The HrpX/HrpY Two-Component System Activates *hrpS* Expression, the First Step in the Regulatory Cascade Controlling the Hrp Regulon in *Pantoea stewartii* subsp. *stewartii*

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A regulatory cascade activating *hrp/hrc* type III secretion and effector genes was delineated in *Pantoea stewartii* subsp. *stewartii*, a bacterial pathogen of corn. Four *hrp* regulatory genes were characterized: *hrpX* and *hrpY* encode the sensor kinase and response regulator, respectively, of a two-component signal transduction system; *hrpS* encodes an NtrC-like transcriptional enhancer; and *hrpL* encodes an alternate sigma factor. Epistasis analysis, expression studies using gene fusions, and genetic reconstruction of each step in *Escherichia coli* were used to delineate the following pathway: HrpY activates *hrpS* and also positively autoregulates the *hrpXY* operon. In turn, HrpS is required for full activation of the σ^{54} -dependent *hrpL* promoter. Finally, HrpL controls expression of all known *hrp* and *wts* genes. In vitro, *hrpS* and all downstream *hrp* genes were regulated by pH and salt concentration. Mutants with in-frame deletions in *hrpX* were still partially virulent on corn but were unable to sense the chemical or metabolic signals that induce *hrp* genes in vitro. Site-directed mutagenesis of HrpY indicated that aspartate 57 is the probable phosphorylation site and that it is needed for activity. These findings suggest that both HrpX and an alternate mechanism are involved in the activation of HrpY in planta.

Additional keywords: *Erwinia stewartii*, PAS domains.

Pantoea stewartii subsp. *stewartii* (synon. *Erwinia stewartii*) causes Stewart's bacterial wilt and leaf blight of sweet corn and maize (*Zea mays* L.). Stewart's wilt is the most serious bacterial disease of sweet corn in the north-central and eastern United States. The pathogen is transmitted by the corn beetle (*Chaetocnema pulicaria*) through feeding wounds (Claflin 1999). Bacteria grow in the xylem vessels of the host plant causing wilt and in the intercellular spaces of the leaves causing "water-soaked" lesions. The primary virulence factor responsible for wilting is the extracellular polysaccharide (EPS) (Dolph

et al. 1988), and the genes required for its production and regulation have been characterized (Coplin and Majerczak 1990; Dolph et al. 1988; Poetter and Coplin 1991; Torres-Cabassa et al. 1987). Water-soaking (Wts) is the result of cell membrane damage and the accumulation of fluids in the leaf tissue.

A cluster of *hrp* genes (Alfano and Collmer 1997) is required by many gram-negative plant-pathogenic bacteria for elicitation of the hypersensitive response (HR) (Klement 1982) in nonhost plants and for pathogenicity in a compatible host. *hrp* genes encode a type III secretion system that is conserved among plant and animal bacterial pathogens (Galan and Collmer 1999). They also encode several regulatory proteins and the secreted effector proteins that determine interactions with susceptible and resistant hosts. *Pantoea stewartii* has a typical *hrp* cluster that spans over 28 kb and that is required for HR elicitation in tobacco and disease in corn (Ahmad et al. 2001; Coplin et al. 1986, 1992; Frederick et al. 2001). In contrast to more necrogenic pathogens such as *Pseudomonas syringae* and *Erwinia amylovora*, *Pantoea stewartii* is only able to elicit a HR in tobacco leaves when the bacteria are pregrown in a Hrp-inducing minimal medium or when *hrpS* is constitutively expressed (Ahmad et al. 2001). Thus, unlike sweet corn and maize, tobacco does not provide all of the signals necessary to induce *hrp* gene expression in this species.

In gram-negative plant pathogenic bacteria, there are two lineages of *hrp* genes that differ significantly in their mode of regulation (Alfano and Collmer 1997). In general, expression of *hrp* genes is environmentally regulated. Both plant factors (Aldon et al. 2000; Schulte and Bonas 1992) and chemical/metabolic stimuli mimicking the plant apoplast (Rahme et al. 1992; Wei et al. 1992; Xiao et al. 1992) have been shown to activate their expression, but the mechanism by which these signals are perceived is still unknown. Group I *hrp* genes in *P. syringae* and *Erwinia* and *Pantoea* spp. are regulated by HrpS and the alternative sigma factor HrpL (Frederick et al. 1993; Xiao and Hutcheson 1994; Xiao et al. 1994; Wei and Beer 1995). Expression of *hrpL* requires the alternate sigma factor σ^{54} in addition to the NtrC-like transcriptional activator HrpS (Frederick et al. 1993; Hendrickson et al. 2000a). In *P. syringae*, HrpR, which is homologous to HrpS, is also required for full activation of the *hrpL* promoter and may form heteromeric complexes with HrpS (Hutcheson et al. 2001). In *Erwinia amylovora*, Wei and associates (2000) described a two-component signal transduction system, HrpX/HrpY, which is orthologous to the one described in this work. They proposed that both HrpS and HrpY regulate *hrpL* and that their effects are addi-

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tive. In contrast, Group II *hrp* genes in *Xanthomonas* and *Ralstonia* spp. are regulated by AraC-like transcriptional activators. In *Ralstonia solanacearum*, a complex regulatory cascade transduces a signal arising from contact with the plant cell wall and activates *hrp* gene expression (Aldon et al. 2000; Brito et al. 2002).

In this report, we describe the sequences and function of genes included in *Pantoea stewartii* *hrp* complementation groups V through VII, which were previously identified as regulatory loci (Frederick et al. 2001). *hrpS* (formerly designated *wtsA*) was characterized by Frederick and associates (1993), and its sequence is analyzed again here in the context of new data. Using mutational and epistasis analysis, we determined the Hrp phenotype of individual regulatory genes and the order of the steps in the regulatory cascade. The results of this study support a model in which phosphorylated HrpY activates *hrpS* in response to environmental stimuli. This is different from the pathway reported for *Erwinia amylovora* but similar to that concurrently discovered in *Erwinia herbicola* (synon. *Pantoea agglomerans*) pv. *gypsophylae* (Nizan-Koren et al. 2002).

RESULTS

Sequence analysis of the *hrp* regulatory region of *Pantoea stewartii*.

The *hrp* regulatory region was identified by transposon mutagenesis and shown to contain three complementation groups (Frederick et al. 2001). The region was subcloned from the cosmid pES1044 into two contiguous plasmids, pRF8 and pRF201SK (Fig. 1). Prior sequencing of pRF8 (Frederick et al. 1993) revealed a single open reading frame (ORF), *hrpS*, that encodes an NtrC-like enhancer protein homologous to HrpS from *P. syringae* pathovars (Rahme et al. 1991; Xiao et al. 1994), *Erwinia amylovora* (Wei et al. 2000), and *Erwinia herbicola* pv. *gypsophylae* (Mor et al. 2001). The main features of *hrpS* have been previously described (Frederick et al. 1993). The sequence of the pRF201SK insert revealed three ORFs between *hrpJ* and *hrpS*, which have been designated *hrpL*, *hrpX*, and *hrpY*. All of the ORFs in the regulatory region are transcribed from right to left, as shown in Figure 1. Sequence analysis of the region upstream of *hrpL* revealed a σ^{54} consensus box (CTGGCA-N₆-TTGCT; probability score = 0.92) at -51 bp from the start codon of *hrpL*. This was preceded by an A+T-rich region containing putative integration host factor (IHF) binding elements. A single dyad-symmetrical site (TGCAA-N₄-TTGCA), weakly matching the NtrC-dependent upstream activating site (TGCACC-N₅-GGTGCA) (Porter et al. 1995), was located at -163 bp. The predicted 21.3-kDa protein encoded by *hrpL* is homologous to alternate sigma factors of the ECF σ^{70} family (Lonetto et al. 1994), including HrpL of *Erwinia amylovora* (E value = 3e-74, 75% identity) (Wei and Beer 1995), *Erwinia herbicola* pv. *gypsophylae* (E = 2e-79, 80% identity) (Mor et al. 2001), and *P. syringae* pathovars (E = 6e-30, 47 to 48% identity) (Rahme et al. 1991; Xiao and Hutcheson 1994).

The *hrpX* ORF is located 144 bp downstream from the stop codon of *hrpL*. A weak σ^{70} promoter consensus sequence (TGTGGA-N₁₇-TAACAA, probability score = 0.87) is located at -22 bp. Only 30 bp separates *hrpX* from the downstream *hrpY* gene. The predicted proteins encoded by *hrpX* (485 aa) and *hrpY* (213 aa) have homology to bacterial two-component signal transduction systems, with HrpX similar to "orthodox" sensor kinases and HrpY similar to bacterial response regulators (Hoch and Silhavy 1995). HrpX has two tandem PAS domains (Taylor and Zhulin 1999) in the input region. HrpY belongs to the FixJ class of response regulators with a conserved aspartate (D57), which is the putative site of phosphorylation,

as inferred from multiple sequence alignments (not shown). The closest PSI-BLAST matches in the GenBank database for these two genes were the ortholog HrpX/HrpY proteins of *Erwinia herbicola* pv. *gypsophylae* (E = 0, 79% identity for HrpX; E = e-103, 88% identity for HrpY) (Mor et al. 2001) and *Erwinia amylovora* (E = 0, 79% identity for HrpX; E = 2e-96, 84% identity for HrpY) (Wei et al. 2000). Interestingly, a putative HrpX and HrpY orthologous pair (E = 3e-40 for HrpX; E = 9e-39 for HrpY) is also present in *P. syringae* DC3000 (as found on the National Center for Biotechnology Information website).

