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The Response Regulator HrpY of *Dickeya dadantii* 3937 Regulates Virulence Genes Not Linked to the *hrp* Cluster

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HrpX/Y is a putative two-component system (TCS) encoded within the type III secretion system (T3SS) gene cluster of *Dickeya dadantii*. A linear regulatory cascade initiated by HrpX/Y that leads to activation of the downstream T3SS genes via HrpS and HrpL was described previously. Therefore, in *D. dadantii*, HrpX/Y plays an important role in regulation of genes involved in bacteria–plant interactions and bacterial aggregation via the T3SS. HrpX/Y is the only TCS shared among the plant-pathogenic enterobacteria that is not also present in animal-associated enterobacteria. To date, the genes known to be regulated by HrpY are restricted to the *hrp* and *hrc* genes and no signal has been identified that triggers HrpY-dependent gene expression. We demonstrated that HrpY interacts with the *hrpS* promoter in vitro. We then used a transposon-based system to isolate previously unidentified HrpY-dependent genes, including genes previously shown to affect virulence, including *kdgM* and *acsC*. HrpY is a dual regulator, positively regulating at least 10 genes in addition to those in the *hrp* gene cluster and negatively regulating at least 5 genes. The regulatory effect on one gene depended on the culture medium used. Of the 16 HrpY-regulated genes identified in this screen, 14 are not present in *Pectobacterium atrosepticum*, the nearest relative of *D. dadantii* with a sequenced genome. None of the newly identified HrpY-regulated genes were required for bacterial aggregation; thus, neither acyl-homoserine lactone-mediated quorum sensing nor the Rcs signal transduction system which regulates colanic acid, a molecule that plays an important role in biofilm formation in other enterobacteria, are required for *D. dadantii* aggregation.

Additional keywords: AHL, arabinose, *Erwinia chrysanthemi,* siderophore.

Bacterial two-component systems (TCS) have been implicated in regulating diverse cellular processes, including cell division, sporulation, chemotaxis, secondary metabolism, and virulence, in both plant and animal bacterial pathogens (Stock et al. 2000). In general, TCS are composed of a transmembrane sensor protein, which perceives environmental stimuli with its N-terminal input domain and then alters the phosphorylation state of the cognate cytoplasmic response regulatory receiver domain. The response regulator has different affinities for its target promoters depending upon its phosphorylation state, and will activate or repress expression of target operons (West and Stock 2001).

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The soft rot pathogen *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) is an opportunistic bacterial plant pathogen that infects a wide range of host species (Ma et al. 2007). A fair number of virulence factors have been identified in *D. dadantii* 3937 and *Dickeya* sp. EC16. The most studied virulence factors are the plant cell-wall-degrading enzymes, which are secreted through the type II secretion (Out) system (Barras et al. 1994; Perombelon 2002; Toth et al. 2003). Many other factors involved in *Dickeya*–plant interactions have been described, including siderophores (Expert 1999), a host antimicrobial peptide detoxifying system (Lopez-Solanilla et al. 1998, 2001), a hemagglutinen adhesin (Rojas et al. 2002), an osmoprotectant (Gloux et al. 2005), and scavengers for reactive oxygen species (Boccara et al. 2005; Hassouni et al. 1999; Reverchon et al. 2002).

The Hrp type III secretion system (T3SS) also is important for virulence in *D. dadantii* 3937 (Yang et al. 2002) and in related enterobacterial plant pathogens, including the soft rot pathogen *Pectobacterium* and the wilt pathogens *Pantoea stewartii* and *E. amylovora* (Grant et al. 2006). In addition to playing a role in plant–microbe interactions, the *D. dadantii* T3SS also is required for formation of bacterial aggregates at the air-liquid interface (Yap et al. 2005, 2006). The regulatory cascade controlling T3SSs (Chatterjee et al. 2003; Feng et al. 2003; Merighi et al. 2003; Nizan-Koren et al. 2003; Wei et al. 2000) and the genetically related flagella secretion systems (Wosten et al. 2004) in many animal and plant pathogens have been shown to include complete or partial TCS signal transduction pairs. This suggests a common strategy for linking the expression of virulence factors to signals indicating the presence of a host.

HrpX/Y is a TCS encoded within the *hrp* cluster, and this TCS is required for activation of *hrp* gene expression in plant-pathogenic enterobacteria, with HrpX acting as a transmembrane sensor protein and HrpY as a response regulator (Merighi et al. 2006; Wei et al. 2000; Yap et al. 2005). HrpY in *E. herbicola* (Nizan-Koren et al. 2003), *P. stewartii* (formerly *E. stewartii*) (Merighi et al. 2003), and *D. dadantii* 3937 (Yap et al. 2005) has been demonstrated to initiate the sequential activation of *hrpS*, which encodes a σ^{54}-enhancer binding protein, and *hrpL*, which encodes a σ factor, subsequently upregulating the T3SS genes encoding structural and secreted proteins. The *hrpS*, *hrpXY*, and *hrpL* operons are clustered and oriented in the same direction in these species. In *P. stewartii*,

the *hrp* regulatory genes participate in a novel regulatory loop that upregulates itself by readthrough transcription of *hrpL* into *hrpXY*. The same phenomenon is likely to occur in related enterobacterial plant pathogens since the gene order of these regulatory genes is conserved (Merighi et al. 2005).

A variety of signals, including osmolarity, nitrogen, nicotinic acid, pH, and temperature changes, have been suggested to be signals perceived by HrpX/Y in *E. amylovora* and *Pantoea stewartii* (Merighi et al. 2003; Wei et al. 2000). The environmental signal sensed by HrpX/Y in *D. dadantii* 3937 that results in *hrp* gene expression is unknown. Until recently, the T3SS genes were the only members of the HrpY regulon identified in plant-associated enterobacteria, although non-T3SS genes have been shown to be regulated by HrpL in *Pseudomonas syringae* (Ferreira et al. 2006). Recently, a 10-bp direct repeat important for *P. stewartii* HrpY regulation of *hrpS* was identified and an integration host factor (IHF) consensus sequence in the *hrpS* promoter also was shown to be important for *hrpS* expression (Merighi et al. 2006). The region upstream of *P. stewartii hrpS* has a 483-bp insertion element (IS) remnant that is not present upstream of *hrpS* in other plant-pathogenic enterobacteria for which sequence is available, including *D. dadantii*, *Pectobacterium atrosepticum*, *Pectobacterium carotovorum*, and *E. amylovora*. How this IS sequence affects regulation of the *P. stewartii hrpS* compared with related species is unknown.

An increasing number of studies have shown that crosstalk among multiple bacterial TCS is more common than previously thought and that a single TCS may exhibit dual functions as both a repressor and an activator (Kobayashi et al. 2001; Oshima et al. 2002; Stock et al. 2000; Worley 2000). The goals of this study were to confirm that the *D. dadantii* HrpY protein can directly interact with the *hrpS* promoter region and to determine whether HrpY regulates non-T3SS genes.

RESULTS

HrpX/Y is the only TCS that is conserved among plant-infecting enterobacteria but lacking in animal-infecting enterobacteria.

