

Analysis of Promoters Recognized by HrpL, an Alternative σ -Factor Protein from *Pantoea agglomerans* pv. *gypsophilae*

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HrpL, an alternative σ factor, activates the transcription of the Hrp regulon by its binding to a common “*hrp* box” promoter. Based on computational techniques, the *hrp* box previously was defined as a consensus bipartite *cis* element, 5'-GGAACC-N₁₅₋₁₆-CCACNNA-3'. The present report combines a quantitative *in vivo* assay for measuring Hrp promoter activity with site-specific mutagenesis to analyze the effect of consensus and nonconsensus nucleotides on promoter activity. The analysis was carried out with Hop effectors of the tumorigenic bacterium *Pantoea agglomerans* pv. *gypsophilae*, in which HrpL is indispensable for gall formation. Mutational analysis indicates that the *hrp* box consensus can be divided into crucial and noncrucial nucleotides. The first 5 nucleotides (nt) of the –35 consensus motif (GGAAC) and the 3 nt of the –10 motif (ACNNA) are crucial, whereas other consensus and adjacent nonconsensus nucleotides exert a significant effect on the promoter's strength. With spacing of 13 or 17 nt between the two motifs, significant activity was still retained. Gel shift assays indicated that deletion of GG from the –35 consensus motif eliminated HrpL binding, whereas mutations in the –10 consensus motif or modification of the spacing, which eliminates promoter activity, did not elicit any effect. The degeneracy in Hrp promoters of four *hrp* and type III effector genes of *P. agglomerans* pv. *gypsophilae* indicated significant differences in promoter activity, whereas increasing the promoter strength of the Hop effector, HsvG, resulted in overexpression of gall formation.

Additional keywords: Hrp gene cluster, type III effectors.

Pantoea agglomerans (formerly *Erwinia herbicola*) pv. *gypsophilae* incites galls on gypsophila (Cooksey 1986), whereas *P. agglomerans* pv. *betae* causes galls on beet and gypsophila (Burr et al. 1991). The pathogenicity of both pathovars is dependent on a plasmid designated as pPATH_{Pag} and pPATH_{Pab} for *P. agglomerans* pvs. *gypsophilae* and *betae*, respectively (Manulis and Barash 2003a). The pPATH_{Pag} has a size of approximately 150 kb and harbors a pathogenicity island (PAI) of approximately 70 kb. This PAI includes a *hrp/hrc* gene cluster (Mor et al. 2001; Nizan et al. 1997), genes encoding type III virulence effectors (Ezra et al. 2000; Guo et al. 2002; Mor et al. 2001; Valinsky et al. 1998), and genes encoding indole 3-acetic acid (IAA) and cytokinin biosynthesis (Clark et al.

1993; Gafni et al. 1997; Lichter et al. 1995). Multiple and highly diverse insertion sequence (IS) elements also are interspersed among the virulence genes of the two plasmids (Guo et al. 2002; Lichter et al. 1996). Simultaneous inactivation of IAA and cytokinin biosynthetic genes substantially reduced gall size but did not eliminate gall initiation (Manulis et al. 1998). In contrast, mutations in *hrp* genes completely abolished gall formation (Nizan et al. 1997), indicating that gall initiation relies on type III effectors, also known as *hrp*-dependent outer protein (Hop) effectors.

The *hrp/hrc* genes control the ability of most gram-negative phytopathogenic bacteria to cause disease and to elicit hypersensitive reaction (HR) in resistant and nonhost plants (Lindgren 1997). The *hrp* gene cluster encodes for components of the type III secretion systems (TTSS), regulatory proteins, and proteinaceous elicitors of HR. The TTSS and Hop effectors both are expressed in response to the environmental conditions in the plant apoplast. The Hop effectors then are delivered directly into the host cells by the TTSS, via the Hrp pilus (Jin et al. 2003). The *hrp* regulatory cascade of *P. agglomerans* pv. *gypsophilae* is composed of the *hrpXY* operon encoding a two-component system, *hrpS* encoding a transcriptional factor of the NtrC family, and *hrpL* encoding an alternative σ factor that is a member of the extracytoplasmic factor (ECF) family of σ factors (Nizan-Koren et al. 2003). A linear signal transduction cascade that leads to activation of the Hrp regulon via activation of HrpL in the presence of σ^{54} was proposed for *P. agglomerans* pv. *gypsophilae* (Nizan-Koren et al. 2003). A model that is essentially similar to that of *P. agglomerans* pv. *gypsophilae* was proposed for the *hrp* regulatory cascade of *P. stewartii* subsp. *stewartii* (Merighi et al. 2003), whereas different modes of signal transduction were suggested for *Erwinia amylovora* (Wei et al. 2000) and *Pseudomonas syringae* (Hutcheson et al. 2002). Regardless of the mechanism involved, the regulatory cascades of all three genera culminate in the activation of HrpL. When HrpL is expressed, it turns on the transcription of *hrp* genes and type III effectors, presumably by binding to a consensus bipartite *cis* element (“*hrp* box”) present in the promoter region of all the *hrp*-controlled genes (Xiao and Hutcheson 1994). Early work on comparative analysis of promoter regions of several *hrp* and *avr* genes identified the consensus *hrp* box sequence as 5'-GGAACCNA-N₁₃₋₁₄-CCACNNA-3' (Innes et al. 1993; Shen and Keen 1993; Xiao and Hutcheson 1994).

Promoter-based screens provide an effective method for identifying Hop effector genes that play a major role in the virulence of phytopathogenic gram-negative bacteria (Greenberg and Vinatzer 2003). The sequencing of the whole genome of

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Pseudomonas syringae pv. *tomato* DC3000 prompted the development of computational and gene expression techniques to identify virulence-implicated genes downstream of HrpL responsive promoters (Fouts et al. 2002; Zwiesler-Vollick et al. 2002). A key step in this functional genomics approach is the identification of a consensus *hrp* box-associated promoter that represents existing variations among the various genes encoding for Hrp and Hop effector proteins. Based essentially on alignment of 12 known *hrp*, *hrc*, and *avr* genes in *Pseudomonas syringae* pv. *tomato* DC3000, the following consensus bipartite *hrp* box was generated by Zwiesler-Vollick and associates (2002) for promoter-based screens: 5'-(T/G) GGA (A/G) C(C/T)-N₁₅₋₁₆-CCACNNA-3'. In another study, 33 promoter regions located upstream of *hrp* functional genes in *Pseudomonas syringae* pv. *tomato* DC3000 and 18 promoter regions identified by reporter transposon screen for HrpL-activated genes were used to create a sequence logo representing the *hrp*-box promoter (Fouts et al. 2002). The latter was obtained by developing a Hidden Markov Model trained with the Hrp promoter sequences to determine the relative frequencies of bases at every position in the consensus sequence (Schneider and Stephens 1990). The resulting sequence logo (Fouts et al. 2002) showed that 5'-GGAACc and 5'-cCACNNA were the most frequent nucleotides in the -35 and -10 motifs, respectively (lower-case letters indicate lower frequency). In both studies (Fouts et al. 2002; Zwiesler-Vollick et al. 2002), the available *Pseudomonas syringae* pv. *tomato* DC3000 genome sequence was searched for genes that contain the *hrp* box-like sequence in their promoter, and the expression of these genes then was examined, with and without HrpL activation, by microarray and Northern blot analyses.