Our sequence analysis did not reveal any *Rho*-independent terminators immediately following the end of *hrpY*, and multiple translational stops are present in the 842-bp *hrpY-hrpS* intergenic region. BLAST searches of the GenBank database indicated that a 483-bp IS-like element is present at -23 bp from the start of *hrpS* (Fig. 1). This putative IS element is flanked by two 8-bp direct repeats and contains two 75-bp imperfect inverted repeats. The latter match sequences flanking unidentified prophage genes and integrases present in the chromosomes and virulence plasmids of *Salmonella typhimurium* and *S. typhi* (GenBank accession numbers AE006471, AE008744, and AL627268) and also flanking virulence ORFs 2 and 3 in *P. syringae* pv. *phaseolicola* plasmid pAV511 (accession number AF141883). However, the IS-like element in *Pantoea stewartii* does not contain an ORF. Several putative σ^{70} promoter sequences are present upstream of and inside the IS-like element. Polymerase chain reaction (PCR) analysis of 11 strains from a geographically representative collection revealed that this IS remnant was present in the same physical position in all strains tested (data not shown).

Null mutant phenotypes and epistasis analysis.

In a previous study, we isolated Tn5 and Tn3HoHoI insertion mutations in *hrpL*, *hrpX*, *hrpY*, and *hrpS* and recombined

Fig. 1. Physical map of the regulatory region of the *Pantoea stewartii* DC283 *hrp* gene cluster showing the *hrpL*, *hrpX*, *hrpY*, and *hrpS* subclones used in this study. Open boxes represent regulatory open reading frames, the cross-hatched box is *hrpJ*, a secretion operon, and the solid box represents an IS remnant in the *hrpS* 5' regulatory region. The direction of transcription is shown by arrows. Solid circles indicate Tn5 insertions, the open circles are point mutations, flags indicate Tn3HoHoI insertions and their orientation, squares denote *aphA-3* insertions, and Δ indicates in-frame deletions. Open triangles in front of genes represent putative σ^{70} promoters, the closed semicircle is a σ^{54} promoter, and the open semicircle is a *hrp* box promoter. *P_{lac}* indicates the position of the constitutive *lac* promoter in the plasmid vector. Solid boxes labeled "*uidA*" indicate transcriptional fusions to the GUS gene in pL6GUS. Restriction enzyme abbreviations: H, *Hind*III, B, *Bam*HI.

them into the genome of *Pantoea stewartii* wild-type strain DC283, producing strains *hrpL*::Tn3HoHoI (MEX105), *hrpX*::Tn3HoHoI (MEX116), *hrpY*::Tn5 (DM064), and *hrpS*::Tn5 (DM758) (Fig. 1) (Frederick et al. 2001). All of these mutants have a Hrp⁻ phenotype, cannot synthesize harpin, and exhibit down-regulation of several cosmid-borne *hrp-lacZ* fusions in secretion genes. Since transposon mutations in *hrpX* are polar on *hrpY*, we constructed two new non-polar, in-frame, deletion mutants of *hrpX*, $\Delta hrpX_{(17-414)}$ (DM2790) and $\Delta hrpX_{(17-470)}$ (DM2788) (Fig. 1). In tobacco, the newly constructed $\Delta hrpX$ mutants were unable to produce HR symptoms, even when infiltrated at high cell densities ($>10^9$ CFU ml⁻¹). On sweet corn seedlings, mean disease severity ratings at 3 days after inoculation were 2.9 ± 0.2 and 1.9 ± 1.0 for DC283 and DM2790, respectively. Although these means are not significantly different at $P = 0.05$, we observed large variations in both infection rates and symptom severity with the mutant but not with the wild-type strain. In general, the $\Delta hrpX$ mutants were capable of attaining wild-type levels of virulence but with variable response times and incidence. This may mean that the *hrpX* mutants are much more sensitive to physiological differences between individual seedlings.

Complementation analysis was done to determine the epistatic interactions between different genes and rule out the possibility that some phenotypes were due to second-site mutations. Low copy number plasmids (pRK415 or pLAFR3) carrying *hrpXY*⁺, *hrpY*⁺, *hrpS*⁺, and *hrpL*⁺ (Fig. 1) were constructed and introduced into different mutants. The ability of these plasmids to restore HR elicitation in tobacco and virulence on sweet corn to the mutants was tested. In all cases where virulence was restored, the complemented mutants were fully virulent (disease severity rating ≥ 2.5 in at least two experiments), and the bacteria did not need to be preinduced in inducing medium (IM) to cause the HR. The *P*_{lac}-*hrpL* plasmid pMM6 restored HR elicitation in tobacco and virulence on sweet corn to all the insertion and deletion mutants in the regulatory region. Next, the *P*_{lac}-*hrpS* plasmid pRF205 was able to restore the Hrp⁺ phenotype to strains with mutations in *hrpX*, *hrpY*, or both (strains MEX116, DM2788, DM2790, DM064) and *hrpS* (DM758) and to a double *hrpY hrpS* mutant (DM733), but not to an *hrpL* mutant (MEX105). Finally, plasmid pMM52, which contains *P*_{lac}-*hrpY*, was able to restore HR elicitation and full virulence to $\Delta hrpX$ (DM2788, DM2790) and *hrpY* (DM064) mutants, but not to *hrpS* (DM758) and *hrpL* (MEX105) mutants. Based on the observations that: i) mutations in *hrpS* were not suppressed by *hrpY*⁺, ii) constitutive expression of *hrpS*⁺ or *hrpL*⁺ restored the Hrp⁺ phenotype to *hrpS* and *hrpY hrpS* mutants, and iii) constitutive expression of *hrpY*⁺ and *hrpS*⁺ alleles did not overcome the Hrp⁻ phenotype of *hrpL* mutants, we propose the following basic model for the Hrp regulatory cascade: *hrpY* $\rightarrow$ *hrpS* $\rightarrow$ *hrpL* $\rightarrow$ *hrp* box promoters.

Expression of *hrp* regulatory genes in different genetic backgrounds.

To confirm the order of the regulatory cascade, *hrpY*::Tn5, *hrpS*::Tn5, and *hrpL*::*aphA3* insertions were introduced into the chromosome of selected *hrp-lacZ* or *hrp-uidA* strains to create double regulatory mutants. In addition, we tested the effects of constitutively expressing various plasmid-borne regulatory genes on chromosomally located *lacZ* and *uidA* transcriptional fusions to *hrpX*, *hrpS*, *hrpL*, and *hrpJ* promoters. Low copy number plasmids carrying *P*_{lac}-*hrpY*, *P*_{lac}-*hrpS*, and *P*_{lac}-*hrpL* were used to overexpress these genes, and β -galactosidase or β -glucuronidase (GUS) activity of the reporter fusions was measured, following induction in IM liquid medium. Results from these experiments are summarized in Figure 2.

A second-site mutation in *hrpY* abolished expression of *hrpS-lacZ* and *hrpL-uidA* (Fig. 2C), and a mutation in *hrpS* decreased expression of *hrpL-uidA* (Fig. 2B), as expected from the epistatic analysis. Overexpressing *hrpY* turned up the *hrpX-lacZ* fusion approximately twofold (Fig. 2A), showing that *hrpXY* is a potentially autoregulated operon. *P*_{lac}-*hrpY* stimulated the *hrpS-lacZ* fusion approximately eightfold in a *hrpY*⁺ background and approximately 20-fold in a *hrpY hrpS-lacZ* double mutant (Fig. 2B). *P*_{lac}-*hrpY* also up-regulated *hrpL* approximately sevenfold in the *hrpY*⁺ chromosomal background and approximately fivefold in the *hrpY hrpL-uidA* double mutant, but it repressed *hrpL* expression in the *hrpS hrpL-uidA* double mutant (Fig. 2C). The inability of HrpY to increase *hrpJ* expression in strains containing a second-site *hrpS* or *hrpL* mutation (strains DM701 and DM786) further indicates that it does not directly activate *hrpLp* or *hrp*-box promoters, respectively (Fig. 2D). In a *hrpS*⁺ *hrpXY*⁺ background, the *P*_{lac}-*hrpS* plasmid up-regulated both *hrpL-uidA* and *hrpJ-lacZ* fusions twelve- and threefold, respectively (Figs. 2C and D). This effect was even more apparent in the *hrpS hrpJ-lacZ* and *hrpY hrpJ-lacZ* double mutants, in which expression increased 44- and 82-fold, respectively (Fig. 2D). However, *P*_{lac}-*hrpS* did not affect *hrpJ* expression in a *hrpL hrpJ-lacZ* double mutant (Fig. 2D), demonstrating that it does not directly affect genes with *hrp* box promoters. Finally, the *P*_{lac}-*hrpL* plasmid up-regulated *hrpJ-lacZ* fusions in all genetic backgrounds (*hrpXY*, *hrpY*, *hrpS*, and *hrpL*; Fig. 2D), thereby confirming that *hrpL* is the last gene in the *hrp* regulatory cascade. Significantly, the expression of the *hrpJ-lac* fusions in this experiment mirrored the virulence of the same strains in the above mutational and epistasis analysis.

Since the lack of apparent terminators following the *hrpL* and *hrpXY* operons means that transposon-induced mutations could have partial polar effects on the level of expression of downstream genes, we repeated the above experiments with plasmid-borne, reporter gene fusions in trans to the tested regulatory mutations. Therefore, we constructed a series of reporter fusions in pPL6GUS. These plasmids contained the regulatory regions upstream of the *hrpX*, *hrpS*, and *hrpL* ORFs fused to a *uidA* reporter gene with its own ribosomal binding site (Fig. 1). Following induction in IM liquid medium, expression of each promoter was assayed in wild-type strain DC283 and in mutants lacking a *hrp* regulatory gene (Table 1). Transcription from the *hrpXp* region, between -198 and +46 bp from the *hrpX* start, was high and constitutive in DC283. Expression of the *hrpS-uidA* fusion, containing the region between -928 and +384 bp, was reduced up to tenfold in *hrpY* and $\Delta hrpX$ mutants, as compared with DC283. The *hrpL-uidA* fusion was reduced approximately 250-fold in *hrpS*, $\Delta hrpX$, and *hrpY* mutants.

