenic region, and the coding sequence for the first 13 amino acids (aa) of *hprS*.  $\beta$ -Galactosidase levels in strains carrying these constructs expressed less than 40 Miller units of  $\beta$ -galactosidase activity. Similar results were obtained when these constructs were tested in *E. coli* MC4100. The R4 construct exhibited minimal if any promoter activity, as was true for the R7 construct (data not shown).

Although the activity of the R1 construct indicates that *hprR* was expressed in *P. syringae* strains under the conditions employed in the preceding experiments, additional experiments were performed using a plasmid-borne *hprR* expression system. Consistent with previous results (63), vector-directed expression of *hprRS* in Pss61 or MC4100 caused at least a 45-fold increase in *hprL* promoter activity (Table 2), showing that the *hprRS* expression system was functioning under the experimental conditions employed. Promoter activity of neither the *hprR* promoter carried by the R1 construct nor the putative *hprS* promoter included in the R4 construct, however, was substan-

TABLE 2. Activities of *hrpR*, *hrpS*, and *hrpL* promoter constructs in *P. syringae* Pss61, *P. syringae* DC3000, and *E. coli* MC4100

Strain	<i>hrpRS</i> expressed <sup>a</sup>	β-Galactosidase activity from promoter fusion <sup>b</sup> :			
		None <sup>c</sup>	<i>hrpR</i> <sup>d</sup>	<i>hrpS</i> <sup>e</sup>	<i>hrpL</i> <sup>f</sup>
Pss61	–	3 ± 1	417 ± 16	31 ± 1	348 ± 16
	+	3 ± 1	615 ± 38	40 ± 1	3,469 ± 184
DC3000	–	4 ± 1	126 ± 2	7 ± 3	57 ± 4
	+	6 ± 1	147 ± 4	9 ± 1	216 ± 8
MC4100	–	1 ± 1	439 ± 23	6 ± 1	5 ± 1
	+	1 ± 1	364 ± 19	5 ± 1	271 ± 5

<sup>a</sup> The indicated strains carried pDSK519 (–) or pNTR3D to ectopically express *hrpRS* (+).

<sup>b</sup> Strains carrying the indicated reporter construct were grown overnight to an OD<sub>600</sub> of 1.0, harvested, washed and used to inoculate inductive M63 fructose (pH 5.5) medium for *P. syringae* strains or King's B medium for *E. coli* MC4100. After 6 h of growth, β-galactosidase activity was determined by the procedures of Miller (36). The data reported are the means of values from a single experiment done in triplicate. Errors represent standard deviations. Each experiment was repeated at least three times with similar results.

<sup>c</sup> Indicated strain carrying pRG970.

<sup>d</sup> Pss61 carrying pDRR1R, DC3000 carrying pJBR6R, or MC4100 carrying pDRR1R.

<sup>e</sup> Pss61 carrying pTSR4R, DC3000 carrying pJBR7R, or MC4100 carrying pTSR4R.

<sup>f</sup> Indicated strain carrying pYXL1R.

tially affected by the presence of the *hrpRS* expression system (Table 2). Levels of activity were similar irrespective of the presence of the *hrpRS* construct. Similar results were obtained when an *hrpR* construct was employed (data not shown). The absence of promoter activity in strains carrying the R4 construct even in the presence of expressed *hrpR* argues that the postulated HrpR-dependent *hrpS* promoter may be only weakly active in Pss61 and DC3000 and is inactive in *E. coli* strains, irrespective of the expression of *hrpR*.