In spite of previous studies on the identification of Hrp promoters, there have been no reports of a direct interaction of HrpL with *hrp* box sequences or determination of essential nucleotides within *hrp* box sequences by site-specific mutagenesis. Such a study might not only unravel the sequence degeneracy present in many known functional Hrp promoters but also could lead to systematic evaluation of promoter activity. The latter cannot be accomplished by comparative studies of Hrp promoters by means of either microarray or Northern blotting (Fouts et al. 2002; Zwiesler-Vollick et al. 2002) because the effect of transcript stability on promoter efficiency cannot be controlled. In the present article, we describe a quantitative in vivo assay for measuring the activity of Hrp promoters based on a direct interaction between HrpL and *hrp* box sequences. This procedure was used to measure the effects of substitutions at each position in the bipartite Hrp promoter and the spacing between the two motifs on promoter activity. Moreover, the promoter sequence required for binding the HrpL σ protein was identified. Finally, we compared the relative efficiency of native *hrp* and Hop effector genes in *P. agglomerans* pv. *gypsophila* and showed that the efficiency of Hrp promoters may exert a significant effect on disease expression.

RESULTS

An in vivo assay for measuring the effect of HrpL on the Hrp promoters.

An assay involving a two-plasmid system in *Escherichia coli* that was based on Minogue and associates (2002) was constructed for measuring the transcription activity of *hrp* box promoters (Fig. 1A). In this assay, plasmid pMBG1 contributed the *hrpL* coding sequence expressed from the *E. coli* P_{BAD} promoter. The latter was controlled by the AraC regulator, which also is encoded by pMBG1 as a function of L-arabinose induction (Guzman et al. 1995). Plasmid pMBG2 carries the tested promoter fused in-frame to *lacZY* reporter gene. Both

plasmids were mobilized into an *E. coli* Top10 host strain and tested for promoter activity.

The virulence genes *hsvG* and *pthG* encode for Hop effectors involved in host specificity and pathogenicity of *P. agglomerans* pv. *gypsophila*. HsvG determines host specificity of *P. agglomerans* pvs. *gypsophila* and *betae* on gypsophila (Valinsky et al. 1998, 2002a). PthG has a dual function, acting as a pathogenicity effector on gypsophila and as an Avr effector on beet (Ezra et al. 2002, 2004). The *hrp* box promoters of these two genes were used to calibrate the abovementioned assay system. Plasmids MBG2-Hs and MBG2-Pt, harboring the promoters of *hsvG* and *pthG*, respectively, were transferred to *E. coli*, grown to the same optical density in broth containing various concentrations of L-arabinose, and harvested and assayed for β -galactosidase. The transcription of both genes started at 0.02% L-arabinose and increased proportionally to the L-arabinose concentration until it reached a maximum at 0.2% (Fig. 1B). Therefore, Luria-Bertani (LB) broth supplemented with 0.2% L-arabinose was employed for bacterial growth in further experiments conducted to determine the promoter activity. The promoter activity of *pthG* was approximately 1.5 times higher than that of *hsvG* (Fig. 1B).

Mutational analysis of the *hrp* box promoter.

The *hrp* consensus sequence was subjected to various modifications and tested for promoter activity by the two-plasmid in vivo assay described above. The bipartite *hrp* box promoter sequence of *pthG*, AAGGAACTG-N₁₅-CCACCGAT (consensus nucleotides are underlined), initially was modified by changing the sixth nucleotide of the -35 motif from T to C (MBG2-PTcc) in order to fit the most common consensus sequence. This substitution resulted in an increase of approxi-

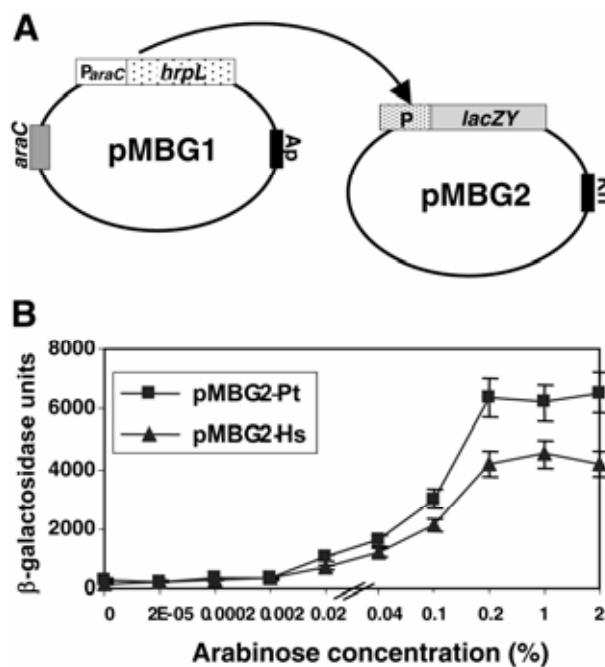


Fig. 1. In vivo assay for measuring activity of Hrp promoter. **A**, Plasmid pMBG1 containing *hrpL* under the promoter of ParaC and plasmid pMBG2 containing modified promoters were transferred to *Escherichia coli* strain (Top10) and used for measuring promoter activity. **B**, Hrp promoter activity expressed by β -galactosidase as a function of L-arabinose. pthGp-pMBG2-Pt (*pthG* promoter); hsvGp-MBG2-Hs (*hsvG* promoter). Cells were grown in RM broth (1 \times M9 salts, 1 mM MgCl₂, 2% casamino acids, 0.4% glucose) supplemented with various concentrations of L-arabinose to optical density (at 600 nm) of 0.5. The cells then were harvested and assayed for activity.

mately 35% in promoter activity (Fig. 2A, lanes 2 and 3). The modified *pthG* consensus sequence then was subjected to site-specific mutational analysis. Mutations in the -35 motif indicated that the five consensus nucleotides (**GGAAC**) were essential for promoter activity. Any substitution or deletion of these nucleotides reduced the promoter activity to the level without arabinose (Fig. 2A, lanes 4 through 12). The sixth nucleotide of the consensus was not essential for promoter activity but exerted a significant effect on the promoter's strength. Thus, C exhibited the highest activity, T and A reduced the promoter's activity by approximately 27 and 45%, respectively, and G exhibited the weakest activity that was only slightly above the control (Fig. 2A, lanes 2, 3, 13, and 14). Interestingly, the first nucleotide downstream to the *hrp* box, although

not considered to be a consensus nucleotide, exhibited a significant effect on the promoter strength. Substitution of any other nucleotide for G significantly decreased (by approximately 77%, in the case of C) the promoter activity in an otherwise perfect consensus *hrp* box (Fig. 2A, lane 15). Replacing the G with A gave similar reductions to its replacement with C (results not shown). Changes of the nucleotides upstream to the *hrp* box did not elicit any significant effect on the promoter activity (Fig. 2A, lane 16).