Since Wei and associates (2000) proposed that HrpS and HrpY both directly activate *hrpL* in *Erwinia amylovora*, we recreated each step of the *Pantoea stewartii* regulatory cascade in *Escherichia coli* SΦ200, a genetic background that would lack other known *hrp*-specific regulatory molecules. We constructed *E. coli* strains that harbored one of the above low copy *hrp-uidA* reporter plasmids along with a high copy number plasmid that overexpressed a regulatory gene (plasmids pMM46, pRF8, pMM22 carrying *hrpY*, *hrpS*, and *hrpL*, respectively). These strains were grown in LB broth, the regulatory gene was induced with IPTG, and the cells were assayed for GUS activity. (The following data are expressed as means $\pm$ standard deviation. They are from representative experiments with at least three replicates each, and statistical significance P was analyzed by ANOVA). The *hrpXp-uidA* fusion in pMM25 was constitutively expressed in *E. coli* but was further stimulated as much as sixfold by overexpression of *hrpY* (from 26 ± 6 to 127 ± 10 units; $P < 0.05$). The *hrpS-uidA* fusion carried by

pMM50 was up-regulated eightfold by HrpY (from 5 ± 2 to 42 ± 5 units; $P < 0.05$), and the *hrpL-uidA* fusion in pDM2785 was activated by HrpS (30-fold from 79 ± 20 to $2,370 \pm 817$ units; $P < 0.001$). In contrast, overexpression of *hrpY* did not enhance *hrpLp* expression, but instead, it consistently repressed its basal level by approximately sixfold (from 79 ± 20 to 12 ± 3 units; $P < 0.001$). Finally, the *hrpJp-uidA* fusion, which contains a promoter with an *hrp*-box, was strongly activated by *hrpL* expressed from plasmid pMM22 (2,000-fold induction from 2 to 3,970 units; $P < 0.001$).

Overall, our data support a model in which HrpY autoregulates *hrpXY* expression, stimulates *hrpS* transcription, and represses *hrpL*. These results further confirm that HrpS activates *hrpL* and then HrpL alone controls expression of *hrp* box promoters.

Role of the conserved aspartate in HrpY.

Based on sequence homology and protein modeling (analysis not shown), the conserved aspartate residue at position 57 is the most likely phosphorylation site in HrpY. To test if D57 is required for activation of HrpY, we mutated this codon to alanine and asparagine. Both substitutions were expected to prevent phosphorylation while minimizing structural effects on the protein, as shown for similar response regulators (Hoch and Silhavy 1995; Jin et al. 1990; Moore et al. 1993). The missense mutant alleles were constructed in vitro by spliced overlap extension PCR and exchanged for the wild-type locus in the chromosome of DC283, using the positive selection, suicide vector pLD55. Both the *hrpY* (D57N) and the *hrpY* (D57A) chromosomal mutants were completely unable to infect corn whorls or to elicit the HR when infiltrated into tobacco leaves; they were complemented for both phenotypes by the *P_{lac}-hrpY* plasmid, pMM52. Unexpectedly, low copy number plasmids carrying the same mutant alleles, pMM74 *P_{lac}-hrpY*(D57A) and pMM118 *P_{lac}-hrpY*(D57N), were also able to complement the null phenotype of the D57A, D57N, and Tn5 *hrpY* chromosomal mutants. As discussed below, this type of overexpression artifact is common with two-component regulators and necessitates the use of single copy mutant alleles for assessing phenotypes.

Effects of environmental conditions on the expression of *hrp* genes in vitro.

The regulatory effects of pH, phosphate, ammonia, and sodium chloride were tested in vitro using chromosomal *hrpX-lacZ*, *hrpS-lacZ*, and *hrpJ-lacZ* fusions (Fig. 3). IM was used as a basal medium to test each variable. Expression of *hrpS-lacZ* and *hrpJ-lacZ* was decreased by neutral to basic pH values (with maximal expression at pH 5.5) and by high potassium phosphate, ammonium chloride, and sodium chloride levels. When we plotted the dose-response curves for each salt expressed as its osmotic pressure (calculated by approximating activities to concentration values), the curves overlapped (data not shown), implying that part of the ability of IM to induce *hrp* genes could be due to its low osmolarity per se rather than to nitrogen or phosphate starvation. In contrast, expression of *hrpX-lacZ* was not affected by pH and was only slightly reduced by addition of the three salts (Fig. 3). In rich culture media, plasmid pRF205 *P_{lac}-hrpS* relieved repression of promoters downstream of *hrpS* in the cascade (data not shown). This was consistent with the ability of pRF205 to enable *Pantoea stewartii* to elicit a HR in tobacco, irrespective of the media used to grow the inoculum. We also tested the effect of 2 mM nicotinic acid on wild-type DC283 cells carrying pDM2531 *hrpJ-uidA* during growth in IM, pH 5.5. Expression of this fusion in IM was reduced fivefold by nicotinic acid (from $7,357 \pm 138$ to $1,439 \pm 60$ units $\pm$ standard error in two experiments).

Fig. 2. β-galactosidase (β-Gal) and β-glucuronidase (GUS) activity of chromosomal *hrp-lacZ* or *hrpL-uidA* fusions in different *Pantoea stewartii* genetic backgrounds, with and without various plasmid-borne *P_{lac}-hrp* regulatory genes. **A**, β-Gal activity in *hrpX-lacZ* strain MEX116. **B**, β-Gal activity in the *hrpS-lacZ* strain MEX1 and *hrpY hrpS-lacZ* strain DM733. **C**, GUS activity in *hrpL-uidA* strain DM2831, *hrpY hrpL-uidA* strain DM2837 and *hrpS hrpL-uidA* strain DM2844. **D**, β-Gal activity in the *hrpJ-lacZ* strain DM711, *hrpY hrpJ-lacZ* strain DM729, *hrpL hrpJ-lacZ* strain DM786, and Δ *hrpS hrpJ-lacZ* strain DM701. Cells were grown in inducing medium, pH 5.5, broth to late log phase, and β-Gal-specific activity was measured (1 unit = 1 pmol MU min⁻¹ OD₆₀₀⁻¹ ml⁻¹ of culture at 25°C). The first column indicates which regulatory gene was constitutively expressed in trans (if any); the second column indicates the second *hrp* mutation (if any) in the strain. Regulatory gene clones: pMM52 *P_{lac}-hrpY* and pMM46 *P_{lac}-hrpY* (C) (low copy pMM330 was used as well, with similar results); pRF205 *P_{lac}-hrpS* (D) and pRF8 *P_{lac}-hrpS* (C); and pMM6 *P_{lac}-hrpL* (D). Controls consisting of the vector plasmids without inserts had no effect on expression of any of the gene fusions.

DISCUSSION

Bacterial virulence factors are usually tightly regulated and controlled by environmental stimuli (Finlay and Falkow 1997). Mediating these signals are complex, often hierarchical, regulatory cascades that integrate multiple inputs and finely tune the infection process. The cascades controlling type III and type IV secretion systems in several mammalian and plant pathogens have been shown to include complete or partial two-component signal transduction pairs. These constitute a common strategy for linking the expression of energy-consuming secretion apparatuses to signals indicating the presence of a host. In this study, we present evidence for a regulatory cascade that activates *hrp/hrc* type III secretion/effector genes in *Pantoea stewartii* in response to in vitro stimuli that mimic the host environment. The cascade involves, from the top down (Fig. 4), a two-component signal transduction pair (HrpX and HrpY), a transcriptional activator (HrpS), and finally an alternative sigma factor (HrpL). In this study, we first confirmed that HrpS and HrpL serve the same functions in *Pantoea stewartii* as they do in *Erwinia amylovora* and *P. syringae* (Hendrickson et al. 2000a, 2000b; Hutcheson et al. 2001; Rahme et al. 1991; Wei and Beer 1995; Wei et al. 2000; Xiao et al. 1992), and then we focused on the role of the HrpX/Y two-component system.

The occurrence of orthologs of HrpX/HrpY in *Erwinia amylovora* (Wei et al. 2000), *Erwinia herbicola* pv. *gypsophylae* (Mor et al. 2001), *Erwinia chrysanthemi* (J. H. Ham, C. M. Rojas, A. Collmer, unpublished data; GenBank accession no. AF448202) and possibly in *P. syringae* pv. *tomato* DC3000 suggests that this two-component system may represent a common mechanism for the environmental regulation of group I *hrp* genes. However, only the functional and genetic analysis of *Erwinia amylovora* *hrpXY* has been published (Wei et al. 2000) to date. The role proposed by Wei and associates (2000) for *Erwinia amylovora* HrpY is different from the one we present here for *Pantoea stewartii* in that HrpY appears to serve as a modulator of HrpS activation of *hrpL* instead of activating *hrpS*. This conclusion was based on their finding that a nonpolar *hrpY::TnphoA* mutation did not affect a chromosomal *hrpS::Tn5-gusA1* reporter and on an earlier observation (Wei and Beer 1995) that a *hrpS* mutation only partially regulates *hrpL*. A similar, independent study on *hrp* gene regulation in *Erwinia herbicola* pv. *gypsophylae* has been published concurrently with ours (Nizan-Koren et al. 2002). Their results with HrpY are in complete agreement with our findings for *Pantoea stewartii*. Other key data in Wei and associates (2000), such as the inability of *hrpY* to complement a *hrpS* mutation, the downregulation of *hrpL* in a *hrpY* background, and the autoregulation of the *hrpXY* operon by HrpY, are consistent with both our results and those in *Erwinia herbicola* pv. *gypsophylae*. This suggests that common regulatory

elements may interact differently in the *pantoeas* and some other *erwinias* than they do in *Erwinia amylovora*. An extreme example of this is found in *Erwinia carotovora*, which has both *hrpS* and *hrpL*. However, *hrpL* expression in this species is constitutive, and the *hrp* genes are posttranscriptionally regulated by RsmA, which affects the stability of *hrpL* mRNA (Chatterjee et al. 2002).