To determine if a transcript extends from *hrpR* into *hrpS*, RT-PCR was performed on total RNA extracted from *P. syringae* Pss61 cells. cDNA synthesis was initiated using the P24200 primer, which is complementary to a specific sequence internal to the *hrpS* coding sequence (Fig. 1). As shown in Fig. 2A, a 0.44-kb fragment was amplified from the P24200-primed cDNA preparation by using *hrpS*-specific primers. This product was identical in size to the expected product amplified from the native DNA template and absent from the control reaction mixture using just the DNase I-treated RNA preparation as a template (–RT). This PCR product confirms that cDNA synthesis occurred and that the RNA preparation was largely free of genomic DNA. When primers were employed to amplify a region encompassing both *hrpR* and *hrpS*, the predicted 1.4-kb fragment indicative of an *hrpRS* transcript was amplified from the same cDNA preparation (Fig. 2B). Since each of the preceding PCRs employed a primer that was also used in the cDNA synthesis, a third PCR was performed to confirm the apparent transcriptional linkage of *hrpR* and *hrpS*. The cDNA preparation generated using the *hrpS* P24200 primer was used as a template for PCR employing the P25591 and P24901 primers, specific to *hrpR*. As shown in Fig. 2C, a 0.7-kb fragment that was indistinguishable from the expected 0.7-kb fragment amplified from genomic DNA was amplified from the cDNA preparation but was absent from the –RT control. Similar results were obtained during parallel experiments with

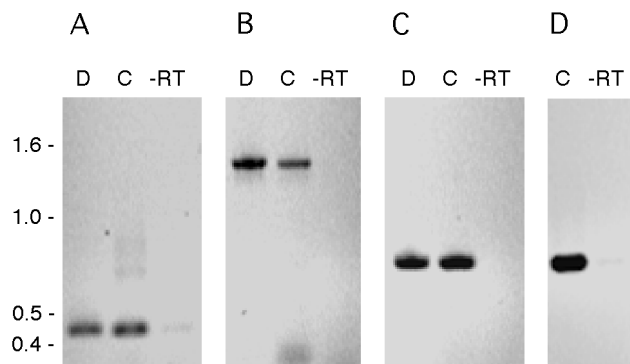


FIG. 2. RT-PCR of the *hrpRS* transcript. Total RNA was extracted using Trizol and precipitated, and 2 μg was treated with DNase I. The DNase I-treated RNA preparation was used for cDNA synthesis using the P24200 primer (Fig. 1) and for a –RT control. Genomic DNA (lanes D), the cDNA preparation (lanes C), and the –RT control (lanes –RT) were used as templates for PCR employing primers P24200 and P24634 to amplify an *hrpS* region (A), primers P24200 and P25591 to amplify an *hrpRS* fragment (B), or primers P24901 and P25591 to amplify an *hrpR* fragment (C). Panel D is similar to panel C except that DC3000 RNA and DC3000-specific primers were employed. Molecular sizes shown on the left in kilobases were estimated using a 1-kb ladder obtained from Invitrogen.

RNA extracted from *P. syringae* strain DC3000. A 0.7-kb *hrpR* fragment could also be amplified from the cDNA preparation generated from DNase-treated DC3000 RNA and DC3000-specific primers equivalent to P24200, P24901, and P25591 (Fig. 2D). The ability to detect the 1.4- and 0.7-kb products after RT-PCR of total RNA extracts of *P. syringae* indicates that a transcript that contains the coding sequences for both *hrpR* and *hrpS* is produced by these *P. syringae* strains.

**Conservation of the *hrpRS* region in *P. syringae* strains.** To determine if the *hrpRS* regulatory sequences might be unique to the strains examined above, the nucleotide sequences of the *hrpRS* regions of several *P. syringae* strains were compared. Within the region carried by the R4 construct, the Pss61 *hrpR* coding sequence exhibited 84% identity at the nucleotide level with the *P. syringae* pv. phaseolicola *hrpR* sequence (data not shown). For comparison, the *hrpS* coding sequence retained 81% identity. The postulated HrpR box (Fig. 3A) and the predicted *hrpS* promoter regions (Fig. 3B) were also conserved in all strains examined. Highest divergence was detected in the noncoding intergenic region between *hrpR* and *hrpS* (Fig. 3C). This 45- to 50-bp region, although large for an intergenic region, lacked motifs known to function as transcriptional terminators. The retention of major features of the region and the absence of significant sequence divergence in the region argue that the means of regulating and expressing *hrpRS* are likely to be similar in all *P. syringae* strains, irrespective of their host range.