An examination of the *Pectobacterium atrosepticum* and *D. dadantii* genomes in the ASAP database (Glasner et al. 2003, 2006) reveals that most of the TCS present in the soft rot enterobacteria also are present in animal-infecting bacteria (Table 1). In total, *Pectobacterium atrosepticum* and *D. dadantii* share 21 TCS with enterobacterial animal pathogens and with each other. Both *Pectobacterium atrospeticum* and *D. dadantii* share an additional two TCS with related animal pathogens but not with each other. Only one TCS is present in *Pectobacterium atrosepticum* but not in *D. dadantii* or closely related animal pathogens. *D. dadantii* has six TCS that are not known to be present in other enterobacteria. The only TCS present in both *Dickeya* and *Pectobacterium* as well as *Erwinia* and *Pantoea* spp. but lacking from enterobacterial animal pathogens is HrpX/Y, which regulates of the T3SS. Therefore, we hypothe-

Table 1. Two component systems (TCS) in *Pectobacterium atrosepticum* SCRI1043 and *Dickeya dadantii* 3937

TCS genes	SCRI 1043[a]	*D. dadanti* 3937[b]
Present in enterobacteria[c]		
arcB/arcA	ECA0314/ECA3893	ABF-0015714 and ABF15715/ABF-0015159[d]
baeS/baeR	ECA3188/ECA3189	ABF-0018812/ABF-0018811
cheA/cheZ/cheY	ECA1689/ECA1695/ECA1694	ABF-0018757/ABF-0018749/ABF-0018750
citA/citB	ECA2577/ECA2578	ABF-0019047/ABF-0019048
cpxA/cpxR	ECA4312/ECA4311	ABF-0019169/ABF-0019170
cusS/copR	Not present	ABF-0018030/ABF-0018032
dcuS/dcuR	ECA4399/ECA4398	ABF-0018408/ABF-0018409
envZ/ompR	ECA4107/ECA4108	ABF-0015086/ABF-0015085
expS/expA	ECA3571/ECA2882	ABF-0014858/ABF-0020512
gnlG/glnL	ECA0027/ECA0028	ABF-0016307/ABF-0016308
kdpD/kdpE	ECA1339/ECA1338	ABF-0016971/ABF-0016972
narQ/narP	ECA1900/ECA1901	Not present
narX/narL	ECA2029/ECA2028	ABF-0020264/ABF-0020263
phoQ/phoP	ECA2446/ECA2445	ABF-0017765/ABF-0017764
phoR/phoB	ECA1111/ECA1110	ABL-0061356/ABF-0017543
pmrB/pmrA	ECA4045/ECA4044	ABF-0015460/ABF-0015459
qseC/qseB	ECA0008/ECA0009	Not present
rcsC/rcsB/rcsD	ECA1201/ECA1203/ ECA1204	ABF-0017295/ABF-0017296/ABF-0017297
rstB/rstA	ECA2012/ECA2013	ABF-0014749/ABF-0014748
tctE/tctD	Not present	ABF-0017792/ABF-0017793
uhpB/uhpA	ECA1496/ECA1497	ABF-0019927/ABF-0019928
yehU/yehT	ECA4152/ECA3353	ABF-0019369/ABF-0019367
yfhK/yfhA	ECA3257/ECA3255	ABF-0019656/ABF-0019654
ypdA/ypdB	ECA2432/ECA2431	ABF-0020311/ABF-0020312
Unnamed	ECA0890/ECA0889	ABF-0016779/ABF-0016778
Shared[e]		
hrpX/hrpY	ECA2088/ECA2089	ABF-0019600/ABF-0019598
Genus-specific		
…	ECA0786/ECA0785	Not present
…	Not present	ABF-0016854/ABF-0016853
…	Not present	ABF-0019562/ABF-0046568/ ABF-0014657
…	Not present	ABF-0015830/ ABF-0015831
…	Not present	ABF-0015809/ ABF-0015810
…	Not present	ABF-0016068/ ABF-0016069
…	Not present	ABF-0014915/ ABF-0014914

[a] *Pectobacterium atrosepticum* SCRI 1043 gene numbers for the TCS are listed.
[b] *D. dadantii* ASAP ID gene numbers for the TCS are listed.
[c] TCS genes present in related animal-associated enterobacteria.
[d] *arcB* is a pseudogene in *D. dadantii* 3937.
[e] TCS genes shared by *D. dadantii* and *Pectobacterium atrosepticum*.

sized that the HrpX/Y TCS is important for responding to an environment shared by plant pathogens (namely, the plant host) and that it may regulate genes important for plant–microbe interactions in addition to those in the T3SS.

HrpY binds the *hrpS* promoter and phosphorylation is crucial for HrpY activity.

Genetic analysis of *D. dadantii* and other related species suggested that HrpY interacts directly with the *hrpS* promoter (Merighi et al. 2003; Yap et al. 2005). His_6-HrpY was overexpressed and purified and the purified His_6-HrpY was phosphorylated by incubation with the phosphodonor acetyl phosphate and used in an electrophoretic mobility shift assay (EMSA) with a 500-bp [α-^{32}P] end-labeled *hrpS* promoter probe in the presence of an excess of nonspecific carrier DNA. HrpY formed two DNA-protein complexes with the *hrpS* promoter (Fig. 1). Two different unlabeled fragments were tested for their ability to compete with the labeled *hrpS* promoter region for HrpY binding in order to test the specificity of HrpY-*hrpS* promoter binding. The unlabeled specific competitor DNA, which was the exact fragment of the *hrpS* promoter probe, prevented the formation of the *hrpS*/HrpY complex (Fig 1B, panel b). A nonspecific competitor derived from the coding region of *hrpS* was not able to titrate the HrpY protein (Fig 1B, panel c), suggesting that binding of HrpY to the *hrpS* promoter is specific. The presence of two DNA-protein species instead of a single DNA-protein complex (Fig 1B, panel a) suggests that there were either two binding sites with different affinities to HrpY binding or a secondary *hrpS*/HrpY complex as a result of HrpY oligomerization. However, we ruled out these possibilities because the upper DNA-protein complex did not appear in the nonspecific competition assay (Fig 1B, panel c), suggesting that the larger complex was, indeed, a result of nonspecific binding. We also found that in vitro phosphorylation is required for HrpY activity because the HrpY protein without acetyl phosphate treatment was unable to bind the *hrpS* promoter DNA fragment (Fig. 1B, panel d).

Recently, the *P. stewartii* HrpY binding site in the *hrpS* promoter was identified as a 10-bp direct repeat AAATCCTTAC-N_{11}-AATTCCTTAC (consensus: AAWCCTTAC) (Merighi et al. 2006). The *P. stewartii* 10-bp sequence is not found as a direct repeat in the *D. dadantii* genome. A similar sequence is present upstream of the *D. dadantii hrpS*, ATTCCTAC-N_{13}-ATTCCTAC. Neither of these sequences is found in *Pectobacterium atrosepticum* upstream of *hrpS*, although a similar repeat is found (CGTTTTCCTG-N_{12}-CGTTTTCCTG). In addition, a putative IHF binding site, CGTTAAGAAATTG, is upstream of the *D. dadantii hrpS*, as in *P. stewartii*. Three other sets of direct repeats were identified in the *D. dadantii hrpS* promoter region—TTGTTAA-N_{17}-TTGTTAA, GGAGCC-N_3-GGAGCC, and CGGGAT-N_{11}-CGGGAT—suggesting that additional regulators bind to this promoter. A putative HrpY-binding site (ATTCCTAC-N_{10}-ATTCCTAC) also is present 178 nucleotides upstream of a *lysR* homolog (ASAP ID ABF-0019114). Merighi and associates (2006) found that just one of the 10-bp direct repeats was sufficient for induction by HrpY to approximately 20% of wild-type levels; therefore, we also searched the *D. dadantii* 3937 genome for single copies of the sequence ATTCCTAC in predicted promoter regions. Three genes, including a catalase homolog (ABF-0015800), a conserved hypothetical protein *yabI* (ABF-0016691), and the oligogalacturonide transporter *kdgM* (ABF-0019629), have putative HrpY binding sites upstream. Because the roles individual nucleotides play in binding to HrpY is unknown, there may be additional HrpY-binding sites in the *Dickeya* genome.