In view of the apparent differences in the role of HrpY between *Pantoea stewartii* and *Erwinia amylovora*, we verified our hypothetical model, using multiple approaches. In particular, the regulatory effect of HrpY on *hrpS* transcription was demonstrated repeatedly by measuring plasmid-borne reporter gene fusions in *hrp* regulatory mutants (Table 1), by using complementation tests with plasmid-borne *hrpY⁺* in *hrpS-lacZ* or *hrpY::Tn5 hrpS-lacZ* strains (Fig. 2B), and by reconstructing individual steps of the cascade in *E. coli*. Moreover, overexpression of *hrpY⁺* repressed the *hrpL-uidA* reporter in both a *hrpS* background (Fig. 2C) and in *E. coli* genetic reconstruction experiments, further indicating that HrpY cannot activate *hrpL*. It is possible that repression of *hrpLp* by unphosphorylated HrpY could provide a way of keeping the basal level of *hrp* gene expression low under noninducing conditions or provide a mechanism to turn it back down late in infection, or both. We are in the process of obtaining biochemical evidence for the function of HrpY; preliminary experiments indicate that *hrpSp* is located between the IS-like element and the end of *hrpY* and that purified HrpY protein can, indeed, retard a *hrpS* promoter fragment in gel-shift experiments and also weakly binds to a *hrpL* promoter fragment (M. Merighi and D. Coplin, unpublished data).

In the course of our complementation experiments, we found that a *P_{lac}-hrpY* plasmid activated *hrpSp* in the absence of *hrpX* and complemented polar *hrpXY* mutants. These observations raised the question as to whether or not HrpY always needs to be phosphorylated in order to activate *hrp* genes. To address this question, we carried out site-directed mutagenesis of the conserved aspartate (D57) in HrpY, which is the putative phosphorylation site, and found that *Pantoea stewartii* mutants carrying either *hrpY(D57N)* or *hrpY(D57A)* chromosomal alleles in single copy were completely Hrp⁻. This result indicated that D57 is required for HrpY to function as a positive regulator and is genetic evidence that phosphorylation is required for HrpY activation. We believe that the avirulence of these mutants reflects the role of D57 in phosphorylation rather than any nonspecific structural changes brought about by the amino acid substitutions. This role is supported by the solvent-exposed position of this residue (based on 3D protein modeling; data not shown), its conserved position, and the type of substitutions performed, as shown for other well studied receiver domains (Jin et al. 1990; Moore et al. 1993). Consequently, we attribute our finding that plasmid-borne wild-type HrpY and D57 mutant alleles were active in the absence of *hrpX* to an

Table 1. Effects of *hrp* regulatory genes on plasmid-borne *hrp::uidA* transcriptional fusions expressed in different genetic backgrounds^a

Plasmid	Promoter fusion ^b	GUS activity (Units ± S. E.) ^c in different genetic backgrounds ^d				
		Wild type	Δ <i>hrpX</i>	<i>hrpY</i>	<i>hrpS</i>	<i>hrpL</i>
pMM25	-198/+46 <i>hrpXp</i>	307 ± 53	370 ± 104	206 ± 2	ND	ND
pMM50	-928/+384 <i>hrpSp</i>	135 ± 9	20 ± 9	21 ± 4	119 ± 12	ND
pDM2785	-207/+182 <i>hrpLp</i>	6053 ± 959	164 ± 5	67 ± 12	36 ± 1	ND
pDM2531	-516/+425 <i>hrpJp</i>	4851 ± 704	94 ± 13	17 ± 5	3 ± 1	0 ± 0
pDM2791	-914/+22 <i>wtsEp</i>	399 ± 125	7 ± 5	1 ± 1	0 ± 0	4 ± 2

^a Strains were grown in inducing medium broth, pH 5.5, to late exponential–early stationary phase and were assayed for GUS activity fluorometrically, using 4-methyl-umbelliferyl- β -D-glucuronide as described by Jefferson (1987), but the assays were scaled down to fit microtiter plates and were analyzed using a Victor 1420-2 multilabel reader.

^b Transcriptional fusion names are preceded by the promoter fragment coordinates in bp (+1 is first base of ORF).

^c One unit = 1 pmol MU min⁻¹ OD₆₀₀⁻¹ ml⁻¹ of culture at 37°C. S. E. = standard error of two to six independent experiments, each with at least two replicates.

^d Strains: wild type = DC283; Δ *hrpX* = DM2790 (mutant DM2788 gave similar results); *hrpY* = DM064; *hrpS* = DM758 or MEX1; *hrpL* = MEX105.

overexpression artifact that is commonly encountered in genetic studies of two-component systems (Hoch and Silhavy 1995). For example, in *E. coli*, overexpression of UhpA (a related FixJ-class response regulator) activated its target promoter *uhpTp*. This overexpression effect was observed with both wild-type UhpA and a D54A mutant protein (Shattuck-Eidens and Kadner 1983; Kadner 1995). Two common explanations for this phenomenon are cross-talk with paralog kinases and the ability of the unphosphorylated response regulator to bind promoters at abnormally high concentrations (Hoch and Silhavy 1995).

In general, *hrp* genes are induced in minimal media that mimic conditions found in planta (Lindgren 1997), but these can vary considerably between species. For example, *hrp* genes in *Erwinia amylovora* are stimulated by low pH and repressed by glucose, ammonium salts, asparagine, histidine, and nicotinic acid, but are unaffected by osmolarity (Wei et al. 1992). *P. syringae* *hrp* genes are likewise induced at low pH in minimal media, but they are nonspecifically repressed by high salt concentrations (Rahme et al. 1992). The results of our study showed that expression of many *hrp* genes in *Pantoea stewartii* was similarly regulated by pH and nitrogen sources but, in contrast to *Erwinia amylovora*, they were also affected by salt concentration (Fig. 4). *hrpS-lacZ* and *hrpJ-lacZ* chromosomal fusions were inhibited at neutral to basic pH and at high concentrations of potassium phosphate, sodium chloride, and ammonium sulfate. In a previous study, we found that nitrates are also strongly repressing but casamino acids increase expression rather than repress it (Coplin et al. 1992).

For our studies on the role of the two-component system in sensing environmental signals, we constructed two *Pantoea stewartii* mutants with different in-frame deletions within the kinase domain of HrpX. In these strains, the expression of *hrpS*, *hrpL*, and *hrpJ* was greatly reduced (to levels comparable to a null *hrpY* mutant) when assayed in IM (Table 1), and the $\Delta hrpX$ mutants could not cause a HR on tobacco, which in *Pantoea stewartii* requires preinduction in IM. This is the first clear indication in any system that HrpX is needed to sense the chemical and physiological signals provided in vitro by an apoplast-mimicking, Hrp-inducing medium. The *hrpXY* operon itself maintained a high basal level of expression in both rich media (data not shown) and in IM medium at neutral pH with added salts (Fig. 3), whereas the *hrpS* promoter was repressed under these conditions. Thus, *hrpS* appears to be the first gene in the regulatory cascade that is under strong environmental control, and its transcriptional regulation by phosphorylated HrpY and global regulators (M. Merighi and D. Coplin, unpublished data) may therefore be the key point in the cascade in which environmental signals are integrated.

The exact nature of the signals detected by HrpX is currently under investigation in our laboratory. At this point, however, the sequence analysis of HrpX offers a possible explanation as to how the *hrp* regulon is able to respond to such a wide range of environmental conditions. The absence of transmembrane regions indicates that HrpX is probably a cytoplasmic protein and the presence of duplicated PAS domains in the N-terminal portion suggests that its signal may be internal. PAS domains are versatile cytosolic protein modules involved in monitoring light, redox potential, oxygen, small ligands, and the energy level inside cells (Stephenson and Hoch 2001; Taylor and Zhulin 1999). Cluster analysis of a diverse set of PAS domains from prokaryotic sensor proteins revealed that the PAS domains of HrpX are most closely related to those of *Azotobacter vinelandii* NifL (data not shown), which is a flavoprotein with FAD as its prosthetic group (Taylor and Zhulin 1999). Therefore, by analogy to NifL, HrpX might sense redox potentials and energy changes within the cell. This hypothesis would explain

how the Hrp regulon is able to respond to most of the conditions known to induce *hrp* genes, i.e., acidity, phosphate, and nitrogen starvation, all of which could affect cellular redox potentials.

The importance of HrpX during infection appears similar in *Pantoea* and *Erwinia* species in that it is not absolutely required for virulence. In *Erwinia amylovora*, a nonpolar *hrpX::TnphoA* mutant was greatly reduced in virulence, response time, and growth in planta, and the expression of *hrpL* was reduced fivefold in IM (Wei et al. 2000). In contrast, a nonpolar *hrpX::mini-Tn10-Km* mutant of *Erwinia herbicola* pv. *gypsophillae* remained fully virulent and did not show any decrease in downstream *hrp* gene expression in planta, but the effect of this mutation was not determined in an inducing medium (Nizan-Koren et al. 2002). Since the basis for the nonpolarity of *TnphoA* and *mini-Tn10* transposons in *Erwinia* spp. is not understood, it is possible that *hrpY* was not expressed at normal levels in these experiments. The two *Pantoea stewartii* $\Delta hrpX$ mutants used in this study were only slightly reduced in virulence, infectivity, and response time, but they exhibited much greater variability in symptom severity and incidence on corn seedlings. This suggests that the ability of the pathogen to sense the nutritional status of its host is very important to insure consistent infection. On the other hand, given that $\Delta hrpX$ mutants still retained significant virulence on sweet corn while nonphosphorylatable mutants of HrpY were totally nonpathogenic, we hypothesize that there is an alternative HrpX-independent pathway leading to HrpY activation in planta. This could reflect either the increased accumulation of small phosphodonor molecules (McCleary et al. 1993) under starvation conditions or the action of an alternative bacterial kinase specifically activated in corn (but not in tobacco or IM). It will be interesting to learn if this alternate phosphorylation pathway responds to host cell contact or to specific plant inducer molecules.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions.