**Maximal activation of the *hrpL* promoter requires both HrpR and HrpS.** As demonstrated above, HrpR and HrpS function as positively acting regulatory factors for the *hrpL* promoter. To determine whether HrpR or HrpS could function as an independent activator of the *hrpL* promoter, coding sequences for *hrpR* and *hrpS* together with their native ribosome binding sites were amplified from pHIR11 by PCR and cloned individually into the IncQ plasmid pDSK600

**A.**

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phaseolicola 361 CCAGCAGGTCCGGGCCCTTTGTGCGCATGAACTGCGCCGCATCCCCGA TCGTTGGCAG
glycinea      173 CCAGCAGGTCCGGGCCCTTTGTCTGCATGAACTGCGCCGCATCCCCGA TCGTTGGCAG
DC3000       431 CCAGCAGGTCCGGGCCCTTTGTGCGCATGAACTGCGCCGCATCCCCGA TCGTTGGCAG
Pss61        431 CGAGCAGGACAGGGGCCCTTTGTGCGCATGAACTGCGCGCCATCCCCGA TCGTTGGCAG

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phaseolicola 421 AAGCCAGTTATTCGGTGTCTTCAATGGCGCATTTACCGGTGTCTGCCGCGCTCGCGAGG
glycinea      233 AAGCCAGTTATTCGGTGTCTTCAATGGCGCATTTACCGGTGTCTGCCGCGCTCGCGAGG
DC3000       491 AAGCCAGTTATTCGGTGTGGTCAACGGTGCATTTACCGCGGTATGCCGCGCTCGCGAGG
Pss61        491 AAGTCAGCTATTCGGCGTGGTCAATGGTGCCTTACCGCGGTGTGCCGCGCTCGCGAGG

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**B.**

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phaseolicola 937 CATGGATGCGCGCGATCCGGTGACAGGTCTGCGCATGCAGATGCGCGGTGATCGAAAACA
glycinea      749 CATGGATGCGCGCGATCCGGTGACAGGTCTGCGCATGCAGATGCGCGGTGATCGAAAACA
Pss61        1007 CCGTCAAGCGCGGTGACCCGGTACAGGACTGCGCATGCAGATGCGCGGTGATCGAAAACA
DC3000       1007 CTCTGGAAGCGCTTGACCTGCCACGGCTGCGCACGCAATGCGCGTTCATCGAAAACA

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**C.**

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phaseolicola 1112 TAGATCGCAGCAACGGCTGGAGTCCAAAAGCTTATGTCTCGGGGTCATGTGATG
glycinea      924 TAGATCGCAGCAACGGCTGGAGTCCAAAAGCTTATGTCTCGGGGTCATGTGATG
DC3000       1210 -TGA-CGCAGGTCTGGTT---ATCCAAC--GTTGATGTCTCGGGGTCATGGGATG
Pss61        1207 TGAATCGCCGCTTCGCTC---GCGCAAC--GCCATGCCATTGGAGTCATGAATG

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FIG. 3. Conservation of the *hrpR* promoter and coding sequence. Sequences were aligned using the CLUSTAL W version 1.8 program (61) and data from GenBank accession numbers AF2322004, U03853, U03852, X77638, and AF069650. (A) Proposed HrpR-responsive *hrpS* promoter element. The dashed lines represent the HrpR box proposed by Grimm et al. (15). (B) Postulated  $\sigma^{54}$ -dependent *hrpS* promoter element. Conserved promoter elements (+) and the transcriptional initiation site (^) identified by Grimm et al. (15) are shown. (C) *hrpRS* intergenic region. The deduced ribosome binding site for *hrpS* is indicated by the ~ symbols.

(pSJS3DS) (Fig. 1) or the IncP-1 plasmid pLAFR3 (pSJR2L) such that the cloned genes were expressed from vector  $P_{lac}$  promoters (Fig. 1). To reconstruct the *hrpRS* operon from the individually cloned fragments and inactivate a potential open reading frame identified on the opposite strand, the *hrpS* fragment was ligated as a 1-kb fragment into pSJR2L to create pSJR2S3L. The resulting construct carried a 30-bp deletion in the intergenic region between *hrpR* and *hrpS* but retained the native ribosome binding site for *hrpS*. The above-named constructs were then transformed individually or in combination into MC4100(pSGL4MS) carrying a  $P_{hrpL}$ -*lacZ* fusion and assayed for  $\beta$ -galactosidase activity.