Mutational analysis of the -10 motif indicated that any change in one of the consensus nucleotides ACNNA either completely eliminated the promoter activity or reduced it to a very low level (Fig. 2B, lanes 4 through 7). Modifications of the consensus nucleotides immediately upstream to the latter sequence,

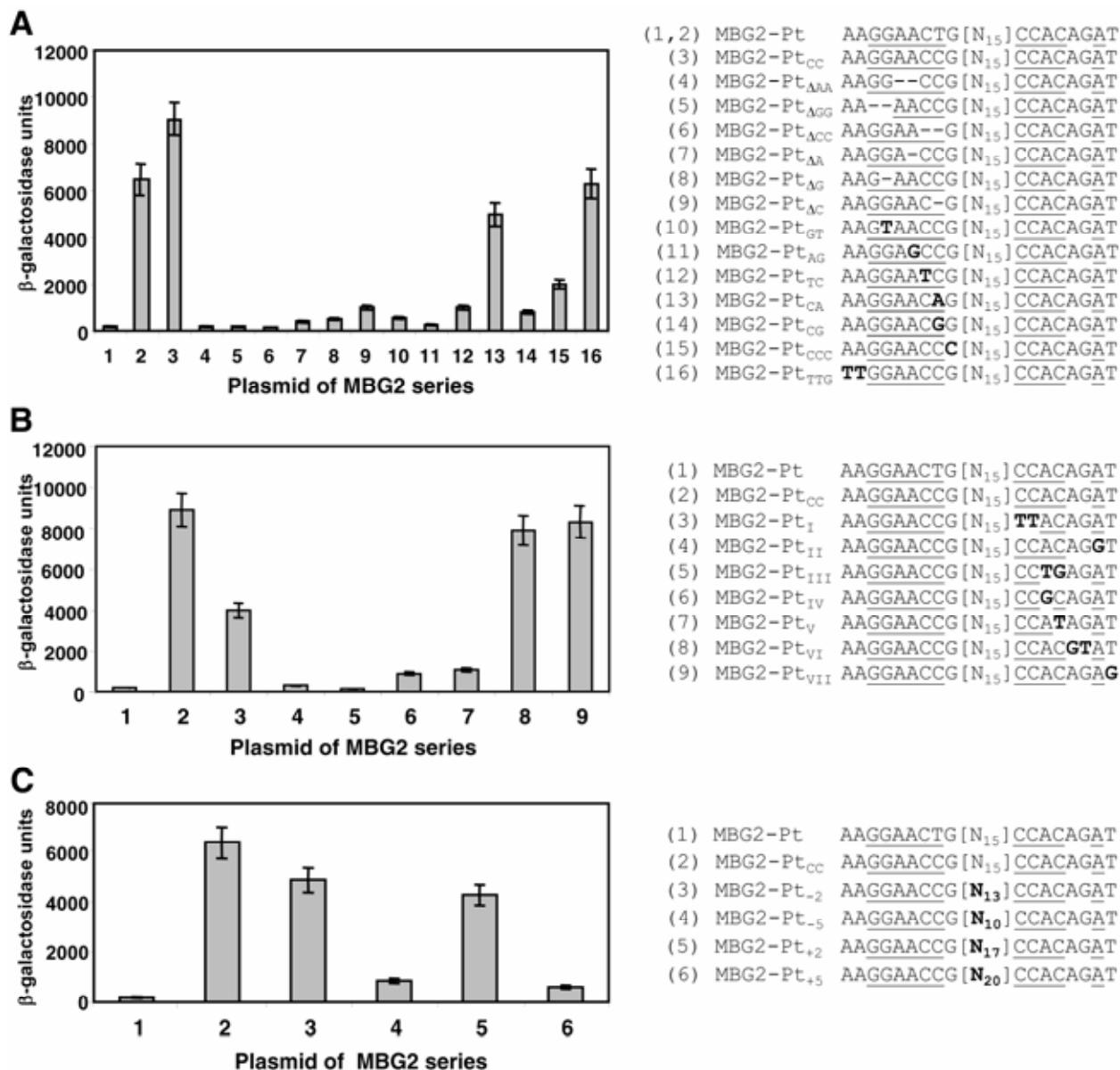


Fig. 2. Mutational analysis of the *pthG* promoter. *Escherichia coli* cells harboring plasmid of the MBG2 series were grown in the presence of 0.2% L-arabinose and assayed for β -galactosidase. Sequence of the tested promoters are listed on the right and their activities monitored by the β -galactosidase reporter are shown on the left. **A**, Effect of site-directed mutagenesis in the -35 motif on promoter activity. Lanes 1, control (MBG2-Pt without arabinose); 2, MBG2-Pt; 3, MBG2-Pt_{CC}; 4, MBG2-Pt_{AAA}; 5, MBG2-Pt_{AGG}; 6, MBG2-Pt_{ACT}; 7, MBG2-Pt_{AA}; 8, MBG2-Pt_{AG}; 9, MBG2-Pt_{AC}; 10, MBG2-Pt_{GT}; 11, MBG2-Pt_{AG}; 12, MBG2-Pt_{TC}; 13, MBG2-Pt_{CA}; 14, MBG2-Pt_{CG}; 15, MBG2-Pt_{CCC}; and 16, MBG2-Pt_{TTG}. **B**, Effect of site-directed mutagenesis in the -10 motif on promoter activity. Lanes 1, control (without arabinose); 2, MBG2-Pt_{CC}; 3, MBG2-Pt_I; 4, MBG2-Pt_{II}; 5, MBG2-Pt_{III}; 6, MBG2-Pt_{IV}; 7, MBG2-Pt_V; 8, MBG2-Pt_{VI}; and 9, MBG2-Pt_{VII}. **C**, Effect of spacing between the -10 and -35 motifs on promoter activity. Lanes 1, control (without arabinose); 2, MBG2-Pt_{CC}; 3, MBG2-Pt₍₋₂₎; 4, MBG2-Pt₍₋₅₎; 5, MBG2-Pt₍₊₂₎; and 6, MBG2-Pt₍₊₅₎. In the sequence alignments, bold letters and hyphens indicate nucleic acid substitutions and deletions, respectively.

CC, significantly influenced the promoter strength but did not eliminate activity. Replacing the CC with TT reduced the promoter activity by approximately 56% (Fig. 2B, lane 3). Changing either the nonconsensus nucleotides (NN) or the first nucleotide downstream to the -10 consensus motif did not have any effect on the promoter activity (Fig. 2B, lanes 8 and 9).

Changing the spacing between the two motifs from 15 to 13 or 17 nucleotides (nt) (Fig. 2, lanes 3 and 5) reduced the promoter activity by 45 to 50%. Further decrease or increase in spacing almost eliminated detectible promoter activity (Fig. 2C, lanes 4 and 6). Transcription rates similar to that of the control were observed with a spacing of 14 or 16 nt (results not shown).

Binding of hHrpL to the *hrp* box DNA.

A gel shift assay was employed for studies on the interaction of HrpL protein with the *hrp* promoter DNA of *pthG*. Purification of HrpL as a histidine-tagged fusion protein (hHrpL), and the gel shift assay are described (discussed below). The presence of a core RNA polymerase (RNAP) obtained from *E. coli* was mandatory for binding of hHrpL to *hrp* box DNA (Fig. 3). Neither core RNAP nor hHrpL showed detectable binding to the *hrp* box DNA in the absence of the other protein (Fig. 3, lanes 1 and 2). No binding was achieved under optimal conditions with control DNA (Fig. 3, lane 3), which indicated specificity in the binding of hHrpL to the promoter sequence. These data are consistent with hHrpL acting as a σ factor (Lonetto et al. 1992).

The promoters of *pthG* and *hsvG* retarded the mobility of the RNAP-hHrpL-DNA complex (Fig. 3, lanes 4 and 5). A mutation in the -10 motif that eliminated *hrp* box promoter activity of *pthG* did not have any significant effect on DNA binding (Fig. 3, lane 6). In contrast, a mutation in the -35 motif (deletion of GG) (Table 1) almost completely eliminated the binding of hHrpL to the *pthG* promoter (Fig. 3, lane 7). A spacing of either 10 or 20 nt, which inactivated promoter activity (Fig. 2C), did not have any significant effect on hHrpL binding (results not shown).

Relationship of *hrp* promoter activity to disease expression.