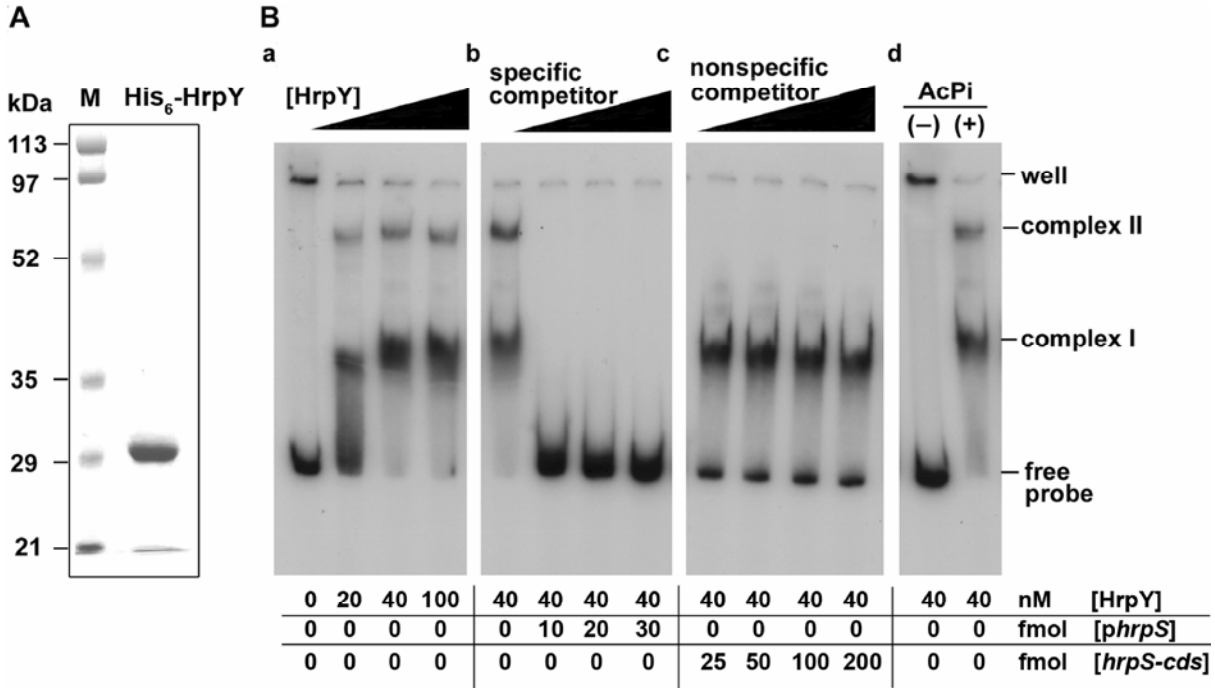

Fig. 1. Purification of His_6-HrpY and electrophoretic mobility shift assay (EMSA) **A,** Sodium dodecyl sulfate polyacrylamide gel elctrophoresis analysis of purified His_6-HrpY after Ni^{2+}-NTA chromatography (Qiagen). The low-range protein standard (BioRad) is denoted as M. **B,** EMSA showing binding of HrpY to the 500-bp *hrpS* promoter region relative to the translation start site, with an increasing HrpY concentration (0, 20, 50, and 100 nM) in the presence of 25 fmol of radiolabeled probe (panel a). In the reactions containing 25 fmol of radiolabeled probe and 40 nM HrpY, competition assays were performed with 10 to 30 fmol of unlabeled specific competitor DNA of the same aforementioned 500-bp *hrpS* promoter fragment (panel b), and 25 to 200 fmol of an unlabeled nonspecific competitor DNA derived from a 250-bp fragment of the *hrpS* coding region (panel c). The requirement for HrpY phosphorylation for DNA-binding was determined by comparison of the DNA-binding affinity of HrpY with and without acetyl phosphate (AcPi) treatment (panel d). Concentrations of HrpY, p*hrpS* DNA, and competitor *hrpS*-cds DNA are shown at the bottom of this figure.

Identification of HrpY-regulated genes.

The strategy for identifying HrpY-regulated genes is depicted in Figure 2A. We used plasmid pCAM140 to construct a mutant library of 8,500 Tn5SS*gusA* insertions in WPP247, which is a *D. dadantii* 3937 derivative with the wild-type *hrpY* deleted and an *hrpY* gene under the control of a tightly regulated arabinose promoter inserted between *lacY* and *prt*. Wild-type 3937 forms pellicles within 3 days when grown in SOBG, whereas an *hrpY* mutant does not (Yap et al. 2005). WPP247 did not form pellicles in SOBG plus 0.2% glucose but did form pellicles when grown in SOBG plus 0.2% arabinose (Fig. 2B).

When WPP247 was allowed to grow for longer periods in SOBG lacking arabinose, pellicles did eventually form. To determine whether this was likely to be due to a low level of expression of *hrpY* from the arabinose-inducible promoter in WPP247, we incubated SOBG cultures of WPP92, WPP96, WPP98, and WPP100, which are *hrpY*, *hrpL*, *hrcJ*, and *hrpA* mutants, respectively (Yap et al. 2005), for up to 1 month at 24°C. The *hrpY* and *hrpL* mutants formed pellicles after at least 3 weeks of incubation, whereas the *hrcJ* and *hrpA* mutants did not. This suggests that a low level of T3SS gene expression, sufficient to allow pellicle formation, occurs in the absence of the known regulatory genes and is consistent with delayed pellicle formation by WPP247 in the absence of arabinose.

Growth of WPP247 in the presence of arabinose resulted in expression of HrpY, whereas growth in its absence or supplementation of the catabolic repressor glucose does not (Fig. 2C). We never were able to detect HrpY protein in wild-type *D. dadantii* 3937, perhaps because only a small amount of HrpY is produced by wild-type cells. Others also have been unable to detect response regulators with immunoassays (Martínez et al. 2005; O'Connor and Nodwell 2005), suggesting that response regulators may be present only at very low levels even under inducing conditions.

WPP247 was mutagenized with *Tn5SSgusA*, generating *gusA* promoter fusions throughout the genome. Of the 8,500 mutants, 20 were chosen randomly for Southern blot analysis and all of the mutants exhibited different fingerprint patterns after digestion with *Pst*I (not shown), demonstrating that our library contained strains with independent mutations. The *gusA* gene in 29 mutants was determined to be differentially regulated on Luria-Bertani (LB) medium plus arabinose compared with LB alone, and these strains were characterized further. LB medium supplemented with glucose was not used to avoid the catabolic repression caused by glucose.

Chromosomal DNA flanking potential HrpY-regulated fusions was obtained and characterized as described in Materials and Methods. Of 29 transposon insertions, 18 were in loci with known or predicted functions and the remaining 11 were inserted into loci with unknown functions (Table 2). Three independent insertions were found in one gene, an O-antigen transporter homolog. Surprisingly, all 29 transposon insertions were outside of the *hrp* cluster. Quantitative β-glucuronidase (GUS) enzyme assays were performed to confirm the initial mutant phenotypes; however, we were unable to obtain consistent quantitative differences using a fluorescence activity assay even though colony phenotypes clearly showed an effect from expression of *hrpY* (Fig. 3).

D. dadantii 3937 encodes *araC*, which is a transcriptional regulator that controls gene expression in response to arabinose; therefore, some of the genes identified could be arabi-