E. coli and *Pantoea stewartii* strains and plasmids used in this study are listed in Table 2. Luria-Bertani (LB) broth and agar (Ausubel et al. 1987) were used for strain maintenance and for growth of *Pantoea stewartii* under Hrp-repressing con-

Fig. 3. Effect of environmental conditions on the expression of various *hrp-lacZ* fusions. The effects of neutral pH, 50 mM sodium chloride, 25 mM ammonium sulfate, and 25 mM potassium phosphate are shown as a percentage of the activity in standard inducing medium (IM), pH 5.5, liquid medium (control). Cells were grown in modified IM, with one component changed, as indicated in the Z-axis of the graph, for 16 h, and β -Gal activity was measured. *hrpX-lacZ* = strain MEX116, *hrpS-lacZ* = strain MEX1, and *hrpJ-lacZ* = DM711.

ditions. Fusaric acid-containing tetracycline-sensitive selection medium was prepared as described (Metcalf et al. 1996). To induce *hrp* genes in *Pantoea stewartii* strains, overnight LB broth cultures were washed twice in IM (Frederick et al. 2001), were inoculated into 2 ml of the same medium at an optical density at 600 nm (OD_{600nm}) equal to 0.05, were grown for 12 to 16 h, and were adjusted to a final OD_{600nm} of approximately 0.5. In the pH experiment, HEPES and Tris-HCl buffers were used for the neutral and basic pH ranges, respectively, in place of MES. Liquid cultures were grown in flasks or tubes shaken at 200 rpm at 37°C for *E. coli* or 29°C for *Pantoea stewartii*. When appropriate, antibiotics were supplied at the following concentrations: ampicillin, 200 $\mu\text{g ml}^{-1}$; kanamycin, 50 $\mu\text{g ml}^{-1}$; and tetracycline, 20 $\mu\text{g ml}^{-1}$ (or 15 $\mu\text{g ml}^{-1}$ for selection of single copy Tet^r cointegrates).

HR and pathogenicity tests.

To test for HR elicitation on tobacco (*Nicotiana tabacum* L. cv. "White Burley") plants, *Pantoea stewartii* strains were grown overnight in IM liquid medium, were washed, and were resuspended in water at $OD_{600nm} = 0.52$ (approximately 1×10^9 CFU ml^{-1}). Cells were infiltrated into tobacco leaves using four replicates per strain as previously described (Frederick et al. 2001), and necrosis was rated 24 to 48 h after infiltration. Pathogenicity tests were performed by inoculating the whorls of five-day-old sweet corn seedlings (*Zea mays* var. *saccharata* cv. "Seneca Horizon") as previously described (Coplin et al. 1986). The plants were held in growth chambers at 29°C (16 h photoperiod, 15,000 lux, 99% relative humidity). After three days, disease severity was rated using a 0 to 3 scale (0 = no symptoms, 1 = scattered small lesions, 2 = numerous lesions, and 3 = extensive lesions that remained water-soaked with ooze forming on leaf surfaces).

General recombinant DNA, genetic, and bioinformatic techniques.

DNA isolation, agarose gel electrophoresis, restriction enzyme digestion, and ligation were done following standard procedures (Ausubel et al. 1987). PCRs used either native *Taq* (for colony PCR screens and construct mapping) or cloned *Pfu* (for molecular cloning) DNA polymerases (Invitrogen, Carlsbad, CA, U.S.A and Clontech, Palo Alto, CA, U.S.A., respectively)

Fig. 4. Working model for the *hrp* regulatory cascade in *Pantoea stewartii*. The HrpX sensor perceives undefined metabolic and stress signals and activates HrpY via phosphorylation (indicated by ~P). A parallel phosphorylation mechanism may also activate HrpY in planta. HrpY then activates *hrpS* and its own operon; HrpS and σ^{54} activate *hrpL*; and finally, HrpL enables RNA polymerase core enzyme (E) to activate promoters containing *hrp* boxes (e.g., the harpin, *wts*, and *hrp* secretion genes).

and standard amplification protocols (Ausubel et al. 1987). For colony PCR, we used cells lysed in 50 mM NaOH for 10 min at 100°C as templates. For pBluescript and pRK415 vectors, the reactions were performed using one primer anchored on either the *lacI* vector gene (primer LACF) or the *alacZ* gene fragment (primer LACR) and a second primer conveniently located in the recombinant insert (Table 3). Standard deprotected oligonucleotide primers were ordered from IDT-DNA (Coralville, IA, U.S.A.). Probes for Southern blot hybridizations were labeled with 11-Dig-dUTP, and colorimetric immunodetection was done as recommended by the manufacturer (Roche Biochemicals, Indianapolis, IN, U.S.A.). Plasmids were introduced into *E. coli* and *Pantoea stewartii* strains by electroporation, chemical transformation (Inoue et al. 1990), or mobilized by triparental mating using pRK2013::Tn7 as a helper plasmid (Ditta et al. 1980).

DNA sequencing was performed using cycle sequencing with fluorescent dye terminator chemistry and the ABI 3700 capillary electrophoresis sequencer (PE-Applied Biosystems, Wellesley, MA, U.S.A.) at The Ohio State University Plant-Microbe Genomic Facility. Database analyses of predicted proteins were performed using BLAST/PSI-BLAST (Altschul et al. 1997), whereas conserved domain analysis used the CD search service (ver. 1.54) (Marchler-Bauer et al. 2002), both available through the National Center for Biotechnology Information. Statistical significance of the matches are reported as E values. Promoter searches were performed using a neural network prediction algorithm (Reese et al. 1996; available on the Berkely Drosophila Genome Project website) and the IHF/Sigma54 promoter-finder program (Goodrich et al. 1990).

Construction

of P_{lac} -*hrpY*, P_{lac} -*hrpS*, and P_{lac} -*hrpL* plasmids.

For complementation and overexpression experiments, the *hrpX*, *hrpY*, and *hrpL* ORFs, including their ribosomal binding sites, were amplified by PCR and were cloned into high and low copy number cloning vectors, such that they were expressed from P_{lac} (Fig. 1). *E. coli* DH10B was used as a cloning host. In all cases, cosmid pES1044 or plasmid pMM58 were used as templates for the amplification reactions. PCR products were gel-purified, were digested with appropriate restriction enzymes, and then, were ligated into pBluescript KS(+) or pBluescript SK(+) (Stratagene, La Jolla, CA, U.S.A.). Clones containing recombinant plasmids were screened by colony PCR, using the LacF primer and the reverse primer used for the PCR cloning. Individual genes were subsequently subcloned into the low copy mobilizable vector pRK415 (Keen et al. 1988) such that they were transcribed from P_{lac} . Vectors and recombinant plasmids are listed in Table 2 and primers in Table 3.

hrpY was amplified using primers YFH and YRH. The 675-bp amplicon was cloned into pBluescript KS(+) to produce pMM46 and then subcloned into pRK415 to produce pMM52. *hrpL*, without its σ^{54} -dependent promoter, was amplified using primers LFB and LRE. The 613-bp PCR fragment was cloned into pBluescript SK(+) to produce pMM22 and then was subcloned into pRK415 to produce pMM6. The construction of P_{lac} -*hrpS* expression plasmids pRF205 (low copy) and pRF8 (high copy) and the *hrpXY* plasmid pRF201 was previously described (Frederick et al. 1993). Plasmid pMM58, containing the entire regulatory region, was constructed by ligating the 1.7-kb *Hind*III insert of pRF8 into pRF201SK digested with *Hind*III.

Construction

of chromosomal *Pantoea stewartii* *hrp* insertion mutants.

Construction of Tn5 and Tn3HoHoI mutants with insertions in *hrpX*, *hrpY*, *hrpS*, and *hrpL* has been previously described (Frederick et al. 2001). Double mutants containing selected

Table 2. Bacterial strains and plasmids.