The observed degeneracy in *hrp* box promoters suggests that differential efficiency may exist among these promoters and may even render some nonfunctional. To gain an insight into the extent to which the activity of Hrp promoters may vary within the same pathogen, we compared the relative efficiency of four native Hrp promoters residing on the pPATH_{pag}. The selected genes included *hrpJ* and Hop effector genes encoding for AvrPphD, HsvG, and PthG. Mutations in each of these genes resulted in either a complete elimination of gall formation or a significant decrease in gall size (Manulis and Barash 2003a). The *hrpJ* gene showed the highest promoter activity and *avrPphD* the lowest (Fig. 4). The activity levels of the Hrp promoters of *pthG*, *hsvG*, and *avrPphD*, relative to that of *hrpJ*, were 0.77, 0.51, and 0.22, respectively.

We also examined whether the observed differences in promoter strength had any effect on disease. To answer this ques-

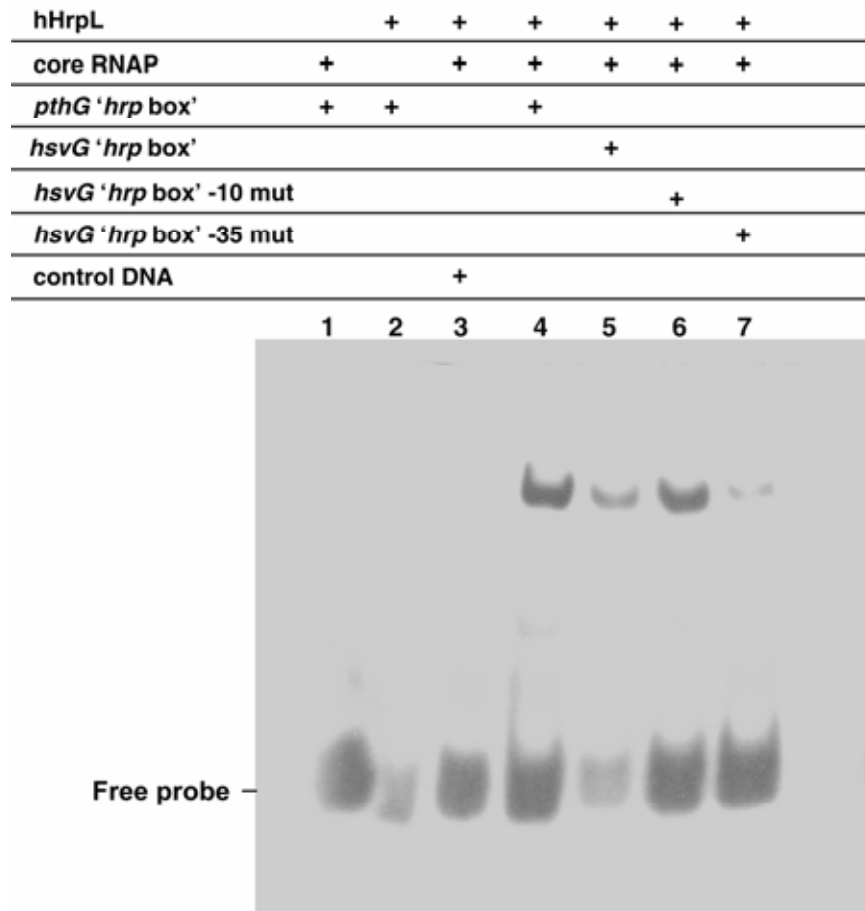


Fig. 3. Gel shift assay of hHrpL with *hrp* promoter DNA of *pthG*. Oligonucleotides used to obtain “*hrp* box” promoters and their mutations are listed in Table 2. Assay procedure is described in Materials and Methods. Lanes 1, Core RNA polymerase (RNAP) with *hrp* box promoter of *pthG* (pt-gr); and 2, hHrpL with *hrp* box promoter (pt-gr). The following lanes contained hHrpL and core RNAP with lane 3, control DNA (DIG Shift Kit, Roche Applied Science); 4, *hrp* box promoter of *pthG* (pt-gr); 5, *hrp* box promoter of *hsvG* (hs-gr); 6, *hrp* box DNA of *pthG* mutated in the -10 motif (pt-gr2); and 7, *hrp* box DNA mutated in the -35 motif (pt-gr1).

tion, we constructed three different sequences based on the *hsvG* promoter: hsp1-d (based on *hrpJ* promoter), hsp2-d (equivalent to *hsvG* promoter), and hsp3-d (based on *avrPphD* promoter) (Table 1). The three promoters were fused in frame with the *hsvG* coding region and cloned into pLAFR3 to yield pHsp1 (strong promoter), pHsp2 (medium-strength promoter), and pHsp3 (weak promoter) (Table 2). These three plasmids then were used to complement the *hsvG* mutant *Pag824-1MxB45*; pHsp1, pHsp2, and pHsp3 were transformed into *Pag824-1MxB45* to yield *Pag824-1MxB45/pHsp1*, *Pag824-1MxB45/pHsp2*, and *Pag824-1MxB45/pHsp3*, respectively (Table 2). *Gypsophila* seedlings then were inoculated with these transconjugants (discussed below). The results clearly demon-

strate that exchanging the *hsvG* promoter with the stronger one caused an increase in gall size (approximately threefold), whereas the weak promoter did not cause gall formation (Table 3; Fig. 5). The appearance of a crown around the cutting edge of the *hsvG* mutant (*Pag824-1MxB45*) and the weak promoter (Fig. 5C and E) previously was reported when high bacterial concentration was employed (Valinsky et al. 1998).

DISCUSSION

In the present study, we have combined a quantitative in vivo assay for measuring Hrp promoter activity with site-directed mutagenesis to analyze the effects of *hrp* box consen-