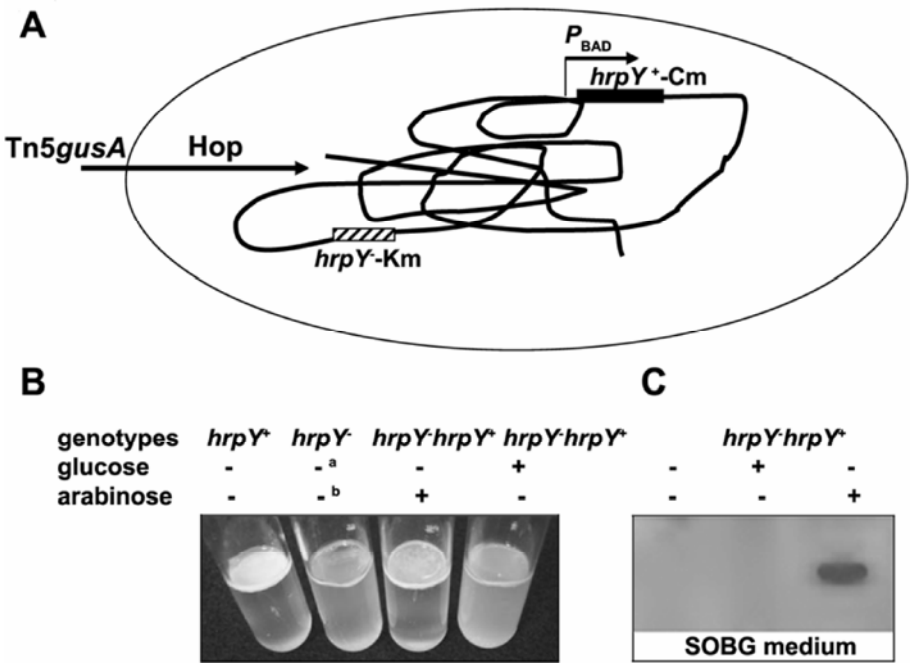

Fig. 2. Genetic screening of HrpY-regulated genes. **A,** Strategy for identifying HrpY-regulated genes. A copy of *hrpY* under the control of an arabinose-inducible promoter was introduced into a chromosomal neutral locus *lacY-prt* of an *hrpY* mutant by homologous recombination, generating the strain WPP247. Growth of WPP247 in the presence of arabinose results in ectopic HrpY expression and complementation of pellicle defective phenotype of *hrpY*⁻, whereas growing this strain either with glucose or in the absence of arabinose results in no HrpY being produced or restoration of the wild-type pellicle formation. Tn5*SSgusA* transcriptional fusions were generated throughout the genome of WPP247. In all, 8,500 independently isolated clones carrying *gusA* promoter fusions were patched in grids onto plates containing Luria-Bertani medium with and without arabinose. The plates were supplemented with chloramphenicol, kanamycin, spectinomycin, and with the chromogenic β-glucuronidase indicator 5-bromo-4-chloro-3-indolyl glucuronic acid, to allow for observation of differential reporter gene expression. **B,** *hrpY* is tightly regulated under the control of an arabinose-inducible promoter. A chromosomal copy of *hrpY* restored the pellicle formation to the *hrpY* mutant only when arabinose was provided. Pellicle assay was performed in static SOBG medium (Yap et al. 2005) at room temperature for 3 days. **C,** Western blot analysis of HrpY production in WPP247. HrpY is overexpressed in the pellicle-inducing SOBG only in the presence of arabinose. Superscripts a and b: pellicle formation did not occur when glucose and arabinose were added in the SOBG culture of *hrpY* mutant (strain WPP92); +A, plus arabinose; –A, minus arabinose; and –, no supplements.

nose regulated rather than HrpY regulated. In all, 27 of the 29 transposon mutants were transformed with pQE31::*hrpY* or with pQE31, which was used as a vector control. We chose to examine only two of the three O-antigen mutants (WPP255 and WPP259) and were unable to transform WPP266. The transformants were tested to determine whether *gusA* gene expression was differentially regulated in transformants with pQE31::*hrpY* compared with the vector control. The transformants were examined on three media, LB agar, LB plus 0.5% glucose agar, and pectate-enrichment medium (PEM). LB was chosen because it was used in the initial mutant screen and LB plus glucose was chosen in an attempt to completely suppress the chromosomal copy of HrpY in WPP247. PEM, a pectate-containing medium, was chosen because pectate, which is a principle component of plant cell walls, is known to induce *D. dadantii* virulence genes. Of the 26 mutants tested, 16 were differentially regulated by the plasmid-borne *hrpY* while the other 10 were not and, presumably, are arabinose regulated (Fig. 3).

The HrpY-regulated genes can be categorized into overlapping functional groups (Fig. 3).

Group 1, metabolic functions. HrpY downregulated diacylglycerol kinase (*dgkA*), which is involved in phospholipid biosynthesis.

Table 2. Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant characteristics[a]	Source
Escherichia coli		
DH5α	*supE44 ΔlacU169* (*Δ80lacZΔM15*) *hsdR17 recA1EndA1 gyrA96 thi-1 relA1*	Clontech
BL21(DE3)	F^-,*ompT, hsdS*$_B$(r_B-m_B-), *dcm, gal, tonA*	Novagen
S17-1/λpir	*thi pro hsdR hsdM*$^+$ *recA* RP4 2-Tc::Mu-Km::Tn7Sm^r λpir	Wilson et al. 1995
Dickeya dadantii		
3937	Wild-type, *Saintpaulia* (African violet) isolate	Lemattre and Narcy 1972
Nx3937	3937 Nx^r derivative	This work
WPP92	Km^r; *hrpY*::Km	Yap et al. 2005
WPP102	Sp^r/Sm^r ; Δ*hrpS*:: ΩSp/Sm	Yap et al. 2005
WPP96	Sp^r/Sm^r; Δ*hrpL*::ΩSp/Sm	Yap et al. 2005
WPP247	Km^{r}Cm^r; *hrpY*::Km; *lacY*:P_{BAD}-*hrpY*$^+$-Cm:*prt*	This work
WPP254-282	Km^r Cm^r Sp^r/Sm^r; *Tn5SSgusA* insertional mutations in the *hrpY*::Km; *lacY*:P_{BAD}-*hrpY*$^+$-Cm:*prt* background	This work
Tn5SSgusA mutants		
WPP254	Histidine kinase, *rcsD*, +1495, ID17297	This work
WPP255	O-antigen transporter,+1833, ID19483	This work
WPP256	Succinate dehydrogenase, *sdhC*, –37, ID16957	This work
WPP257	Unknown, +171, ID20186	This work
WPP258	Unknown, +545, ID19724	This work
WPP259	O-antigen transporter,+143, ID19483	This work
WPP260	LysR homolog, +465, ID16499	This work
WPP261	Diacyglycerol kinase, *dgkA*, –37, ID18675	This work
WPP262	Unknown, +218, ID20188	This work
WPP263	N-acyl-homoserine lactone synthase, *expI*, +209, ID19415	This work
WPP264	Unknown, +145, ID17231	This work
WPP265	ABC transporter, *untM*, +1690, ID16070	This work
WPP266	Nicotinic acid phosphoribosyl transferase (*pcnB*), +445, ID19864	This work
WPP267	Achromobactin synthetase, +564, ID18859	This work
WPP268	Unknown, +37, ID19175	This work
WPP269	Unknown, +1297, ID19186	This work
WPP270	Xylose isomerase, *xylA*, +249, ID16103	This work
WPP271	ATP-binding protein of glutamate/aspartate transport system, +253, ID18323	This work
WPP272	LysR homolog, +1495, ID16817	This work
WPP273	Unknown, +592, ID19347	This work
WPP274	Unknown, +33, ID46578	This work
WPP275	Maltodextrin phosphorylase, *malP*, +1497, ID15074	This work
WPP276	Unknown, +337, ID15544	This work
WPP277	Chitinase, +1246, ID19302	This work
WPP278	Unknown, +1445, ID17229	This work
WPP279	O-antigen transporter,+676, ID19483	This work
WPP280	Oligogalacturonate porin, *kdgM*, +406, ID19629	This work
WPP281	Unknown, +875, ID20803	This work
WPP282	Soluble lytic murein transglycosylase, *slt*, +1496, ID15166	This work
Plasmids		
pGEMT-Easy	Ap^r; *lacZ*',cloning vector	Promega Corp.
pBluescript SK(±)	Ap^r, *lacZ*', cloning vector	Stratagene
pJN105	Gm^r; arabinose inducible expression vector	Newman and Fuqua 1999
p105Y	Gm^r; 0.6-kb *hrpY* without its native promoter cloned into *Spe*I and *Sac*I sites of pJN105.	This work
pCAM140	Sm^r/Sp^r, Ap^r; mTn5SSgusA40 (promoterless *gusA* for transcriptional fusions) in pUT/mini-Tn5 Sm/Sp	Wilson et al. 1995
pQE31	Ap^r; 6×His-tag overexpression vector	Qiagen
pQhrpY	Ap^r; 0.6-kb *hrpY* cloned into *Bam*HI and *Hin*dIII sites, overexpression plasmid of N-terminal 6×His-tagged HrpY	This work
pTCLSCm	Ap^r, 2.8-kb *lacY-prt* region on pGEMT-Easy with 1-kb Cm resistance cassette cloned into *Bgl*II of the *lacY-prt* integenic space	This work
pCLSP$_{BAD}$YCm	Ap^r,Cm^r P_{BAD}-*hrpY*$^+$-Cm allele integrated to the *lacY-prt* intergenic space on the pGEMT-Easy	This work

[a] Ap = ampicillin; Cm = chloramphenicol; Gm = gentamicin; Km = kanamycin; Nx = nalidixic acid; Sm = streptomycin; Sp = spectinomycin; Tc = tetracycline; and ^{r} = resistance. For the *Tn5SSgusA* mutants, mutated genes, insertion sites, and ASAP ID numbers for each mutant are presented.