The *hrpL* promoter construct carried by pSGL4MS was inactive in MC4100 in the absence of *hrpRS* (Table 3) as observed above. Vector-directed expression of *hrpR* by the pSJR2L construct alone had no detectable effect on the activity of the *hrpL* promoter. Consistent with the observations of Grimm et al. (15), expression of *hrpS* partially activated *hrpL* promoter activity. MC4100(pSGL4MS)(pSJS3DS) expressing just *hrpS*, however, accumulated only 83 Miller units of  $\beta$ -galactosidase activity during logarithmic growth. Significantly higher levels of *hrpL* promoter activity were detected in strains expressing (i) *hrpR* and *hrpS* from separate plasmids [MC4100(pSGL4MS)(pSJR3L)(pSJS2D)], (ii) the reconstructed *hrpRS* operon [MC4100 (pSGL4MS)(pSJR3S2L)], and (iii)

*hrpRS* as a native construct [MC4100 (pSGL4MS)(pYXRS1D)]. Transformants expressing *hrpR* and *hrpS* in *trans* on separate plasmids expressed greater than 4,700 Miller units of  $\beta$ -galactosidase activity. In these constructs, the proposed *hrpS* promoter and regulatory sequences were physically separated from the *hrpS* coding sequence. The reconstructed *hrpRS* operon produced a similar level of activation. Transformants carrying the native RS construct exhibited greater than 13,000 Miller units of activity. As these activities were all more than

TABLE 3. Activation of the *hrpL* promoter in *E. coli* MC4100 by cloned *hrpR* and *hrpS*

MC4100 (pSGL4MS) transformant plasmid(s) <sup>a</sup>	Expressed gene(s)	Promoter activity <sup>b</sup>
pDSK519	None	1 ± 1
pYXRS1D	Native <i>hrpRS</i>	13,162 ± 745
pSJR2L	<i>hrpR</i>	1 ± 1
pSJS3DS	<i>hrpS</i>	83 ± 9
pSJR2L, pSJS3DS	<i>hrpR</i> + <i>hrpS</i> in <i>trans</i>	4,725 ± 524
pSJR2S3L	Reconstructed <i>hrpRS</i> operon	3,077 ± 111

<sup>a</sup> The indicated plasmids are described in Table 1.

<sup>b</sup> Promoter activity as estimated by  $\beta$ -galactosidase activity (in Miller units) expressed in strains carrying the indicated plasmids as described in Materials and Methods. Values are means ± standard deviations.

TABLE 4. Interaction of HrpR and HrpS detected by yeast two-hybrid analysis

L40 transformant plasmid(s)	LexA'-BD fusion	GAL4'-AD fusion	$\beta$ -Galactosidase activity <sup>a</sup>
pDWR3BTM	HrpR	Absent	<1
pDWS4BTM	HrpS	Absent	<1
pBTM-lamin, pDWR3GAD	Lamin	HrpR	<1
pBTM-lamin, pDWS4GAD	Lamin	HrpS	<1
pDWR3BTM, pGAD424	HrpR	None	<1
pDWS4BTM, pGAD424	HrpS	None	1.4 $\pm$ 0.6
pDWR3BTM, pDWR3GAD	HrpR	HrpR	<1
pDWS4BTM, pDWS4GAD	HrpS	HrpS	3 $\pm$ 0.3
pDWR3BTM, pDWS4GAD	HrpR	HrpS	29 $\pm$ 10
pDWS4BTM, pDWR3GAD	HrpS	HrpR	28 $\pm$ 10
pDWS4BTM, pTSR4GAD	HrpS	HrpR $\Delta$ N	26 $\pm$ 3
pDWS4BTM, pTSR5GAD	HrpS	HrpR $\Delta$ C	1.4 $\pm$ 0.5
pDWS4BTM, pTSR6GAD	HrpS	HrpR $\Delta$ N $\Delta$ C	1.0 $\pm$ 0.2