Table 1. Sequence of fragments and primers used in this study

Fragment, primer	Sequence	Used for
Fragments		
hs-gr	5'-TTTATGCCGGAACCGCCGGGCGGTTTTTCGTTACAAA 3'-AAATACGGCCTTGGCGGCCGCCAAAAGCAATGTTTT	Mobility-shift Assay of <i>hsvG</i> <i>hrp</i> box promoter
pt-gr	5'-TGCAA <u>GGA</u> ACTGAATCCATCCCAGATGCCACAGATTAAG 3'-ACGTTCC TTGAC TTAGGTAGGGTCTACGGTGTCTAATTTCC	Mobility-shift Assay of <i>pthG</i> <i>hrp</i> box promoter
pt-gr2	5'-TGCAA <u>AA</u> CTGAATCCATCCCAGATGCCACAGATTAAG 3'-ACGTTTT GACTTAGGTAGGGTCTACGGTGTCTAATTTCC	Mobility-shift Assay of <i>pthG</i> <i>hrp</i> box promoter mutated in the -35 box
pt-gr1	5'-TGCAA <u>GGA</u> ACTGAATCCATCCCAGATGCCACAGATTAAG 3'-ACGTTCTTGACTTAGGTAGGGTCTACGGTGTCTAATTTCC	Mobility-shift Assay of <i>pthG</i> <i>hrp</i> box promoter mutated in the -10 box
Primers		
hrp1-d	GGCATCCATGGTTAAAGATAGC	Cloning <i>hrpL</i> into: pBAD22 pQE60
hrp1-r1	GTTAGATCTAAAGACGGACTGTT	
hrp1-r2	GTAAGATCTGGACTGTTTTAACG	
hsp1-d	GGGAAGCTTTTATGCCGGAACCGCCGGGCGGTTTTTCGCCACAAAAGAGG	Cloning modified promoter region into pKS-HsvG
hsp2-d	GGGAAGCTTTTATGCCGGAACCGCCGGG	
hsp3-d	GGGAAGCTTTTATGCCGGAACCGCCGGGCGGTTTTTCGCCACAAAAGAGG	
hsp-r	CCGGCGGTACCCTGAGGATGCGGG	
MBG2 series		
avp-d	GAGTCAAGCTTTGGAACCC	Creating: pMBG2-avr
avp-r	TTGTAAGCTTTTCATAGCGCTATC	
hrpj-d	GGAAAAAGTTTAGGGAACCGCAGCG	pMBG2-hrpj
hrpj-r	AACGGAAGCTTTTTCATCTGGCG	
phs-d	GGGAAGCTTTTATGCCGGAACCGCCGGG	pMBG2-Hs
phs-r	CCGTAAGCTTTAATGTTGTTCC	
ppt-d	GGCCAAGCTTTGCAAGGAACCTGAATC	pMBG2-Pt
ppt-r	CGAAGCTTTGAAAACATCCC	
ppt-cc	GGCCAAGCTTTGCAAGGAACCGAATC	pMBG2-Pt _{CC}
ppt-Δgg	GGCCAAGCTTTGCAAAAACCGAATC	pMBG2-Pt _{ΔGG}
ppt-Δaa	GGCCAAGCTTTGCAAGGCCGAATC	pMBG2-Pt _{ΔAAA}
ppt-Δct	GGCCAAGCTTTGCAAGGAAAGAATC	pMBG2-Pt _{ΔACT}
ppt-Δg	GGCCAAGCTTTGCAAGAACCGAATC	pMBG2-Pt _{ΔAG}
ppt-Δa	GGCCAAGCTTTGCAAGGACCGAATC	pMBG2-Pt _{ΔA}
ppt-Δc	GGCCAAGCTTTGCAAGGAACGAATC	pMBG2-Pt _{ΔAC}
ppt-Δg	GGCCAAGCTTTGCAAGGAGCCGAATC	pMBG2-Pt _{ΔAG}
ppt-Δt	GGCCAAGCTTTGCAAGGAATCGAATC	pMBG2-Pt _{ΔT}
ppt-Δc	GGCCAAGCTTTGCAAGGAACGAATC	pMBG2-Pt _{ΔCA}
ppt-Δg	GGCCAAGCTTTGCAAGGAACCGAATC	pMBG2-Pt _{ΔCG}
ppt-ccc	GGCCAAGCTTTGCAAGGAACCCAATC	pMBG2-Pt _{ΔCCC}
ppt-ttg	GGCCAAGCTTTGCTTGGAACCGAATC	pMBG2-Pt _{ΔTTG}
ppt-I	GGCCAAGCTTTGCAAGGAACCGAATCCATCCCAGATGTTACAGATTAAAGT	pMBG2-Pt _I
ppt-II	GGCCAAGCTTTGCAAGGAACCGAATCCATCCCAGATGCCACAGGTTAAAGT	pMBG2-Pt _{II}
ppt-III	GGCCAAGCTTTGCAAGGAACCGAATCCATCCCAGATGCCATAGATTAAAGT	pMBG2-Pt _{III}
ppt-IV	GGCCAAGCTTTGCAAGGAACCGAATCCATCCCAGATGCCGAGATTAAAGT	pMBG2-Pt _{IV}
ppt-V	GGCCAAGCTTTGCAAGGAACCGAATCCATCCCAGATGCCATAGATTAAAGT	pMBG2-Pt _V
ppt-VI	GGCCAAGCTTTGCAAGGAACCGAATCCATCCCAGATGCCACGTATTAAAGT	pMBG2-Pt _{VI}
ppt-VII	GGCCAAGCTTTGCAAGGAACCGAATCCATCCCAGATGCCACAGATTAAAGT	pMBG2-Pt _{VII}
ppt-(-2)	GGCCAAGCTTTGCAAGGAACCGAATCCATCCCAGATGCCACAGATTAAAGT	pMBG2-Pt ₍₋₂₎
ppt-(-5)	GGCCAAGCTTTGCAAGGAACCGAATCCATGCCACAGATTAAAGT	pMBG2-Pt ₍₋₅₎
ppt-(+2)	GGCCAAGCTTTGCAAGGAACCGAATAACCATCCCAGATGCCACAGATTAAAGT	pMBG2-Pt ₍₊₂₎
ppt-(+5)	GGCCAAGCTTTGCAAGGAACCGAATAACCATCCCAGACATGCCACAGATTAAAGT	pMBG2-Pt ₍₊₅₎

sus and nonconsensus nucleotides on promoter activity. The dependency of the promoter activity on arabinose concentration in our two-plasmid system suggests a direct interaction between HrpL and the promoter. The same transcript (*lacZY*) was employed with the different promoters; therefore, the assay was independent of transcript stability. The consensus bipartite *hrp* box promoter (i.e., GGAACC-N₁₅₋₁₆-CCACNNA) has been generated previously by alignment of promoter regions from functional *hrp/hrc* and *avr* genes obtained from *Pseudomonas syringae* strains (Fouts et al. 2002; Innes et al. 1993; Shen and Keen 1993; Xiao and Hutcheson 1994; Zwiesler-Vollick et al. 2002). In the present study, the analysis was performed with HrpL of *Pantoea agglomerans*, which exhibits only 52% similarity to HrpL of *Pseudomonas syringae* pv. *tomato*. Nevertheless, the results obtained are relevant to *hrp* box promoters of *Pseudomonas syringae* as well as to *Er-*

winia spp., because the *hrp* box consensus nucleotides seem to be common to all the HrpL-dependent phytopathogenic bacteria. Moreover, some type III effectors (e.g., AvrPphD, HopPmaB, and HopPmaH) are common to *Pantoea agglomerans* pv. *gypsophylae* and *Pseudomonas syringae* and, presumably, have been acquired by horizontal gene transfer (Manulis and Barash 2003b).

The present study demonstrates that the *hrp* box consensus can be divided into crucial and noncrucial nucleotides. The first 5 nt of the -35 consensus motif (GGAAC) and the last 3 nt of the -10 consensus motif (ACNNA) cannot be replaced. The sixth nucleotide of the -35 consensus motif (C) and the first 2 nt of the -10 consensus motif (CC) can be replaced with other nucleotides; however, their replacement caused a significant reduction of promoter activity in a differential manner. An unexpected result was the effect of the first nu-

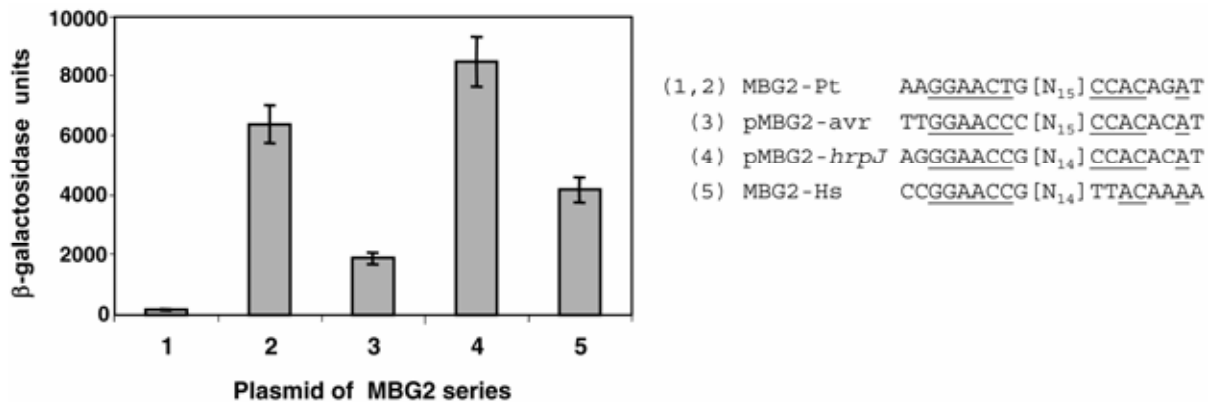


Fig. 4. Comparative activity of native “*hrp* box” promoters from *Pantoea agglomerans* pv. *gypsophylae*. Sequence of the tested promoters are listed on the right and their activities monitored by the β-galactosidase reporter are shown on the left. In the sequence alignments, bold letters and hyphens indicate nucleic acid substitutions and deletions, respectively; 1 = *pthGp* (without arabinose), 2 = *pthGp* (MBG2-Pt), 3 = *avrPphDp* (MBG2-avr), 4 = *hrpJp* (MBG2-hrpJ), and 5 = *hsvGp* (MBG2-Hs).