Group 2, regulation. HrpY upregulated a putative transcriptional factor belonging to the LysR family.

Group 3, acquisition and transport. HrpY suppressed four genes involved in acquisition and transport, including homologs of an O-antigen transporter, a glutamate/aspartate transporter, an oligogalacturonate porin required for galacturonate uptake (*kdgM*) (Blot et al. 2002), and an achromobactin siderophore synthesis gene (*acsC*) (Franza et al. 2005). The effects of HrpY on *kdgM* and *acsC* were most apparent when cells were grown on pectate enrichment medium. Conversely, HrpY upregulated an ABC-transporter permease (*untM*) and the effect of HrpY on this gene was not apparent on pectate enrichment medium.

Group 4, virulence-related. Two of the genes identified in this screen, *kdgM* (Blot et al. 2002) and *acsC* (Franza et al. 2005), are required for full virulence of *D. dadantii*. In addition, based on results from other species, the O-antigen transporter is likely to contribute to *Dickeya* spp.–host interactions, through both aiding in attachment to plant cells (Barak et al. 2007) and affecting other surface structures, including the T3SS (Augustin et al. 2007; West et al. 2005).

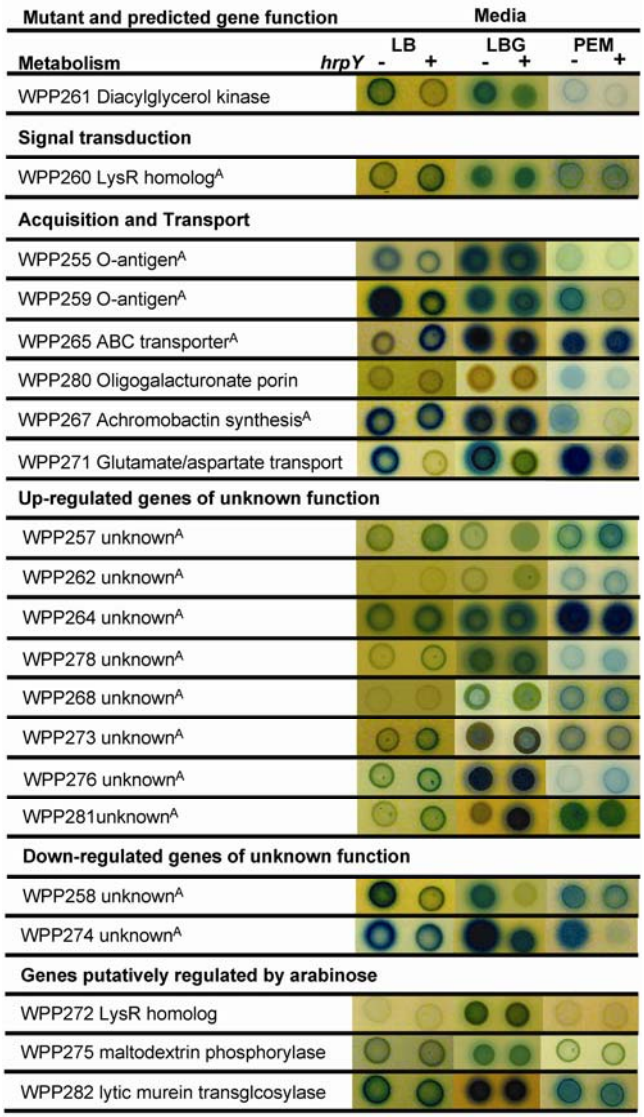

Fig. 3. Regulatory effects of HrpY on the β-glucuronidase reporter gene in *Tn5SSgusA* mutants generated from WPP247. All mutants were transformed with either a vector control, pQE31, or a plasmid expressing *hrpY*, pQE31::*hrpY*. Bacteria from liquid cultures were spotted onto Luria-Bertani (LB), LB plus 0.5% glucose (LBG), or pectate-enrichment medium (PEM). All of the media were supplemented with ampicillin, isopropyl-β-D-thiogalactoside, and 5-bromo-4-chloro-3-indolyl glucuronic acid. The colonies were grown overnight at 24°C, then photographed. The mutants are divided into functional categories described in the text. Three of the 10 mutants recovered in our initial screen that appear to be arabinose regulated rather than HrpY regulated are also shown. Superscript A: genes not present in the closely related soft rot pathogen *Pectobacterium atrosepticum*.

It is notable that, of the 16 HrpY-regulated genes, only 2 are also present in the closely related soft rot pathogen *Pectobacterium atrosepticum*; thus, of the genes regulated by HrpY, a high proportion are likely to contribute to biological differences between *D. dadantii* and closely related genera. It also is notable that, even though not all 16 genes are highly expressed on LB or LB plus glucose, they all are expressed on PEM, a pectate-containing medium. Of the 10 genes of unknown function, there are two sets that are clustered. ASAP ID 17229 (WPP264) and 17231 (WPP278) are the first and third genes in a four gene-operon. None of the genes in this operon have a known function. ASAP ID 20186 (WPP257) and 20188 (WPP262) are also adjacent to each other, although they may not be in an operon. Only one of the genes, ASAP ID 46578 (WPP274) is relatively near the T3SS gene cluster, located 38 kb from *hrcU*, which a core component of the T3SS encoded on the edge of the T3SS. The rest of the HrpY-regulated genes are scattered throughout the chromosome, at least 120 kb from the nearest neighboring gene identified in our screen.

These *hrpY*-regulated genes could be directly regulated by HrpY or by other regulators induced by HrpY, such as HrpS or HrpL, the LysR homolog identified in this screen, or the LysR homolog that contains a putative HrpY binding site upstream of its coding region (ASAP ID ABF-0019114). Both the HrpL and HrpY binding sites have been characterized (Nissan et al. 2005) whereas the HrpS binding site remains unknown. Of the genes identified in this screen, only *kdgM* has a putative HrpY binding site upstream of its coding region. We were unable to identify putative σ^{54} binding sites in front of any of the genes identified in our screen; thus, they are not likely to be directly regulated by HrpS.

We aligned promoter sequences from the core T3SS gene cluster that most closely matched the consensus HrpL-binding

Table 3. Sequences similar to Hrp boxes upstream of HrpY-regulated operons

Gene	Predicted HrpL-binding sites[a]	Distance from translational start
Consensus[b]	GGAACnG–N14-nnACnnA	…
hrpA	GGAACCC–N14-CTACTTA	86
hrpF	GGAACCG–N14-CCACACA	64
hrcN	GGAACCG–N14-CCACATA	48
hrpK	GGAACCT–N14-CCACTCA	46
hrpN	GGAACCG–N14-TCACTCA	99
hrpW	GGAACTA–N14-TCACTTA	69
dspE	GGAACCG–N14-CCACTCA	122
19010	GGAACCG–N14-TTACTCA	44
Consensus[c]	GGAACYN–N14-YYACWYA	72
16069[d]	GCAAC**A**G–N15-CCACGGA	97
17229/17331[e]	GGATC**G**C–N14-TCACGTA	75

[a] Nucleotides matching the consensus are shaded. Promoter nucleotides that can differ from the consensus sequence described by Nissan and associates (2005) but allow the promoter to retain activity are in bold. Nucleotides that differ from the consensus and that were not tested by Nissan and associates (2005) are not shaded.