<sup>a</sup> The indicated *S. cerevisiae* L40 transformants were grown overnight at 25°C in defined medium lacking Leu and/or Trp (as required to maintain selection for plasmids) to an OD<sub>600</sub> of 1.0. Cells were harvested and lysed by three freezing cycles in liquid N<sub>2</sub>, and  $\beta$ -galactosidase activity was determined as indicated in Materials and Methods.

37-fold higher than the activity induced by *hrpS* alone, these results indicate that maximal activation of the *hrpL* promoter requires expression of both *hrpR* and *hrpS*.

**Physical interaction of HrpR and HrpS is detected by yeast two-hybrid studies.** One possible interpretation for the requirement of both HrpR and HrpS in the activation of the *hrpL* promoter is that the two proteins physically interact. A yeast two-hybrid assay (3) was used to determine if HrpR and HrpS could interact. Fragments carrying codons 2 to 314 of *hrpR* and codons 2 to 302 of *hrpS* (Fig. 1) were amplified by PCR from pHIR11 and cloned as *EcoRI*-*Bam*HI fragments into pBTM116 and pGAD424 to create translational fusions with the LexA' DNA BD or the GAL4' AD carried by each respective plasmid. The resulting constructs were verified by sequencing and transformed into the yeast reporter strain L40.

Yeast L40 transformants carrying either the LexA' BD-HrpR or LexA' BD-HrpS fusion alone did not exhibit a  $\beta$ -galactosidase-positive phenotype, indicating that neither protein alone could activate the reporter construct in yeast (Table 4). None of the HrpR or HrpS fusions exhibited an interaction with the GAL4' AD expressed by pGAD424 or with a LexA'-lamin fusion routinely used to detect nonspecific interactions (3). A weak interaction was detected in strains carrying both HrpS constructs, but this activity was about 10% of the activity observed when both translational fusions were expressed in the yeast indicator strain. Strong interactions were detected when HrpR and HrpS fusions were expressed in the same strain, irrespective of the fusion domain. Lysed colony lifts floated on X-Gal solutions exhibited a positive phenotype within 15 min, and quantitative analyses detected relatively high levels of  $\beta$ -galactosidase activity. Twenty-five to 40 U of  $\beta$ -galactosidase

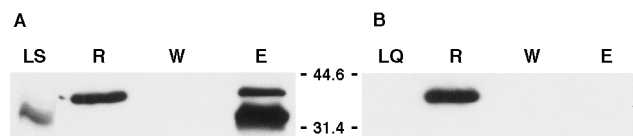


FIG. 4. Interaction of HrpR and HrpS detected by column-binding experiments. Clarified lysate from an IPTG-induced DH5 $\alpha$ (pREP4) (pSHS23Q30) culture expressing HrpS was loaded onto a Ni<sup>+</sup>-nitrilotriacetic acid column (Qiagen), and the column was washed as described in Materials and Methods to release weakly bound proteins. A clarified lysate from an IPTG-induced DH5 $\alpha$ (pTSR8CTC) culture was then applied to the column, and the column was washed with sufficient buffer to produce an HrpR-free elutant. Bound proteins were eluted using 250 mM imidazole in lysis buffer. Proteins in the indicated samples were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed using anti-His and anti-FLAG antibodies simultaneously as described in Materials and Methods. (A) HrpR eluted from an HrpS-loaded column. Lane LS, DH5 $\alpha$ (pREP4) (pSHS23Q30) lysate applied to the column (contrast enhanced); lane R, DH5 $\alpha$ (pTSR8CTC) lysate applied to the column; lane W, final wash prior to elution; lane E, imidazole-eluted proteins. (B) HrpR binding to an *E. coli* protein-loaded column. Lane LQ, DH5 $\alpha$ (pREP4) (pQE30) lysate applied to the column; lane R, DH5 $\alpha$ (pTSR8CTC) lysate; lane W, final wash; lane E, imidazole-eluted proteins. Molecular masses shown in kilodaltons were estimated using Kaleidoscope prestained markers (Bio-Rad).

activity was routinely detected in strains carrying these constructs.