Table 2. Bacterial strains, cosmids, and plasmids used in this study

Strains, plasmids ^a	Relevant characteristics ^b	Reference or source
Strains		
Top10	<i>Escherichia coli</i> , <i>lacZ</i> ΔM15 Δ <i>lacX</i> 74 <i>araD</i> 139 Δ(<i>ara-leu</i>) 7697	Invitrogen
DH5α	<i>E. coli</i> , <i>lacZ</i> ΔM15 Δ (<i>lacZYA-argF</i>)U169 <i>gyrA</i> 96	Invitrogen
M15 (pREP4)	Lac, ara, gal, mtl, recA ⁺ , uvr ⁺ , (pREP4: <i>lacI</i> , kan ^r)	Qiagen
MBG2 series	<i>E. coli</i> Top10 (pMBG1, pMBG2)	This study
<i>Pag</i> 824-1	Wild-type pathogenic strain with Rif ^r , <i>gypsophila</i> pathovar	Manulis 1991
<i>Pag</i> MxB45	<i>Pag</i> 824-1, marker exchange mutants on <i>hsvG</i>	Valinsky et al. 1998
<i>Pag</i> 824-1Mx B45/pHsp1	<i>Pag</i> 824-1Mx B45 transformed with pHsp1	This study
<i>Pag</i> 824-1Mx B45/pHsp2	<i>Pag</i> 824-1Mx B45 transformed with pHsp2	This study
<i>Pag</i> 824-1Mx B45/pHsp3	<i>Pag</i> 824-1Mx B45 transformed with pHsp3	This study
Cosmids		
pLAFR3	Tc ^r , broad-host-range vector <i>IncP-1</i> rixRK2+ <i>lacZa</i> Tra- Mob+ cos	Staskawicz 1987
pHsp1	Tc ^r , <i>hsvG</i> under strong <i>hrp</i> box promoter cloned into pLAFR3 as an <i>Hind</i> III/ <i>Bam</i> HI fragment	This study
pHsp2	Tc ^r , <i>hsvG</i> under medium <i>hrp</i> box promoter cloned into pLAFR3 as an <i>Hind</i> III/ <i>Bam</i> HI fragment	This study
pHsp3	Tc ^r , <i>hsvG</i> under weak <i>hrp</i> box promoter cloned into pLAFR3 as an <i>Hind</i> III/ <i>Bam</i> HI fragment	This study
Plasmids		
pQE60	Ap ^r , His-tag expression vector	Qiagen
pBluescript-II KS+	Ap ^r , cloning vector, ColE1 ori	Stratagene
pBAD22	Ap ^r , arabinose inducible expression vector	Guzman et al. 1995
pBBR1MCS-3	Tc ^r , broad range expression vector	Kovach et al. 1995
pLKC480	Source of <i>lacZY</i> -Km ^r cassette	Tiedeman and Smith 1988
pHrpL-His	PCR-amplified <i>hrpL</i> coding sequence cloned into pQE60 as a <i>Nco</i> I/ <i>Bam</i> HI fragment	This study
pMBG1	PCR-amplified <i>hrpL</i> coding sequence cloned into pBAD22 as a <i>Nco</i> I/ <i>Hind</i> III fragment	This study
pBB-lacZ	The <i>lacZYKm</i> cassette cloned as an <i>Xma</i> I fragment into pBBR1MCS-3	This study
pMBG2 series	Various PCR-amplified <i>hrp</i> box promoter and partial coding sequence cloned into pBB-lacZ as <i>Hind</i> III fragments	This study
pKS-HsvG	PCR-amplified <i>hsvG</i> coding sequence and promoter cloned into pBluescript-II KS as an <i>Hind</i> III/ <i>Bam</i> HI fragment	This study

^a *Pag* = *Pantoea agglomerans* pv. *gypsophylae*.

^b Tc = tetracycline, Rif = rifampicin, Ap = ampicillin, Km = kanamycin, PCR = polymerase chain reaction.

cleotide downstream to the -35 consensus motif on promoter activity: G exhibited the highest activity, whereas all the other nucleotides exhibited substantially reduced promoter activity (by 70 to 85%) even in the presence of a perfect nucleotide consensus in the -35 motif. It is noteworthy that G appears in this site of Hrp promoters more frequently than the other nucleotides. For example, of 33 Hrp promoters of *hrpI* functional genes in *Pseudomonas syringae* pathovars and 18 promoter regions identified in *Pseudomonas syringae* pv. *tomato* 3000 by a reporter transposon screen for HrpL-activated genes, 54% contained G immediately downstream to the -35 motif, whereas the percentages of C, A, and T were 20, 20 and 6%, respectively (Fouts et al. 2002). Our promoter assay analyses indicate that, although the highest activity was obtained when there was a spacing of 14 to 16 nt between the two conserved motifs, this spacing was not critical, contrary to what has been reported elsewhere (Zwiesler-Vollick et al. 2002). Although a decrease of the spacing to 13 nt or an increase to 17 nt caused a significant reduction in promoter activity, the promoters still retained approximately 65% of their maximal activity.

The absolute need of RNAP for HrpL binding to *hrp* box promoters is in accordance with its function as a σ factor. Interestingly, deletion of GG from the -35 consensus motif was sufficient to eliminate HrpL binding completely, whereas neither mutation in the -10 motif nor reduction or extension of the spacing between the two motifs had any effect on HrpL binding, although it entirely inactivated promoter activity.

Table 3. Effect of Hrp promoter activity on gall size

Mutant strain ^a	Gall fresh weight (mg) ^b
<i>Pag</i> 824-1 (wild-type)	43 (16)
<i>Pag</i> 824-1Mx B45 (<i>hsvG</i> ⁻ mutant)	19 (11)
<i>Pag</i> 824-1Mx B45/pHsp1	139 (22)
<i>Pag</i> 824-1Mx B45/pHsp2	38 (14)
<i>Pag</i> 824-1Mx B45/pHsp3	17 (7)

^a *Pag* = *Pantoea agglomerans* pv. *gypsophila*. pHsp1, pHsp2, and pHsp3 denote strong, medium-strength, and weak Hrp promoters, respectively. The promoters were cloned into pLAFR3 and used to complement *Pag* 824-1Mx B45 (*hsvG*⁻ mutant).

^b Results are expressed as fresh weight of gall tissue taken 10 days after inoculation and represent one of two different experiments and are given as a mean \pm standard deviation of eight plants.