[b] Consensus sequence of *Pantoea stewartii* HrpL-binding sites identified through mutational analysis (Nissan et al. 2005). *P. stewartii* is closely related to *Dickeya dadantii*.

[c] Consensus sequence of putative HrpL-binding sites identified in *D. dadantii* through sequence comparisons of promoters in the core type III secretion system gene cluster.

[d] Probable regulator for *untM*.

[e] These two genes are part of the same four-gene operon.

site described by Nissan and associates (2005) and also searched upstream of the genes identified in our screen. We found that the *D. dadantii* HrpL-binding site appears to differ from the consensus described by Nissan and associates (2005) in that the terminal G nucleotide in the –35 region is not conserved (Table 3). We were unable to identify any consensus HrpL-binding sites upstream of the genes identified in our screen. However, we found that the promoter region upstream of the clustered genes 17229 (WPP264) and 17231 (WPP278) was similar to the HrpL-binding site consensus, as was a sequence in the promoter of a regulatory gene adjacent to the operon encoding *untM* (WPP265) (Table 3). The nucleotides in these sequences that diverge from the consensus either matched mutations that retain ability to be HrpL induced or were not tested in a previous study (Nissan et al. 2005).

Virulence and biofilm assays with the *Tn5SSgusA* mutants.

The 27 strains with transposon insertions that were within operons (all strains except WPP256 and WPP261) were inoculated into Yukon Gold potato tubers to determine if any of the mutated operons were required for plant tissue maceration. All of the mutants could macerate potato tubers and none were significantly reduced in ability to initiate infection compared with WPP247 with a potato tuber assay; thus, none of these genes were required for plant tissue maceration (not shown). Although HrpY is required for wild-type pellicle formation, presumably due to its upregulation of the T3SS, none of the 29 transposon mutants recovered in this screen were impaired in pellicle formation when grown in SOBG plus arabinose; thus, none of the insertions were in genes required for pellicle formation. Therefore, the quorum sensing molecule acyl-HSL, the Rcs signal transduction system, and the O-antigen transporter homolog, all of which play roles in aggregation or adhesion in other species, are not required for biofilm formation in *D. dadantii* in these growth conditions (Barak et al. 2007; Majdalani and Gottesman 2005).

DISCUSSION

Recently, Zhou and associates (2003) used a comprehensive phenotypic microarray analysis to examine the phenotypes of mutations in each of the *Escherichia coli* TCS. The results showed that the effects of a single TCS deletion were generally pleiotropic, suggesting that each TCS controls multiple cellular processes, possibly through integration of different signal transduction modules (Zhou et al. 2003). Of the TCS in *D. dadantii*, only HrpXY is shared by the enterobacterial plant pathogens and is not present in related animal-associated bacteria. Therefore, we reasoned that elucidation of the HrpY regulon would provide information on how gene regulation networks differ between plant and animal pathogens and would help to identify genes important for plant pathogenesis. In total, our results show that the plant-pathogen-specific HrpXY TCS is similar to those in *E. coli* in that HrpY regulates genes with diverse functions throughout the *D. dadantii* chromosome. Importantly, the majority of the genes identified in our screen are not common in enterobacteria, and 14 of the 16 are found mainly in plant-pathogenic enterobacteria or have been found so far only in *Dickeya* spp. Because these genes are regulated by HrpY, which is the only TCS shared among the plant-pathogenic enterobacteria and absent from related animal-associated enterobacteria, these genes are likely to play a role in *D. dadantii*–plant interactions.

Most studies regarding the HrpY-dependent gene regulation have used reporter gene fusion approaches or phenotypic analysis of regulatory mutants (Lehtimaki et al. 2003; Nizan-Koren et al. 2003; Yap et al. 2005). In all cases, HrpY was shown to be a positive transcriptional regulator of *hrpS*. Recently, *P. stewartii* HrpY was shown to bind to the *hrpS* promoter and a binding site was identified (Merighi et al. 2006). HrpY also weakly retards the promoter region of *hrpL* in vitro (Merighi 2004; Merighi et al. 2003). The hierarchical order of Hrp regulon in *D. dadantii* 3937 is similar to that of *P. stewartii* (Yap et al. 2005); this prompted us to propose that HrpY activates *hrpS* by directly binding the *hrpS* promoter, and this was verified by in vitro gel-shift assays (Fig. 1).

We previously showed that the putative sensor kinase HrpX of *D. dadantii* 3937 is dispensable for HrpY activity, and that it is not crucial for activation of the downstream *hrp* gene expression (Yap et al. 2005). Many response regulators can be phosphorylated by small metabolites such as acetyl phosphate in vitro and in vivo in the absence of their cognate sensor kinase (Lukat et al. 1992). In our EMSA experiments, we showed that acetyl phosphate was able to modify the affinity of HrpY for *hrpS* promoter, and this was interpreted as an indication of its ability to phospharylate HrpY, as was found with *P. stewartii* HrpY (Merighi et al. 2006). Acetyl phosphate is the central intermediate molecule in the acetate metabolism pathways in *E. coli* and functions as a global regulator of genes involved in biofilm formation (Wolfe et al. 2003). As in other plant-pathogenic enterobacteria, the *D. dadantii* 3937 HrpY contains a conserved aspartate-57, which is important for HrpY activity in *P. stewartii* and *Erwinia herbicola* (Merighi et al. 2006; Nizan-Koren et al. 2003).

To investigate the extent to which HrpY affects gene expression in *D. dadantii* 3937, we used a promoterless *Tn5SSgusA* mutagenesis approach to identify candidates that might be controlled by HrpY. We identified 27 different putative HrpY-regulated genes, with an O-antigen transporter identified by three different insertions, one operon identified by two different insertions (WPP264 and WPP278), and one gene cluster identified by two different insertions (strains WPP257 and WPP262). Because our approach relied on arabinose induction of *hrpY*, we then determined which of these genes were regulated by HrpY as opposed to arabinose by expressing *hrpY* from a plasmid promoter not controlled by arabinose in 26 of the mutants. Of these, 16 were confirmed to be regulated by HrpY while the other 10 could not be unambiguously confirmed and may be arabinose regulated. Arabinose is an important component of hemicellulose, a polymer found in plant cell walls; therefore, it is not surprising that some *D. dadantii* genes would be induced by this monosaccharide.

This study was complicated by the high proportion of false positives found. In addition, we had difficulty quantifying the effects of HrpY on these genes with a fluorescence-based assay even though the colony phenotypes clearly showed an effect of HrpY. Our difficulties with the fluorescence-based quantitative assay may be due to high autofluorescence of some of the media used in this study (Billinton and Knight 2001). In addition, the *D. dadantii* T3SS is regulated in a bistable fashion; thus, even when cells are grown in an apparently homogeneous culture under inducing conditions, only a portion of the cells express the T3SS (Peng et al. 2006). The possible lack of homogenous expression of the reporter genes in our mutants may have complicated the quantitative assay.

The T3SS, which is regulated by HrpY, contributes to initiation of infection and virulence of *D. dadantii*, but it is not required for pathogenicity (Bauer et al. 1994; Yang et al. 2002). Similarly, none of the genes identified in this screen are required for maceration of plant tissue, although *kdgM* and *acsC* both contribute to virulence (Blot et al. 2002; Franza et al. 2005; Nasser et al. 1998). Previous studies in *D. dadantii* have shown that these genes are controlled by multiple regulators that have varying effects on gene regulation depending upon environ-

mental conditions. For instance, *kdgM* expression is controlled by the general repressor of pectinolytic genes, KdgR; catabolite repression via CRP; the repressor of hexuronate catabolism genes, ExuR; and the pectinase gene repressor, PecS (Blot et al. 2002).