To determine whether a specific domain was involved in the interaction between HrpR and HrpS, truncated derivatives of HrpR lacking the 15 N-terminal aa and/or the 70 C-terminal aa (includes the DNA BD [37]) were constructed in pGAD424. Such constructs have been stable in other systems (47). The resulting constructs were transformed into the yeast reporter strain carrying the LexA BD'-HrpS construct in pBTM116. Yeast transformants carrying the HrpR derivative lacking just the 15 N-terminal amino acids (R $\Delta$ N) exhibited a  $\beta$ -galactosidase-positive phenotype (Table 3), whereas the derivatives lacking the C terminus (R $\Delta$ C, R $\Delta$ N $\Delta$ C) exhibited little or no  $\beta$ -galactosidase activity. These results suggest that a C-terminal domain may control the interaction between HrpR and HrpS or that the C-terminal deletion renders the fusion protein unstable.

**Copurification of His-tagged HrpS and FLAG-tagged HrpR in column-binding experiments.** The requirement for both *hrpR* and *hrpS* in the activation of the *hrpL* promoter and the yeast two-hybrid experiments are highly suggestive of a physical interaction between HrpR and HrpS. To confirm the apparent interaction of HrpR and HrpS, column-binding experiments were performed. An N-terminal six-His fusion to HrpS was constructed in pQE30 (pSHS23Q30), and a C-terminal FLAG-tagged HrpR was constructed in pFLAG-CTC (pTSR8CTC) (Fig. 1). The His-tagged HrpS was collected from lysates of DH5 $\alpha$ (pREP4)(pSHS23Q30) on Ni<sup>+</sup>-agarose matrix, washed, and allowed to interact with clarified lysates of DH5 $\alpha$ (pTSR8CTC) containing the C-terminal FLAG-tagged HrpR. The matrix was washed, and bound proteins were eluted using 250 mM imidazole. As shown in Fig. 4, both His-tagged HrpS, which migrated as a 34-kDa protein, and FLAG-tagged HrpR, detected as a 40-kDa protein, were eluted from the column with the imidazole wash. Although the apparent 40-

kDa mass of HrpR was larger than expected for the FLAG-tagged derivative (35 kDa), His-tagged HrpR also migrated as a 40-kDa protein (data not shown). The reason for this aberrant behavior of HrpR during SDS-PAGE has not been established. FLAG-tagged HrpR did not bind to an Ni<sup>+</sup>-agarose matrix loaded with lysates of DH5 $\alpha$ (pREP4)(pQE30). The copurification of HrpS and HrpR under these conditions is consistent with the strong physical interaction detected in the yeast two-hybrid analyses.

## DISCUSSION

The *hrp*-encoded type III PEC is central to the pathogenicity of *P. syringae* strains. Although the characterization of the regulatory system controlling assembly of the *hrp*-encoded PEC is still incomplete, it is clear that expression of *hrp* genes in *P. syringae* is coordinated by the activity of HrpL. HrpL is an alternative sigma factor required for transcription of the operons encoding structural elements of the PEC as well as the genes for the secreted effector proteins (24). As the only factor currently thought to affect HrpL activity is protein turnover, expression of *hrpL* is likely to be critical to the assembly of the *hrp*-encoded PEC of *P. syringae*. The results presented above indicate that the expression of *hrpL* is controlled in part at the transcriptional level by the interaction of two unusual enhancer-binding proteins, HrpR and HrpS.

HrpR and HrpS retain most of the structural features conserved in other members of the enhancer-binding protein family that function in transcriptional regulation of  $\sigma^{54}$ -dependent promoters (37, 40). Consistent with these features, HrpR and HrpS activated the  $\sigma^{54}$ -dependent *hrpL* promoter (63). This promoter contains a  $\sigma^{54}$  promoter consensus sequence (63), and transcription of *hrpL* initiates 12 bp downstream of this promoter motif (S. Heu and S. Hutcheson, unpublished results). *hrpL* expression in *P. syringae* pv. *maculicola* was recently reported to be dependent upon *rpoN* (17, 18).