Strain or plasmid	Relevant phenotypes and genotypes	Source or reference
<i>Escherichia coli</i>		
BW20339	F ¹ 128::Tn10-12(Kan ^r) <i>lacI</i> ^r Δ lacZ _{M15} <i>pro</i> (BA) ⁺ /DE3(<i>lac</i>) _{X74} <i>uidA</i> (Δ MluI):: <i>pir</i> ⁺ <i>recA1</i> Δ phoA532 Δ (<i>phnCADEFGHIJKLMNOP</i>)33-30	Metcalf et al. 1996
DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80d <i>lacZ</i> Δ M15 Δ lacX74 <i>endA1</i> <i>recA1</i> <i>deoR</i> Δ (<i>ara,leu</i>)7697 <i>araD</i> 139 <i>galU</i> <i>galK</i> <i>nupG</i> <i>rpsL</i> λ ⁻	Gibco BRL
HB101	F ⁻ <i>thi-1</i> <i>hsd20</i> (r ⁻ _B m ⁻ _B) <i>sup</i> <i>E44</i> <i>recA13</i> <i>ara-14</i> <i>leuB6</i> <i>proA2</i> <i>lacY1</i> <i>rpsL20</i> (Sm ^r) <i>xyl-5</i> <i>mtl-1</i>	Boyer and Roulland-Dussoix 1969
HB101Rif	spontaneous Rif ^r strain	Lab collection
S ϕ 200Rif	<i>metB</i> <i>strA</i> <i>purB</i> Δ (<i>agg-uidA-man</i>) Rif ^r	Wei et al. 2000
<i>P. stewartii</i> subsp. <i>stewartii</i>		
SS104	Wild type, Wts ⁺ , HR ⁺	ICPPB ^a
DC283	SS104 Nal ^r	Coplin et al. 1986
DM064	DC283 <i>hrpY1296</i> ::Tn5 (Kan ^r)	Frederick et al. 2001
DM701	DC356 Δ <i>hrpS</i> <i>hrpJ268</i> ::Tn3HoHoI (Amp ^r , Rif ^r)	This study
DM711	DC283 <i>hrpJ79</i> ::Tn3HoHoI (Amp ^r)	Frederick et al. 2001
DM729	DC283 <i>hrpY1296</i> ::Tn5 <i>hrpJ79</i> ::Tn3HoHo (Kan ^r , Amp ^r)	This study
DM733	DC283 <i>hrpY64</i> ::Tn5 <i>hrpS1</i> ::Tn3HoHoI (Kan ^r , Amp ^r)	This study
DM758	DC283 <i>hrpS224</i> ::Tn5 (Kan ^r)	Frederick et al. 2001
DM786	DC283 <i>hrpJ79</i> ::Tn3HoHoI <i>hrpL2560</i> :: <i>aphA-3</i> (Kan ^r , Amp ^r)	This study
DM2788	DC283 Δ <i>hrpX</i> ₍₁₇₋₄₇₀₎	This study
DM2790	DC283 Δ <i>hrpX</i> ₍₁₇₋₄₁₄₎	This study
DM2831	DC283 <i>hrpL-uidA</i> (Tet ^r)	This study
DM2837	DC283 <i>hrpY</i> <i>hrpL-uidA</i> (Kan ^r Tet ^r , Tp ^r)	This study
DM2844	DC283 <i>hrpS</i> <i>hrpL-uidA</i> (Kan ^r , Tet ^r , Tp ^r)	This study
MEX1	DC283 <i>hrpS1</i> ::Tn3HoHoI (Amp ^r)	Frederick et al. 2001
MEX105	DC283 <i>hrpL105</i> ::Tn3HoHo (Amp ^r)	Frederick et al. 2001
MEX116	DC283 <i>hrpX116</i> ::Tn3HoHoI (Amp ^r)	Frederick et al. 2001
MM211	DC283 <i>hrpY</i> (D57A)	This study
MM254	DC283 <i>hrpY</i> (D57N)	This study
Plasmids		
pBluescript		
KS and SK (+)	ColE1 <i>dlacZ</i> (Ap ^r)	Stratagene
pLAFR3	IncP <i>cos</i> <i>dlacZ</i> (Tc ^r)	Staskawicz et al. 1987
pRK415	IncP <i>dlacZ</i> (Tc ^r)	Keen et al. 1988
pVK100	IncP <i>cos</i> (Tc ^r)	Knauf and Nester 1982
pLD55	R6K <i>ori</i> , <i>dlacZ</i> , <i>tetAR</i> (Ap ^r)	Metcalf et al. 1996
pUC18K	promoter/terminator-less <i>aphA-3</i> cassette	Menard et al. 1993
pR751	IncP, (Tp ^r , Sp/Sm ^r)	Ruvkun and Ausubel 1981
pSU19	pACYC187 replicon (Cm ^r)	Bartolome et al. 1991
pPL6GUSC	pLAFR6 derivative carrying a promoterless <i>uidA</i> gene (Tc ^r)	Knoop et al. 1991
pRK2013::Tn7	ColE1 <i>mob</i> ⁺ <i>tra</i> _{KK2} Δ <i>rep</i> _{KK2} <i>repE</i> <i>kan</i> ::Tn7 (Tp ^r , Sm ^r , Sp ^r)	Ditta et al. 1980
pDM1296	pES1044 <i>hrpY</i> ::Tn5	Coplin et al. 1992
pDM2560	pRF201 <i>hrpL</i> :: <i>aphA-3</i>	This study
pDM2842	pBKS (+) with PCR-made <i>hrpX</i> allele deleted from aa 17 to 444	This study
pDM2846	pBKS (+) with PCR-made <i>hrpX</i> allele deleted from aa 17 to 470	This study
pDM2848	pLD55 with the 0.98-kb <i>SstI-XhoI</i> fragment from pDM2842	This study
pDM2851	pLD55 with the 1.1-kb <i>SstI-XhoI</i> fragment from pDM2846	This study
pES1044	cosmid clone in pVK100 containing left half of <i>hrp</i> cluster	Coplin et al. 1986
pES411	cosmid clone in pVK100 containing right half of <i>hrp</i> cluster	Coplin et al. 1992
pMM6	pRK415 with a 0.6-kb <i>BamHI-EcoRI</i> <i>hrpL</i> ⁺ PCR fragment, transcribed from P _{lac}	This study
pMM22	pBSK(+) with a 0.6-kb <i>BamHI-EcoRI</i> <i>hrpL</i> ⁺ PCR fragment, transcribed from P _{lac}	This study
pMM46	pBKS(+) with a 0.7-kb <i>HindIII</i> <i>hrpY</i> fragment made by PCR, transcribed from P _{lac}	This study
pMM52	pRK415 with the 0.7-kb <i>BamHI-EcoRI</i> <i>hrpY</i> ⁺ fragment from pMM46, transcribed from P _{lac}	This study
pMM57	pBKS(+) with a 0.7-kb <i>HindIII</i> <i>hrpY</i> (D57A) fragment made by PCR-SOE	This study
pMM58	pRF201SK with the 1.7-kb <i>HindIII</i> fragment from pRF8 and containing <i>hrpL</i> ⁺ , <i>hrpXY</i> ⁺ , <i>hrpS</i> ⁺	This study
pMM74	pRK415 with the 0.7-kb <i>HindIII</i> <i>hrpY</i> (D57A) fragment from pMM74, transcribed from P _{lac}	This study
pMM92	pBKS(+) with a 0.7-kb <i>HindIII</i> <i>hrpY</i> (D57N) fragment made by PCR-SOE	This study
pMM118	pRK415 with a 0.7-kb <i>HindIII</i> <i>hrpY</i> (D57N) fragment from pMM92, transcribed from P _{lac}	This study
pMM245	pLD55 with the 0.7-kb <i>SstI-XhoI</i> fragment from pMM57	This study
pMM246	pLD55 with the 0.7-kb <i>SstI-XhoI</i> fragment from pMM92	This study
pMM330	pSU19 with the 0.7-kb <i>Sall-SstI</i> <i>hrpY</i> ⁺ fragment from pMM46, transcribed from P _{lac}	This study
pRF201	pLAFR3 with the 3.6-kb <i>BamHI-HindIII</i> fragment containing <i>hrpL</i> ⁺ , <i>hrpX</i> ⁺ and <i>hrpY</i> ⁺ from pES1044	Coplin et al. 1992
pRF201SK	pBKS(+) with the 3.6-kb <i>BamHI-HindIII</i> fragment from pRF201	This study
pRF205	pVK100 with the 1.7-kb <i>HindIII</i> fragment containing <i>hrpS</i> from pES1044	Frederick et al. 1993
pRF8	pBKS(+) with the 1.7-kb <i>HindIII</i> fragment containing <i>hrpS</i> from pRF205	Frederick et al. 1993
pDM2531	pPL6GUSC with a 0.9-kb <i>BamHI</i> fragment to create a <i>hrpJ-uidA</i> fusion	This study
pDM2785	pPL6GUSC with a 389-bp <i>BamHI</i> PCR fragment to create a <i>hrpL-uidA</i> fusion	This study
pDM2791	pPL6GUSC with a 1.1-kb <i>HindIII-BamHI</i> fragment from pES411 to create a <i>wtSE-uidA</i> fusion	This study
pMM25	pPL6GUSC with a 244-bp <i>HindIII-BamHI</i> PCR fragment to create a <i>hrpX-uidA</i> fusion	This study
pMM50	pPL6GUSC with a 1.3-kb <i>BamHI</i> PCR fragment to create a <i>hrpS-uidA</i> fusion	This study

^a International Collection of Plant Pathogenic Bacteria.

null mutations and *lacZ* gene fusions were constructed by allele exchange of Tn5-mutagenized cosmids, as previously reported (Frederick et al. 2001), by using an incompatible plasmid, pR751 (Ruvkun and Ausubel 1981), to force the first cross-over event. Nonpolar *hrpL* mutations were constructed by ligating the *aphA-3* cassette from pUC18K (Menard et al. 1993) into *hrpL* in pRF201. The resulting plasmid, pDM2560, was used to exchange the *hrpL::aphA-3* allele into the *Pantoea stewartii* chromosome as described above. Several strategies to construct strains with usable chromosomal *hrpL-lacZ* transcriptional fusions were unsuccessful. Therefore, we created a strain with a single-copy chromosomal *hrpL-uidA* transcriptional fusion by a Campbell-type single crossover event. Plasmid pDM2785, which contains *hrpLp* cloned into pPL6GUSC, was forced to recombine with the *hrpL* locus on the chromosome, following introduction of an incompatible plasmid, pR751, to produce *hrpL-uidA hrpL⁺*, *Tet^r*, and *Tp^r* strains. This plasmid was recombined into wild-type (DC283), *hrpY* (DM064), and *hrpS* (DM758) backgrounds to produce strains DM2831, DM2837, and DM2844, respectively.

Site-directed mutagenesis of *hrpY* codon D57 by overlap extension PCR and construction of missense mutant strains.

To evaluate the role of the conserved aspartate residue at position 57 in HrpY, we performed site-directed mutagenesis by a modification of the megaprimer overlap extension PCR technique (Aiyar and Leis 1993). D57 was changed by nonconservative substitution to alanine (D57A) or by conservative substitution to asparagine (D57N). Five primers were designed: YFH and YRH delimited the *hrpY* ORF; YR2 was the antisense primer; and YD57N and YD57A were the sense-orientation mutagenic primers for each substitution. The mutagenic primers contained missense base changes at D57 (bold font in Table 3) and a near silent restriction site (*NheI* and *DraI* for YD57A and YD57N, respectively). PCRs were performed us-

ing pMM46 as template DNA. The first round of reactions used primer pairs YFH+YR2 and YRH+YD57A (or YD57N) to produce 350- and 500-bp (or 508-bp) overlapping amplicons. Gel-purified PCR products from the two reactions were mixed, annealed, and used as templates for a second round of PCR, using primers YFH and YRH. PCR products were gel-purified and cloned into pBluescript KS(+). Recombinant colonies were screened by PCR and by restriction enzyme analysis for the presence of the marker restriction site, and the inserts were sequenced. Plasmids pMM57 and pMM92 contain the *hrpY*(D57A) and *hrpY*(D57N) alleles, respectively. These inserts were then subcloned into pRK415 to produce plasmids pMM74 and pMM118, respectively. To construct missense *hrpY* mutants, the *hrpY*(D57A) and *hrpY*(D57N) ORFs were subcloned from pMM57 and pMM92 into suicide vector pLD55 (Metcalf et al. 1996) as *XhoI/SstI* inserts. The unmarked mutations were introduced into the *Pantoea stewartii* genome using the procedure described by Metcalf and associates (1996) and were confirmed by Southern blot hybridization.