Our data show that HrpY acts as both a positive and negative regulator, as has been observed for TCS in other species (Gao et al. 2005; Leoni et al. 2005). The new members of the HrpY regulon identified in this work include genes that encode proteins not directly involved in the functioning of the T3SS but that, nonetheless, might be associated with virulence. In addition to the two genes previously described as important for plant–microbe interactions (*kdgM* and *acsC*), one gene (*untM*) is in a large *D. dadantii* pathogenicity island (Nasser et al. 2005; Praillet et al. 1997; Reverchon et al. 1994, 2002). Two genes with putative functions, homologs of an O-antigen transporter and a glutamate/aspartate transporter, also may contribute to pathogenicity. For example, O-antigen contributes to attachment to plant cells in the related bacterial species *Salmonella enterica* (Barak et al. 2007) and affects efficacy of the T3SS (West et al. 2005). Because this extracellular polymer affects host–microbe interactions in many other species, it is not surprising that it is co-regulated with the T3SS. Recently, glutamate was shown to be a key signal molecule for plant cells, capable of inhibiting root tip growth (Walch-Liu et al. 2006) and possibly modulating calcium flux (Dennison and Spalding 2000). HrpY represses expression of this transporter, suggesting that glutamate levels may affect how *D. dadantii* interacts with host plants.

For most of the genes identified, it was not clear how HrpY is affecting their regulation because most do not have putative HrpY, σ^{54}, or HrpL binding sites in their promoters. However, because there is an HrpY binding site upstream of a *lysR* homolog (ASAP ID ABF-0019114), and a second lysR homolog was identified in our screen, HrpY could be affecting regulation of genes indirectly through these regulators. Two putative HrpL binding sites were associated with three of the genes found in this study, including two genes of unknown function that probably are encoded in the same operon and a regulator adjacent to the ABC transporter gene *untM* (Table 3).

HrpY and the T3SS also are required for *D. dadantii* to form a biofilm at the air–liquid interface in culture. However, none of the mutations affected biofilm formation. Thus, AHL-based quorum sensing (WPP264) and *rcsD* (WPP254), a regulator of colanic acid synthesis, which is a polymer that contributes to bacterial aggregation and virulence in related species (Majdalani and Gottesman 2005; Mouslim et al. 2004; Prigent-Combaret et al. 2000; Venecia and Young 2005), are not required for *D. dadantii* biofilm formation.

Although a single gene encoding a putative O-antigen transporter was trapped by three different insertions (Table 2; Fig. 3.), we did not test enough mutants to saturate the genome, which may explain why none of the known *hrp* genes was detected. Similarly, *pelD*, which is regulated by several proteins, including HrpL (Peng et al. 2006), a σ factor upregulated by HrpY, was not detected in our screen. It also is possible that the screening method was not appropriate for finding *hrp* genes, perhaps because it was not sensitive enough or because the culture conditions were not optimal for identification of T3SS mutants.

Regulators important for expression of plant cell-wall-degrading enzymes and the active oxygen scavenger indigoidine recently were shown to regulate T3SS genes (Nasser et al. 2005; Reverchon et al. 1994). This work demonstrates that the co-regulation of multiple virulence gene clusters is reciprocal, with regulatory genes in the T3SS gene cluster affecting multiple genes not directly related to function of the T3SS, and that, with some promoters, such as the *kdgM* and *acsC* promoters, the level of the HrpY regulatory effect is altered by growth conditions. Most notably, HrpY appears to regulate a contingent of genes found primarily in plant pathogens and, in many cases, not found even in the closest sequenced relative, *Pectobacterium atrosepticum*. Thus further characterization of the HrpY regulon is likely to provide new insights into distinctive features of *D. dadantii* virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains are listed in Table 2 and were grown routinely in LB medium at 37°C for DNA isolation and mutagenesis unless otherwise noted. ASAP gene identifications are given for genes of unknown function. In some cases, *hrp*-inducing minimal medium (HMM) (Wei et al. 1992), PEM (Schaad et al. 2001), M9 (Sambrook and Russell 2001), and M63-glycerol (Miller 1972) media were used for *D. dadantii* carrying promoter-probe constructs. SOBG was used to induce biofilm formation as described previously (Yap et al. 2005). When required, other chemicals and antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; gentamicin, 25 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, μ0 μg/ml; spectinomycin, 50 μg/ml; tetracycline, 10 μg/ml; 5-bromo-4-chloro-3-indolyl glucuronic acid (X-GlcA) (Research Products International Corp., Prospect, IL, U.S.A.); 100 μg/ml; isopropyl-β-D-thiogalactoside (IPTG), 20 μg/ml; 5-bromo-3-indoyl-β-D-galactopyranoside (X-Gal), 50 μg/ml; and arabinose, 0.2% (wt/vol).

DNA manipulation and mutagenesis.

Standard techniques for DNA manipulation and bacterial transformation were performed basically as described by Sambrook and Russell (2001). Plasmids used are shown in Table 2. Primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, U.S.A.) as listed in Table 4. Allelic-exchange mutagenesis was done according to the methods describe by Ried and Collmer (1987).

Construction of inducible chromosomal *hrpY* allele.

A 2-kb P_{BAD}-*hrpY* was released from p105Y (Table 2) with *Cla*I and *Eco*RI, blunt ended, and ligated to *Stu*I-digested pTCLSCm (Table 4), generating pCLSP_{BAD}YCm. This plasmid was electrotransformed and allelic exchanged into the *lacY-prt* locus (ASAP feature ID 19138-19137) of an *hrpY* mutant (WPP92). The recombinant, WPP247, was confirmed by Southern blot and polymerase chain reaction analysis (not shown). The *lacY* and *prt* genes are predicted to be convergently transcribed. Raffinose (Segall 1971) and protease assays (Dow et al. 2003) were performed to ensure that the *lacY-prt* integration site was neutral by confirming that the adjacent genes were still expressed (not shown). The resulting recombinant strain WPP247 allows the tight regulation of *hrpY* via the arabinose-inducible pBAD promoter (Newman and Fuqua 1999). All HrpY-regulated *Tn5SSgusA* fusions are derivatives of WPP247.

***Tn5SSgusA* mutagenesis.**

A mutant library consisting of approximately 8,500 potentially independent *Tn5SSgusA* transposon insertion mutants of WPP247 was constructed by biparental mating between WPP247 and *Escherichia coli* S17-1//λpir (pCAM140) (Wilson et al. 1995). Both strains were grown in LB at 37°C until the optical density at 600 nm (OD_{600}) = 0.8 and 1 ml of culture was harvested. The pellets were washed twice with sterile water and resuspended in 1 ml of sterile water. The strains were mixed in a 1:2 ratio (donor/recipient) and spotted on a mannitol-glutamate (MG) agar plate (MG medium: 30 g of mannitol, 6 g of glutamic acid, 1.5 g of potassium phosphate monobasic, 0.6 g of NaCl, 0.6 g of $MgSO_4$, and 15 g of agar per liter) (Keane et

al. 1970), followed by 18 h of incubation at room temperature (approximately 25°C). The resulting mating spot was suspended in sterile water and the conjugation mixture was dilution plated onto LB plus kanamycin, chloramphenicol, and spectinomycin plates. The mutant library was replicated onto LB agar plates containing X-GlcA with and without 0.5% arabinose. These plates were placed at 30°C and corresponding colonies from each collection were monitored each day for the accumulation of blue pigment for 3 days. Mutants that displayed different blue color development in the presence and absence of arabinose were colony purified and tested for HrpY regulation in quantitative enzyme assays. The randomness of mutagenesis was confirmed by Southern blot analysis (Sambrook and Russell 2001) using *Pst*I digestion (not shown).

Identification of *Tn5SSgusA* insertion sites.

Genomic DNA from each transposon mutant was cut with either *Pst*I or *Sac*I and cloned into the same site of pBluescript SK (±), transformed into *E. coli* DH5α, and selected for spectinomycin resistance colonies. The flanking sequences of the insertion site were obtained by sequencing from the I-end and O-end of the Tn5SS*gusA40* with the primers P0449 and P0450 (Table 4). DNA sequencing reactions were performed with a Big-Dye Terminator kit (Perkin-Elmer, Norwalk, CT, U.S.A.) and were analyzed at the University of Wisconsin–Madison Biotechnology Center. The insertion sites were identified using the BLAST service at the Genome Center at the University of Wisconsin–Madison and verified by comparison with the complete *D. dadantii* 3937 genome sequence at ASAP database (Glasner et al. 2003, 2006).