In contrast to other known enhancer-binding proteins, both HrpR and HrpS were required for maximal activation of the *hrpL* promoter. HrpS expressed from a strong promoter on a multicopy plasmid could function only as a weak activator of *hrpL* promoter activity. This activity was less than 2.5% of the activity detected when both *hrpR* and *hrpS* were expressed in a cell, irrespective of the promoter construct used to drive expression. As the proposed HrpR-linked *hrpS* regulatory sequences internal to *hrpR* (15) were physically separated from *hrpS* in these experiments, it appears unlikely that HrpR directly influences transcription of *hrpS* in these constructs. The simplest explanation for these results is that both proteins are required to fully activate the *hrpL* promoter. The observation that HrpS can act as a weak activator of the *hrpL* promoter provides an explanation for the reported plant response-positive phenotype of a *P. syringae* *hrpR* mutant carrying an *hrpS* expression construct (15). Relatively little *hrp* expression appears to be necessary to assemble the *hrp*-encoded PEC (65). Ectopic expression of *hrpS* would have induced at least some expression of the *hrp* regulon and thus allowed the *hrp*-encoded PEC to be assembled.

Consistent with the requirement for both proteins in the activation of *hrpL* expression, *hrpR* and *hrpS* were shown to be expressed as an operon. The only fragment from the *hrpRS*

region with significant promoter activity was 5' to *hrpR*, and a transcript encompassing both *hrpR* and *hrpS* was detected by RT-PCR analysis. Although some sequence divergence was detected in the *hrpRS* region, most involved silent codon substitutions. The conservation of the *hrpRS* region argues that *hrpR* and *hrpS* are transcribed as an operon in all *P. syringae* strains. Regulation of *hrpRS* expression in distinct strains, however, may be different, as sequences upstream of the *hrpR* promoter were not well conserved.

A requirement for both HrpR and HrpS in the activation of the *hrpL* promoter may indicate that HrpR either activates HrpS or forms a stable complex with HrpS. In either model, HrpR and HrpS would be expected to physically interact. The yeast two-hybrid experiments demonstrated that a physical interaction between HrpR and HrpS can occur. This apparent strong interaction was confirmed in column-binding experiments. A FLAG-tagged HrpR derivative in crude cell lysates was retained by immobilized His-tagged HrpS. This complex formed under the relatively high-salt conditions (100 mM KCl) of the lysis buffer and was stable during washes exceeding 5 column volumes. Another enhancer-binding protein, NtrC, has been proposed to form a homodimer that upon phosphorylation assembles into a larger oligomeric activator complex (62). Dimerization involves the C terminus of the protein (31). The ability of HrpR and HrpS to form a stable complex during column-binding experiments in the absence of a target promoter suggests that these proteins form a heteromeric complex prior to activation of the *hrpL* promoter. The yeast two-hybrid assay results suggest that the formation of this complex may involve the C-terminal domain of HrpR, as reported for NtrC.

HrpR and HrpS lack the 130-aa receiver (AB) domain that is typically found in most other members of the protein family (37, 41). The receiver domain has been proposed to be a repressor of ATP hydrolysis in the absence of kinase-mediated phosphorylation or binding of a regulatory effector molecule (52). The absence of the receiver domains argues that HrpR and HrpS do not require posttranslational modification, such as phosphorylation or the binding of an effector molecule, to activate the target promoter. Consistent with this hypothesis, vector-directed expression of *hrpRS* as a minimal coding sequence produced a functional activator complex in *E. coli* transformants. HrpR and HrpS are thus functionally similar to *E. coli* PspF (27, 28) or truncated derivatives of DctD (33) and XylR (42). These proteins lack the AB receiver domain and are also constitutively active. The activity of HrpR and HrpS thus appears to be independent of a direct posttranslational modification mechanism, such as phosphorylation, but posttranslational modification by a broadly conserved mechanism cannot be fully excluded at this time.