Construction of *Pantoea stewartii* strains with in-frame deletions in *hrpX*.

Two unmarked in-frame deletions in *hrpX* were constructed in vitro by PCR. The deletions span amino acids 17 to 414 and 17 to 470, as indicated in the subscripts. To construct $\Delta hrpX_{(17-414)}$, DNA fragments corresponding to the ends of *hrpX* and its flanking sequences were amplified by PCR from cosmid pES1044, using primer pairs EdX1+BdX1 and BdX4+XdX3 (Table 3). $\Delta hrpX_{(17-470)}$ was similarly constructed using the same primers, except BdX3 was substituted for BdX4. PCR fragments were ligated into pBluescript KS(+) as *EcoRI-BamHI* and *BamHI-XbaI* contiguous fragments. The deleted alleles were then ligated into pLD55 as *XhoI/SstI* inserts, transformed into *E. coli* BW20339 (Metcalf et al. 1996), mobilized to *Pantoea stewartii*, and introduced into the chromosome as described above. Cosmid pDM1296 *hrpX⁺ hrpY::Tn5*, which

Table 3. Primers

Primer	Sequence (5' → 3') ^a	Sequence coordinates (5' → 3') ^b	Source
pBluescript and pPL6GUSC vectors			
LacF	ACCATGATTACGCCAAGCTC	813-794	This study
LacR	GCGGATTAAGTTGGGTAACG	577-558	This study
5GUS	CATTTACAGGGTTGGGGTTTCT	15-36	Jefferson 1987
<i>Pantoea stewartii</i> subsp. <i>stewartii</i>			
XFH	ACGGAAGCTTACTTCAAGGAGGCGAGATGC	1364-1383	This study
XRH	ACGGAAGCTTGTCTTCGCGCATGATGACG	2924-2905	This study
YFH	CCGAAGCTTTTCGATAACAATATGGATAACAC	2855-2877	This study
YRH	CCGAAGCTTATTAAGAGCAATCCTAAGAGA	3521-3501	This study
LFB	CGCGGATCCACATTAAGCCAAACGGCAAAA	662-642	This study
LRE	CCGGAATTCCTGTTTTACCCCGTTCA GTT	1255-1237	This study
201.3	CCTGGCGAACCTCATACAGG	411-430	This study
201.1	CGCTATCATCTCGCCCTGTG	1691-1672	This study
YD57A	TCCTTTACTGCTAGCTATGTGTATGCCTGG	3022-3051	This study
YD57N	CAGTCGATCTTTTACTTTTAAATATGTGTATGCCTGG	3014-3051	This study
YR2	GTTCAGGATCGTTATCTTTTCGTGATG	3204-3178	This study
EdX1	CGCGAATTCCTTCGGTATTGCCCTGAACCT	919-938	This study
BdX1	CGCGGATCCCTTTGATTGGCGTATCACTGCT	1427-1407	This study
BdX2	CGCGGATCCAACGAGGTTCTCATAGAGGT	2622-2641	This study
BdX3	CGCGGATCCACCAATGGAACCCGTGTGACT	2794-2813	This study
XdX3	CGCTCTAGATTTGCTCGGCGAGCATTTGG	3272-3253	This study
XFHG	CCCAAGCTTACGTTTCGCGTTTGTACACGGA	1182-1201	This study
XRBG	CGCGGATCCTGATTGGCGTATCACTGCTA	1425-1406	This study
SFBG	CGCGGATCCATAAAGCCCGCTGATGGAA	3423-3442	This study
SR3BG	CGCGGATCCTCTAGATGGCATGCTATCGATCTCGT	4735-4715	This study
JFBG	CGCGGATCCGAGCTCTTTCCCAGGCAACAGAGTTAA	481-501	This study
LRBG	CGCGGATCCTCTAGAAATGACTTCCAGCCAGGTCAT	869-850	This study

^a Underlined bases represent engineered restriction sites; mutant codons are shown in bold character.

^b Positions of LacF/R and 5GUS primers are according to GenBank accession X52331 (pBluescriptKS+) and U12639 (*uidA* locus), respectively; positions for *Pantoea stewartii* primers correspond to GenBank accession AF282857 and do not include the 5'-end restriction sites and anchoring tail.

expresses *hrpX* from its native promoter, restored HR elicitation to these mutants, thereby genetically confirming that these strains are, in fact, bona fide nonpolar *hrpX* mutants.

Construction of *hrp*-promoter reporter plasmids.

hrp promoter fragments containing the 5' regulatory regions of *hrpX*, *hrpS*, *hrpL*, *hrpJ*, and *wtSE* were either produced by PCR, using the primers described in Table 3, or isolated as restriction fragments from cosmids pES411 or pES1044. To amplify the *hrpL* promoter region from -207 to +182 (coordinates refer to the first base of the ORF), primers JFBG and LRBG were used (Table 3). An *hrpX* promoter fragment containing from -198 to +46 bp was produced with primers XFHG and XRBG. The *hrpS* promoter region from nucleotides -928 to +384 bp was amplified with primers SFG and SR3BG. To isolate two promoter regions containing *hrp* boxes, a 0.9-kb *Bam*HI insert containing *hrpJp* and a 1.1-kb *Hind*III-*Bam*HI fragment containing *wtSEp* were purified from plasmid pRF201::Tn5seq118 and cosmid pES411, respectively. The former plasmid has a Tn5seq insertion at +252 bp in *hrpL* (R. Frederick and D. Coplin, unpublished data). The DNA fragments, digested with *Bam*HI, *Hind*III, or both as required were ligated into the low-copy, promoterless, *uidA* fusion vector pL6GUSC (Knoop et al. 1991). *hrp-uidA* reporter plasmids are listed in Table 2.

Measurement of reporter gene activity.

GUS activity was assayed fluorometrically using 4-methylumbelliferyl- β -D-glucuronide as described by Jefferson (1987), but the assays were scaled down to fit microtiter plates and were analyzed using a Victor 1420-2 multilabel reader (PE-Applied Biosystems). Net GUS activity of each strain was corrected for the basal fluorescence of *Pantoea stewartii* DC283 or *E. coli* S Φ 200 carrying pL6GUSC without an insert. Specific activity was expressed in GUS units (pmol MU min⁻¹ OD₆₀₀⁻¹ ml⁻¹ of culture at 37°C). β -galactosidase (β -Gal) activity was measured fluorometrically as described by Miller (1992) with the following modifications to adapt the protocol to the multiplate reader. Aliquots of cultures (100 μ l) were mixed with 100 μ l of a 2 $\times$ β -Gal assay buffer (2.5 mM 4-methylumbelliferyl- β -D-galactopyranoside, 30% DMSO, and 200 mM NaCl in 200 mM potassium phosphate buffer, pH 7), and data was collected as described above. β -Gal activity units were pmol MU min⁻¹ OD₆₀₀ nm⁻¹ ml⁻¹ of culture at 25°C.

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LITERATURE CITED

Ahmad, M., Majerczak, D. R., Pike, S., Hoyos, M. E., Novacky, A., and Coplin, D. L. 2001. Biological activity of harpin produced by *Pantoea stewartii* subsp. *stewartii*. Mol. Plant-Microbe Interact. 14:1223-1234.

Aiyar, A., and Leis, J. 1993. Modification of the megaprimer method of PCR mutagenesis: Improved amplification of the final product. Biotechniques 14:366-369.

Aldon, D., Brito, B., Boucher, C., and Genin, S. 2000. A bacterial sensor of plant cell contact controls the transcriptional induction of *Ralstonia solanacearum* pathogenicity genes. EMBO (Eur. Mol. Biol. Organ.) J. 19:2304-2314.

Alfano, J. R., and Collmer, A. 1997. The type (III Hrp) secretion pathway of plant pathogenic bacteria: Trafficking harpins, Avr proteins, and

death. J. Bacteriol. 179:5655-5662.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. A., and et al. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds). 1987. Current Protocols in Molecular Biology, Vol. 1. New York: John Wiley & Sons.

Bartolome, B. Y., Jubete, Y., Martinez, E., and de la Cruz, F. 1991. Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. Gene 102:75-78.

Boyer, H. W., and Roulland-Dussoix, D. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 41:459-472.

Bruto, B., Aldon, D., Barberis, P., Boucher, C., and Genin, S. 2002. A signal transfer system through three compartments transduces the plant cell contact-dependent signal controlling *Ralstonia solanacearum* *hrp* genes. Mol. Plant-Microbe Interact. 15:109-119.

Chatterjee, A., Cui, Y., and Chatterjee, A. K. 2002. Regulation of *Erwinia carotovora* *hrpL_{ECC}* (sigma-L_{ECC}), which encodes an extracytoplasmic function subfamily of sigma factor required for expression of the Hrp regulon. Mol. Plant-Microbe Interact. 15:971-980.

Claflin, L. E. 1999. Stewart's bacterial wilt. Pages 3-4 in: *Compendium of Corn Diseases*. White, D. G. (ed). American Phytopathological Society Press, St. Paul, MN, U.S.A.

Coplin, D. L., and Majerczak, D. R. 1990. Extracellular polysaccharide genes in *Erwinia stewartii*: Directed mutagenesis and complementation analysis. Mol. Plant-Microbe Interact. 3:286-292.

Coplin, D. L., Frederick, R. D., Majerczak, D. R., and Haas, E. S. 1986. Molecular cloning of virulence genes from *Erwinia stewartii*. J. Bacteriol. 168:619-623.