HrpL is a member of the ECF σ factors that regulate and respond to extracytoplasmic functions and direct transcription of specific regulons during unusual physiological conditions, such as stress response and pathogenesis (Bashyam and Hasnain 2004). Members of the ECF subfamily are composed of small (approximately 20 kDa) proteins that exhibit remarkable diversity in their primary structures (Lonetto et al. 1994) and that have been classified as a subfamily of the σ^{70} family (Bashyam and Hasnain 2004; Helmann and Chamberlin 1988).

Limited proteolysis studies have led to a view of the σ factor structure as a series of compact domains connected by flexible linkers of variable lengths and sequences (Lonetto et al. 1992; Severinova et al. 1996). This type of architecture lends itself to conformational changes, and biochemical and biophysical probes have indicated that σ undergoes substantial conformational changes during the transcription initiation process (Callaci et al. 1999). The σ^{70} family of proteins comprises four domains (Helmann and Chamberlin 1988; Lonetto et al. 1992) but we could identify only two (σ_2 and σ_4) in the HrpL of *Pantoea agglomerans* pv. *gypsophila* or *Pseudomonas syringae* pv. *tomato*. There are distinct differences between the respective DNA binding domains; σ_2 binds to the -10 promoter region, whereas σ_4 , which encodes for helix-turn-helix motif, binds to the -35 promoter region (Lonetto et al. 1992). The promoter specificity is determined by the -35 motif, which differs among promoters that are recognized by different ECF σ factors in the same bacterial species (Lonetto et al. 1992). HrpL-dependent phytopathogenic bacteria share identical -35 motifs of *hrp* box promoters; therefore, a high similarity among the σ_4 domain of HrpL would be expected. Thus, the percentage similarities of amino acids between the σ_4 domain of HrpL from *Pantoea agglomerans* pv. *gypsophila* and those from *Erwinia amylovora*, *E. chrysanthemi*, and *Pseudomonas syringae* pv. *tomato* are 85, 75, and 60%, respectively. In contrast, the similarity with *pvdS*, an ECF factor essential for siderophore biosynthesis in *Pseudomonas aeruginosa*, is 47% related (Wilson et al. 2001). It is noteworthy that a deletion of GG in the -35 *hrp* box motif prevents hHrpL binding, presumably via the σ_4 domain, indicating a high degree of specificity. A crystal structure of the σ_4 domain complexed with the -35 element recently has been demonstrated (Campbell et al. 2002).

Because coordination of virulence factors is necessary for a successful pathogenic process, the expression of these fac-

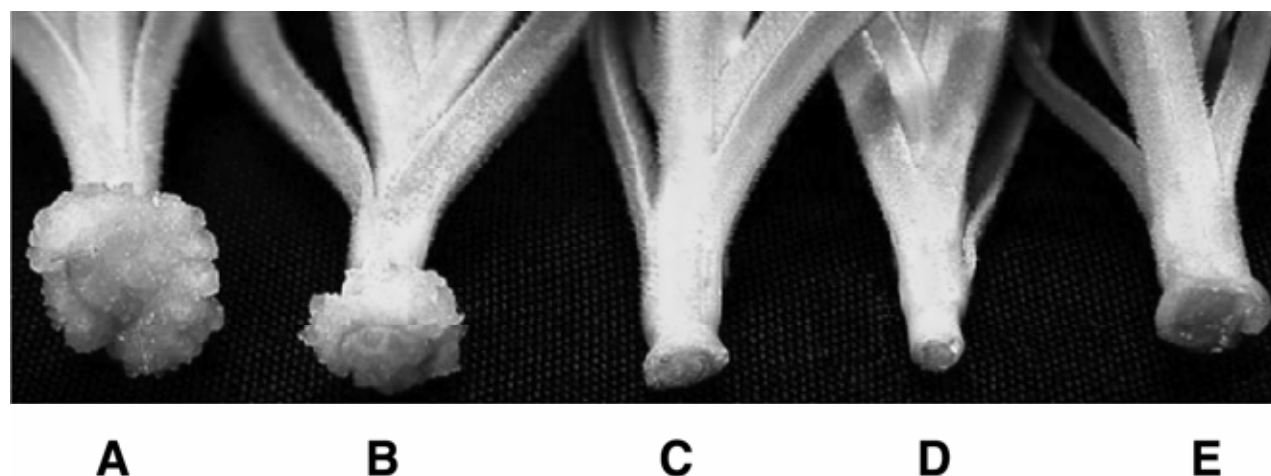


Fig. 5. Phenotypic expression of *hsvG* under different promoters. *Gypsophila* cuttings were inoculated with *hsvG* mutant (*Pantoea agglomerans* pv. *gypsophila* [*Pag*]824-1MxB45) and its transconjugants. **A**, *Pag*824-1MxB45/pHsp1 (strong promoter equivalent to *hrpI*); **B**, *Pag*824-1MxB45/pHsp2 (medium-strength promoter based on *hsvG*); **C**, *Pag*824-1MxB45/pHsp3 (weak promoter equivalent to *avrPphD*); **D**, water control; **E**, *Pag*824-1MxB45. Inoculations were carried out with 10^9 CFU/ml and the photograph was taken after two weeks.

tors generally is tightly regulated. Transcriptional regulation is a major mechanism for control of gene expression in bacteria and is influenced mainly by the efficiency of the promoter and the steady-state transcript levels. The degeneracy observed in Hrp promoters could represent a mechanism for fine tuning that is necessary for expressing components of the type III system and Hop effectors in a concerted manner. This adjustment may be provided by the nonconsensus or consensus nucleotides that affect the promoter strength without impeding its function. Genes controlled by the Hrp regulon generally are located in PAIs that presumably have been acquired by the pathogen through one or more horizontal genes transfers (Arnold et al. 2003; Hacker and Kaper 1999). It is reasonable to hypothesize that during the evolution or horizontal gene transfer of the PAI, its virulence genes underwent genetic rearrangements that were followed by selection pressure that affected the composition of Hrp promoters. Thus, it is not surprising to find that the bacterial genome contains Hrp promoters that differ in their activity, as well as nonfunctional promoters. However, functional Hrp promoters have to comply with appropriate transcript stability and the role of the virulence gene in pathogenicity. Comparison of four native Hrp promoters obtained from the pPATH_{pag} (Fig. 4) illustrates the variation in promoter efficiency within the same organism. Thus, the promoter of *hrpJ* is 4.5 times as efficient as that of *avrPphD*. Both promoters have identical and perfect consensus in the two motifs of the *hrp* box, and the difference in strength can be attributed to a difference of a single nucleotide downstream to the -35 consensus motif (G in *hrpJ* versus C in *avrPphD*), which could account for a decrease of approximately 77% in promoter strength. The difference in promoter activity between *hrpJ* and *hsvG* can be attributed to a change in the -10 motif (from CC to TT).

The relationship between gall size and promoter activity of *hsvG* implies that expression of this gene is a limiting factor in gall development. HsvG was characterized in pPATH_{pag} and pPATH_{pab} as a host-specific Hop effector (Valinsky et al. 1998, 2002a,b). Mutations in *hsvG* eliminated the ability of *Pantoea agglomerans* pvs. *gypsophila* and *betae* to cause gall formation on gypsophila but did not diminish the pathogenicity of *P. agglomerans* pv. *betae* on beet or of HR of both pathovars on tobacco. The function of HsvG is not yet known, but preliminary results of studies in which HsvG was used as a bait for screening the cDNA library of *P. agglomerans* pv. *gypsophila*-infected gypsophila seedlings in a yeast two-hybrid system indicated the presence of an activation domain (G. Nissan, S. Manulis, and I. Barash, unpublished data). This led us to hypothesize that HsvG may act as a DNA-binding protein and a potential transcription factor. In the latter case, it might exert its effect by activating several genes simultaneously. Overgrowth of gall tissue in gypsophila cuttings previously was reported to result from overproduction of cytokinins (Lichter et al. 1995), and it is attractive to speculate that HsvG may influence either the production of cytokinins or the sensitivity of plant tissues to these hormones.