GUS assays.

Cultures of *D. dadantii* WPP247 derivatives containing the chromosomal Tn5SS*gusA40* fusion were grown overnight in LB and subcultured into the same medium with and without 0.2% (wt/vol) arabinose at 1:100 fold dilution and grown for another 14 h. A fluorometric assay for GUS activity was performed essentially following the method of Jefferson (1987). Briefly, 50 µl of cell suspension was added to 200 µl of 4-methylumbelliferyl β-D-glucuronide (MUG) (Hach Company, Loverland, CO, U.S.A.) extraction buffer prewarmed to 37°C (50 mM sodium phosphate buffer [pH 7.0], 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, [pH 8.0], 0.1% sodium lauryl sarcosine, 0.1% Triton X-100, and 2 mM MUG). After 1 h, 10 µl samples were taken and the reaction was stopped by adding the sample into 240 µl of 0.2 M Na_2CO_3 stop buffer. Fluorometric measurements were performed using a Wallac Victor^2 1420 multilable counter (Perkin-Elmer) with a 355-nm excitation lamp filter and a 460-nm emission filter. The fluorescence readings were converted to nM of 4-methylumbelliferone (MU) (MP Biomedicals, Inc, Solon, OH, U.S.A.) liberated from enzymatic reaction using a MU standard curve. Protein concentrations were determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) using bovine serum albumin as a standard. The GUS activity was defined as the nanomole of MU produced per minute per milligram of total protein. Enzyme assays were repeated at least three times with independent cultures. For strains carrying pQE31 and pQhrpY, cultures were grown overnight at 24°C in LB and 5 µl of each culture was spotted onto LB, LB plus 0.5% glucose, or PEM agar. All of the agar media were supplemented with ampicillin, X-GlcA, and IPTG. Two to four replicates were spotted for each strain and the experiment was repeated at least three times for each strain.

Virulence assays.

Yukon gold potatoes were purchased from a local market, surface sanitized with 10% bleach, and air dried. Bacteria were grown overnight on LB agar and suspended in sterile water to an approximate OD_{600} of 0.2. Potato tubers were stabbed approximately 15 mm deep with a pipette tip and 5 µl of bacterial suspension was placed into each wound. The potato tubers were incubated in plastic bags at 28°C for 2 to 3 days, then cut open to determine if maceration had commenced. In all, 8 to 10 potato tubers were inoculated with each strain and the experiment was repeated three times.

His_6-HrpY purification and immunoblots.

An overnight LB culture of *E. coli* BL21 (DE3) carrying plasmid pQhrpY (Table 2) was subcultured into fresh LB plus ampicillin and grown at 30°C for 6 h. Cells were harvested 4 h after addition of a final concentration of 1 mM IPTG. His_6-HrpY was purified according to the manufacturer's protocol (Qiagen, Inc., Valencia, CA, U.S.A.) under denaturing conditions with slight modification of the buffers: lysis buffer = 100 mM NaH_2PO_4, 10 mM Tris-Cl, 10 mM imidazole, and 8 M urea (pH 8.0); wash buffer A = 100 mM NaH_2PO_4, 10 mM Tris-Cl, 20 mM imidazole, and 8 M urea (pH 6.3); wash buffer B = 100 mM NaH_2PO_4, 10 mM Tris-Cl, 20 mM imidazole, and 8 M urea (pH 5.9); wash buffer C = 100 mM NaH_2PO_4, 10 mM Tris-Cl, 250 mM imidazole, and 8 M urea (pH 4.5). Centricon 10 columns (Millipore, Inc., Bedford, MA, U.S.A.) were used for desalting, buffer exchange (in 10 mM Tris-HCl, pH 8.0), and concentrating the purified protein. Purified 28-kDa His_6-HrpY was analyzed by sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (Laemmli 1970). Chicken anti-HrpY antibodies were customized made by GeneTel Laboratories, LLC (Madison, WI, U.S.A.). Western blotting analysis was performed based on the manufacturer's instructions. A 1:8,000 fold dilution of both primary antibody (immunoglobulin 7 [IgY]8 mg/ml) and rabbit anti-chicken IgY (GeneTel Laboratories, LLC, Madison, WI, U.S.A.) secondary antibody was used, fol-

Table 4. Oligonucleotides used in this study

Primer	Sequence (5′→3′)	Restriction site	Amplified region
P0263	gagctcggatccagcgtttaatcgcctga	*Sac*I, *Bam*HI	
P0311	tcattgctattccttatcgtcat	…	0.5 kb 5′ upstream of *hrpS*
P0277	tctggaggaggatccgcgaatg	*Bam*HI	
P0278	tacaagctttcatcaggcga	*Hind*III	0.6-kb *hrpY*
P0315	tgcaatgacagctactgatggc	…	
P0380	tcacgtcgttggatttgtcgc	…	127 bp 5′ upstream of *hrpS*
P0371	ataccgactagtatagattttctc	*Spe*I	
P0372	acgaagggagagctccagacta	*Sac*I	0.6-kb *hrpY*
P0409	atggacatcagctctgcgacc	…	
P0410	agatctcgcgaggcctcgagtgccgcaatgacggcacgg	*Xho*I, *Stu*I, *Nru*I, *Bgl*II	
P0411	ctcgaggcctcgcgagatcttgccgggacggtgccggcg	*Xho*I, *Stu*I, *Nru*I, *Bgl*II	
P0412	tatctgatgggtgctggcat	…	2.8-kb *lacY-prt*
P0449	tttctacaggacgtaacataaggg	…	
P0450	gggaattcggcctaggcgg	…	O-end and I-end of Tn5SS*gusA40*

lowed by detection with the Immuno-Star-AP substrate (Bio-Rad Laboratories, Inc.).

EMSA.

To facilitate end labeling with [α-^{32}P]dCTP, DNA fragments used for EMSA were subcloned into pGEMT-Easy and excised with *Not*I which generated 5′ overhanging ends. DNA fragments were gel purified and end labeled with [α-^{32}P]dCTP by DNA polymerase I large (Klenow) Fragment (Promega Corp., Madison, WI, U.S.A.), following the manufacturer's manual. Unincorporated labels were removed by passing though the G-25 spin column (Amersham Bioscience, Inc., Piscataway, NJ, U.S.A.). To activate DNA-binding of His_6-HrpY, 10 μg of purified His_6-HrpY protein was treated with 32 mM acetyl phosphate in 20 μl of phosphorylation buffer (50 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; and 3 mM dithiothreitol) for 75 min at 37°C, as described by McCleary (1996). EMSA was performed essentially based on the manufacturer's instructions from the gel-shift assay systems (Promega Corp.). Briefly, various amount of acetyl phosphate-treated His_6-HrpY ranging from 20 to 100 nM was incubated with 2,500 counts per min of end-labeled probe in 15 μl of binding buffer containing a final concentration of 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM NaCl, 500 μM EDTA (pH 8.0), 500 μM dithiothreitol, 4% glycerol, and 1 μg of poly (dI-dC) DNA carrier (Amersham Bioscience, Inc.). After 1 h at room temperature, 2 μl of 10× loading buffer (250 mM Tris-HCl [pH 7.5], 0.2% bromophenol blue, and 40% glycerol) was added, and the samples were loaded onto a 4% polyacrylamide gel (Sambrook and Russell 2001). After electrophoresis, the gels were dried and autoradiographed.

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AUTHOR RECOMMENDED INTERNET RESOURCES

University of Wisconsin's ASAP database:
asap.ahabs.wisc.edu/asap/home.php

University of Wisconsin-Madison's E. coli Genome Project:
www.genome.wisc.edu/tools/blast2.htm