Regulation of the *P. syringae* *hrp* PAI shares some similarities to the regulatory system controlling flagellar biosynthesis. Flagellar biosynthesis has been proposed to be a form of a type III PEC, and three classes of promoters have been identified for genes involved in the assembly of flagella (6). At the top of the regulatory system is the class 1 promoter for *flhCD*. FlhC and FlhD, once expressed, interact to form an FlhD-FlhC complex that then activates expression of class 2 promoters. FliA, expressed from a class 2 promoter, functions as an alternative sigma factor to direct expression of class 3 promoters. Like FlhD-FlhC, HrpR and HrpS are expressed as an operon



and form a complex. There is, however, little if any sequence similarity between FlhD-FlhC and HrpR-HrpS. At present the only known target for the HrpR-HrpS complex appears to be the *hrpL* promoter, but other HrpR-HrpS-dependent promoters may exist in cells. HrpL is a sigma factor related to FliA that directs expression of the HrpL-dependent regulon. The HrpL-dependent promoters are analogous to the class 3 promoters of flagellar biosynthesis. Although the HrpR- or HrpS-HrpL regulatory system is superficially similar to the FlhD- or FlhC-FliA regulatory system, the genes controlled at each level of these regulatory systems are distinct. In flagellar biosynthesis, the genes encoding the type III PEC are considered to be class 2 operons, although there is some influence of FliA on their expression (35), whereas the *hrp* counterparts could be considered to be equivalent to the class 3 operons.

The HrpR-HrpS regulatory system also shares some similarity to the RcsB-RcsA system regulating capsular biosynthesis in several bacterial species (14). RcsB interacts with RcsA to regulate *cps* expression. RcsB is part of a two-component regulatory system involving RcsC. The RcsB-RcsC system can activate low-level expression of the *cps* genes but acts synergistically with RcsA. RcsA is present at limiting levels in which RcsA levels are regulated by turnover mediated by Lon protease. HrpS appears to be able to activate low-level expression of the *hrp* regulon but requires HrpR for maximal activity. A similar situation occurs in the regulation of *Erwinia amylovora* *hrp* genes. The *Erwinia* HrpS can initiate expression of the *hrpL* promoter but requires HrpX for maximal activity (57). HrpX is an enhancer-binding protein that is part of a classic two-component regulatory system involving a phosphorelay. As mentioned above, there is no evidence at present to indicate that HrpR or HrpS functions as part of a two-component regulatory system, and in contrast to *rscA* and *rscBC*, *hrpR* and *hrpS* are expressed as an operon.

Unresolved at present is the mechanism by which environmental signals generated during pathogenesis are transduced to alter *hrp* expression. The proposed regulatory system appears to represent a regulatory cascade in which expression of the Hrp regulon may be controlled by the expression of *hrpRS* in a manner analogous to that of PspF in the regulation of stress genes in *E. coli* (28) and *flhCD* in flagellar biosynthesis (6). *hrpS* transcript levels have been reported to be repressed in DC3000 during growth in repressive media (57). Other results suggest that *hrpRS* expression is constitutive in several strains because *hrpRS* transcripts could be detected by primer extension and RT-PCR, irrespective of the growth conditions (J. Bretz and S. Hutcheson, unpublished results). Interestingly, significant differences in *hrpR* promoters were observed between *P. syringae* strains as described above. This opens the possibility of strain-specific regulation of *hrpRS* expression. In contrast to the *hrpR* promoter, the *hrpL* promoter was observed to be environmentally regulated (24). This finding argues that additional factors must mediate the environmental regulation of the *hrp* cluster in addition to HrpR-HrpS and HrpL. One such candidate is HrpV, which has been reported to function as a negative regulator of *hrp* expression (44), and preliminary results suggest that it functions analogously to PspA (10) in the regulation of HrpR-HrpS activity (T. Sussan, X. Wei, and S. Hutcheson, unpublished results).

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