Interestingly, AvrPphD previously was reported as a virulence effector of *P. agglomerans* pv. *gypsophila* on gypsophila, because a mutant of this gene reduced gall size (Guo et al. 2002). Nevertheless, an equivalent promoter of this gene was insufficient to express HsvG function during gypsophila infection by *P. agglomerans* pv. *gypsophila* (Table 3). These results could be attributed to either a difference in mRNA stability between the two genes or a need for higher effector concentration for expressing a phenotype, or both. Regardless of the mechanism involved, the present results demonstrate that the efficiency of Hrp promoters may serve as a significant factor in disease expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains, cosmids, and plasmids used in the present study are listed in Table 2. A rifampicin-resistant wild-type strain of *P. agglomerans* pv. *gypsophila* (Pag824-1) and its derivatives were used throughout. Bacteria were grown either in LB broth and agar or in a minimal A medium (Ausubel et al. 1995) with appropriate antibiotics, at 28°C. *Escherichia coli* strains DH5 α (Life Technologies, Gaithersburg, MD, U.S.A.), M-15 (Qiagen, Basel, Switzerland), and Top10 (Invitrogen, Basel, Switzerland) were used as cloning hosts and grown at 37°C on nutrient agar plates, LB broth, or RM medium (1 \times M9 salts, 1 mM MgCl₂, 2% casamino acids, 0.4% glucose) in the presence of ampicillin (Ap) at 100 μ g ml⁻¹ and kanamycin (Km) at 10 μ g ml⁻¹, where applicable. Additional antibiotics were used in the following concentrations: rifampicin (Rif) at 100 μ g/ml, spectinomycin (Spec) at 100 μ g/ml, and tetracycline (Tc) at 15 μ g/ml.

Recombinant DNA techniques and plasmid constructions.

DNA isolation, agarose gel electrophoresis, subcloning, electroporation, Southern blotting, and polymerase chain reaction (PCR) were performed according to standard procedures (Ausubel et al. 1995; Sambrook et al. 1989) or as recommended by the relevant supplier. DNA fragments were amplified by PCR, with *Taq* polymerase (Sigma-Aldrich, St. Louis) and synthetic oligonucleotides (Table 1), synthesized according to specification (Sigma-Aldrich). For plasmid constructions, the *hrpL* gene was amplified with PCR primers hrpl-d and hrpl-r2 (Table 1) to create a 5' *NcoI* cloning site that overlapped and included the ATG translation initiation codon, and a 3' *HindIII* site downstream of the stop codon. This fragment was inserted into the similarly digested expression vector, pBAD22 (Guzman et al. 1995), resulting in plasmid pMBG1 (Fig. 1A). The *lacZYKm'* cassette from plasmid pLKC481 (Tiedman and Smith 1988) was inserted into the broad-host-range vector, pBBR1MCS-3 (Kovach et al. 1995), and cloned as an *XmaI* fragment into the internal *SmaI* site to generate plasmid pBB-lacZ (Table 2). This plasmid was used to create an in-frame reporter gene fusion by inserting PCR-amplified promoters and partial coding sequences of *hsvG*, *pthG*, or other tested promoters with an *HindIII* primer site used to generate the pMBG2 series (Table 2). MBG1 and MBG2 were electrophoresed into an *E. coli* Top10 host strain that was used for analyzing promoter activity.

HrpL was purified as an His-tagged fusion protein as follows. A DNA fragment extending from the ATG start codon of *hrpL* to 3 bp upstream to the stop codon was amplified by PCR with primers hrpl-d and hrpl-r1 (Table 1) and cloned into the His-tag expression vector pQE60 (Qiagen, Basel, Switzerland) to generate pHrpL-his (C-terminus). The C-terminal tagging was preferred because this region is highly divergent in ECF factors, both in length and in sequence, indicating that structural tolerance is to be expected in this protein domain.

β -Galactosidase activity assay.

E. coli Top10 harboring a plasmid of the MBG2 series was grown overnight in RM minimal medium. The cells then were diluted to an optical density at 600 nm (OD₆₀₀) of 0.05 in fresh RM medium and allowed to grow to an OD₆₀₀ of 0.1 before L-arabinose was added. β -Galactosidase activity, expressed in enzyme units (Sambrook et al. 1989), was measured after the cells reached an OD₆₀₀ of 0.5. Each experiment was performed in triplicate and was repeated at least twice.

Expression and purification of hHrpL protein.

pHrpL-his was transformed into *E. coli* M-15 and the bacteria were grown in LB (100 ml) containing Ap until an OD₆₀₀ of 0.5 was reached. Following addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM, the culture was incubated for an additional 3 h to induce the expression of the *hrpL*-his gene. The bacteria then were harvested by centrifugation, resuspended in 60 ml of water, and lysed by freezing and thawing. The lysate was sonicated on ice and centrifuged (22,000 × g, 20 min). The insoluble pellet was washed twice with a washing buffer (25% [wt/vol] sucrose, 5 mM EDTA, 1% [vol/vol] Triton X-100 in phosphate-buffered saline). The pellet then was suspended in 5 ml of denaturing solution (6 M guanidinium hydrochloride; 0.1 M Tris-HCl buffer, pH 8.0; 1 mM EDTA, pH 8.0; and 0.03 M β-mercaptoethanol for 1 h at 25°C) (Turkov et al. 1997). Protein renaturation was initiated by 20-fold dilution in 0.2 M ammonium acetate (pH 8.5). After 48 h at 15°C, the insoluble protein was removed by filtration through a 1-mm Whatman filter paper and the soluble protein was precipitated with 55% saturated ammonium sulfate overnight. The precipitate was collected by filtration through a GF/C Whatman filter and solubilized in 7 ml of 10 mM Tris-HCl (pH 8.0).

Gel retardation assay.

The synthetic DNA fragments used in these assays are listed in Table 1. Double-stranded DNA was obtained by mixing complementary oligodeoxyribonucleotides, heating to 95°C, and slowly cooling to room temperature. These DNAs were labeled with digoxigenin (DIG) with a DIG-labeling kit (Roche Molecular Biochemicals, Mannheim, Germany). Core RNAP (Epicentre Technologies, Madison, WI, U.S.A.) was incubated on ice for 10 min with the purified HrpL protein. Core σ mixtures then were added to reaction tubes containing the DNA probe (100 fmol), assay buffer (100 mM Hepes, pH 7.6; 5 mM EDTA; 50 mM (NH₄)₂SO₄; 5 mM dithiothreitol; 1% [wt/vol] Tween 20, and 150 mM KCl) to give a final volume of 20 ml. Following incubation at 28°C for 15 min and standing at room temperature for 5 min, the samples were loaded onto a 5% native polyacrylamide gel containing Tris-borate EDTA (TBE) (22 mM Tris-HCl, pH 8.0, 23 mM boric acid, 0.65 mM EDTA) and 5% bis-acrylamide, and were electrophoresed in TBE. DNA probes were transferred from the gel to an N⁺ nylon membrane and detected with anti-DIG antibodies according to the manufacturer's protocol (Roche Molecular Biochemicals).

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