Coplin, D. L., Frederick, R. D., Majerczak, D. R., and Tuttle, L. D. 1992. Characterization of a gene cluster that specifies pathogenicity in *Erwinia stewartii*. Mol. Plant-Microbe Interact. 5:81-88.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. U.S.A. 77:7347-7351.

Dolph, P. J., Majerczak, D. R., and Coplin, D. L. 1988. Characterization of a gene cluster for exopolysaccharide biosynthesis and virulence in *Erwinia stewartii*. J. Bacteriol. 170:865-871.

Finlay, B. B., and Falkow, S. 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. 61:136-169.

Frederick, R. D., Majerczak, D. R., and Coplin, D. L. 1993. *Erwinia stewartii* WtsA, a positive regulator of pathogenicity gene expression, is similar to *Pseudomonas syringae* pv. *phaseolicola* HrpS. Mol. Microbiol. 9:477-485.

Frederick, R. D., Ahmad, M., Majerczak, D. R., Arroyo-Rodriguez, A. S., Manulis, S., and Coplin, D. L. 2001. Genetic organization of the *Pantoea stewartii* subsp. *stewartii* *hrp* gene cluster and sequence analysis of the *hrpA*, *hrpC*, *hrpN*, and *wtSE* operons. Mol. Plant-Microbe Interact. 14:1213-1222.

Galan, J. E., and Collmer, A. 1999. Type III secretion machines: Bacterial devices for protein delivery into host cells. Science 284:1322-1328.

Goodrich, J. A., Schwartz, M. L., and McClure, W. R. 1990. Searching for and predicting the activity of sites for DNA binding proteins: Compilation and analysis of the binding sites for *Escherichia coli* integration host factor (IHF). Nucleic Acids Res. 18:4993-5000.

Hendrickson, E. L., Guevera, P., and Ausubel, F. M. 2000a. The alternative sigma factor RpoN is required for *hrp* activity in *Pseudomonas syringae* pv. *maculicola* and acts at the level of *hrpL* transcription. J. Bacteriol. 182:3508-3516.

Hendrickson, E. L., Guevera, P., Penaloza-Vazquez, A., Shao, J., Bender, C., and Ausubel, F. M. 2000b. Virulence of the phytopathogen *Pseudomonas syringae* pv. *maculicola* is *rpoN* dependent. J. Bacteriol. 182:3498-3507.

Hoch, J. A., and Silhavy, T. J. (eds.) 1995. Two-Component Signal Transduction. American Society for Microbiology, Washington, DC.

Hutcheson, S. W., Bretz, J., Sussan, T., Jin, S., and Pak, K. 2001. Enhancer-binding proteins HrpR and HrpS interact to regulate *hrp*-encoded type III protein secretion in *Pseudomonas syringae* strains. J. Bacteriol. 183:5589-5598.

Inoue, H., Nojima, H., and Okayama, H. 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene 96:23-28.

Jefferson, R. A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5:387-405.

Jin, S. G., Prusti, R. K., Roitsch, T., Ankenbauer, R. G., and Nester, E. W. 1990. Phosphorylation of the VirG protein of *Agrobacterium tumefaciens* by the autophosphorylated VirA protein: Essential role in biological activity of VirG. J. Bacteriol. 172:4945-4950.

- Kadner, R. J. 1995. Expression of the Uhp sugar phosphate transport system of *Escherichia coli*. Pages 263-274 in: Two-Component Signal Transduction. J. A. Hoch, and T. J. Silhavy, (eds.) American Society for Microbiology, Washington, DC.
- Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* 70:191-197.
- Klement, Z. 1982. Hypersensitivity. Pages 150-178 in: Phytopathogenic Prokaryotes. Vol. 1. M. S. Mount, and G. S. Lacy, eds. Academic Press, New York.
- Knauf, V. C., and Nester, E. W. 1982. Wide host range cloning vectors: A cosmid clone bank of an *Agrobacterium* Ti plasmid. *Plasmid* 8:45-54.
- Knoop, V., Staskawicz, B., and Bonas, U. 1991. Expression of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria* is not under the control of *hrp* genes and is independent of plant factors. *J. Bacteriol.* 173:7142-7150.
- Lindgren P. B. 1997. The role of *hrp* genes during plant-bacterial interactions. *Annu. Rev. Phytopathol.* 35:129-152.
- Lonetto, M. A., Brown, K. L., Rudd, K. E., and Buttner, M. J. 1994. Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase sigma factors involved in the regulation of extracytoplasmic functions. *Proc. Natl. Acad. Sci. U.S.A.* 91:7573-7577.
- Marchler-Bauer, A., Panchenko, A. R., Shoemaker, B. A., Thiessen, P. A., Geer, L. Y., and Bryant, S. H. 2002. CDD: A database of conserved domain alignments with links to domain three-dimensional structure. *Nucleic Acids Res.* 30:281-283.
- McCleary, W. R., Stock, J. B., and Ninfa, A. J. 1993. Is acetyl phosphate a global signal in *Escherichia coli*? *J. Bacteriol.* 175:2793-2798.
- Menard, R., Sansonetti, P. J., and Parsot, C. 1993. Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J. Bacteriol.* 175:5899-5906.
- Metcalfe, W. W., Jiang, W., Daniels, L. L., Kim, S. K., Haldimann, A., and Wanner, B. L. 1996. Conditionally replicative and conjugative plasmids carrying *lacZa* for cloning, mutagenesis, and allele replacement in bacteria. *Plasmid* 35:1-13.
- Miller, J. H. 1992. A short course in bacterial genetics: A laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Moore, J. B., Shiau, S. P., and Reitzer, L. J. 1993. Alterations of highly conserved residues in the regulatory domain of nitrogen regulator I (NtrC) of *Escherichia coli*. *J. Bacteriol.* 175:2692-2701.
- Mor, H., Manulis, S., Zuck, M., Nizan, R., Coplin, D. L., and Barash, I. 2001. Genetic organization of the *hrp* gene cluster and *dspAE/BF* operon in *Erwinia herbicola* pv. *gypsophila*. *Mol. Plant-Microbe Interact.* 14:431-436.
- Nizan-Koren, R., Manulis, S., Mor, H., Iraki, N.M., and Barash, I. 2003. The regulatory cascade that activates the Hrp regulon in *Erwinia herbicola* pv. *gypsophila*. *Mol. Plant-Microbe Interact* 16:249-260.
- Poetter, K., and Coplin, D. L. 1991. Structural and functional analysis of the *rcsA* gene from *Erwinia stewartii*. *Mol. Gen. Genet.* 229:155-160.
- Porter, S. C., North, A. K., and Kustu, S. 1995. Mechanism of transcriptional activation by NtrC. Pages 147-158 in: Two-Component Signal Transduction. J.A. Hoch and T. J. Silhavy, eds. American Society for Microbiology Press, Washington, D.C., U.S.A.
- Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. 1991. Genetic and transcriptional organization of the *hrp* cluster of *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* 173:575-586.
- Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. 1992. Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* 174:3499-3507.
- Reese, M. G., Harris, N. L., and Eckman, F. H. 1996. Large scale sequencing specific neural networks for promoter and splice site recognition. In: Biocomputing: Proceedings of the 1996 Pacific Symposium. L. Hunter and T. E. Klein, eds. World Scientific Publishing Co., Singapore.
- Ruvkun, G. B., and Ausubel, F. M. 1981. A general method for site-directed mutagenesis in prokaryotes. *Nature* 289:85-88.
- Schulte, R., and Bonas, U. 1992. Expression of the *Xanthomonas campestris* pv. *vesicatoria hrp* gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. *J. Bacteriol.* 174:815-823.
- Shattuck-Eidens, D. M., and Kadner, R. J. 1983. Molecular cloning of the *uhp* region and evidence for a positive activator for expression of the hexose phosphate transport system of *Escherichia coli*. *J. Bacteriol.* 155:1060-1070.
- Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *J. Bacteriol.* 169:5789-5794.
- Stephenson, K., and Hoch, J. A. 2001. PAS—A domain of phosphorelay sensor kinase A: A catalytic ATP-binding domain involved in the initiation of development in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U.S.A.* 98:15251-15256.
- Taylor, B. L., and Zhulin, I. B. 1999. PAS domains: Internal sensors of oxygen, redox potential, and light. *Microbiol. Mol. Biol. Review.* 63:479-506.
- Torres-Cabassa, A., Gottesman, S., Frederick, R. D., Dolph, P. J., and Coplin, D. L. 1987. Control of extracellular polysaccharide synthesis in *Erwinia stewartii* and *Escherichia coli* K-12: A common regulatory function. *J. Bacteriol.* 169:4525-4531.
- Wei, Z. M., and Beer, S. V. 1995. *hrpL* activates *Erwinia amylovora hrp* gene transcription and is a member of the ECF subfamily of sigma factors. *J. Bacteriol.* 177:6201-6210.
- Wei, Z. M., Sneath, B. J., and Beer, S. V. 1992. Expression of *Erwinia amylovora hrp* genes in response to environmental stimuli. *J. Bacteriol.* 174:1875-1882.
- Wei, Z., Kim, J. F., and Beer, S. V. 2000. Regulation of *hrp* genes and type III protein secretion in *Erwinia amylovora* by HrpX/HrpY, a novel two-component system, and HrpS. *Mol. Plant-Microbe Interact.* 13:1251-1262.
- Xiao, Y., and Hutcheson, S. W. 1994. A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. *J. Bacteriol.* 176:3089-3091.
- Xiao, Y., Lu, Y., Heu, S., and Hutcheson, S. W. 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. *J. Bacteriol.* 174:1734-1741.
- Xiao, Y., Heu, S., Yi, J., Lu, Y., and Hutcheson, S. W. 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. *J. Bacteriol.* 176:1025-1036.

AUTHOR-RECOMMENDED INTERNET RESOURCES

The National Center for Biotechnology Information: www.ncbi.nlm.nih.gov.
The Institute for Genomic Research website: www.tigr.org.
The Berkeley Drosophila Genome Project: www.fruitfly.org/seq_tools/promoter.html