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SlyA Regulates Type III Secretion System (T3SS) Genes in Parallel with the T3SS Master Regulator HrpL in *Dickeya dadantii* 3937

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The hypersensitive response and pathogenicity (*hrp*) genes of *Dickeya dadantii* 3937 encode a type III secretion system (T3SS) which is essential for its full virulence. Previous studies of the T3SS regulation in *D. dadantii* 3937 revealed that the expression of the *hrp* genes is regulated by a master regulator, HrpL, through the HrpX-HrpY-HrpS-HrpL and GacS-GacA-*rsmB*-RsmA pathways. In this work, we identified a novel regulator of the SlyA/MarR family, SlyA, which regulates *hrp* genes of the HrpL regulon in parallel with HrpL in *D. dadantii*. SlyA regulates the T3SS in a two-tier manner. It negatively regulates the expression of *hrpL* by downregulating *hrpS* and upregulating *rsmA*. Interestingly, concomitant with its downregulation of the *hrpL*, SlyA positively regulates the expression of *hrpA* and *hrpN*, two *hrp* genes located in the HrpL regulon. In contrast to *Pectobacterium carotovorum*, the expression of *slyA* is not controlled by ExpR and ExpI in *D. dadantii* 3937. We further show that SlyA is involved in controlling swimming motility and pellicle formation in *D. dadantii* 3937.

Dickeya dadantii 3937 (formerly named *Erwinia chrysanthemi* 3937) is an enterobacterium that causes soft-rot, wilts, and dwarfing diseases in a wide range of plant species, including many economically important crops (22). It secretes a set of plant cell wall-degrading enzymes, such as pectinases, cellulases, polygalacturonases, and proteases, through the type I and type II secretion systems that together with the *hrp*-encoded type III secretion system (T3SS) are important for bacterial full virulence (13, 32, 40). Additionally, T3SS is required for pellicle formation and cell aggregation in *D. dadantii* 3937 (32, 37, 39).

Previous reports have described the genetic regulation of the group I T3SS genes in *Pectobacterium carotovorum*, *Pseudomonas syringae*, *Dickeya dadantii*, and *Erwinia amylovora* (27). In *Dickeya dadantii*, the expression of the *hrp* genes that encode the T3SS structural and functional proteins, such as *hrpA*, *hrpN*, and *dspE*, is controlled by the master regulator HrpL (24, 36). HrpL is an alternative sigma factor that binds to the *hrp* box in the promoter region of *hrp* genes to activate their expression (27, 36). Genes involved in T3SS are regulated by two sensory/regulatory pathways. First, the transcription of *hrpL* is regulated by the HrpX-HrpY-HrpS-HrpL pathway (40). HrpX and HrpY compose a two-component system (TCS). HrpX, a sensor histidine kinase, perceives environmental signals and in turn activates HrpY by phosphorylation (40, 41). HrpY is a response regulator which binds to the promoter region of *hrpS* to activate its expression (41). HrpS, an NtrC family enhancer protein, initiates the transcription of *hrpL* in an RpoN-dependent manner (27, 41). In addition to the transcriptional regulation, the amount of *hrpL* mRNA is controlled by the regulator of a secondary metabolism (Rsm) system through the GacS-GacA-*rsmB*-RsmA pathway (35, 43). The Rsm system in *D. dadantii* is composed of RsmA and *rsmB*. RsmA is a small RNA-binding protein that binds to *hrpL* mRNA and promotes its degradation. Alternatively, an untranslated regulatory small RNA, *rsmB*, binds to RsmA and neutralizes its negative regulatory effect by forming an inactive ribonucleoprotein complex (3–7, 35, 43). GacS/A is another TCS involved in the regulation of T3SS genes in *D. dadantii* 3937 (35). Under T3SS-inducing conditions, GacA positively regulates the transcription of *hrpA*, *hrpN*, and *dspE* through the Rsm posttranscriptional regulatory pathway by increasing the expression of *rsmB*, which thereby inhibits the degradation effect of RsmA on *hrpL* mRNA (35, 43).

SlyA is a member of the SlyA/MarR family transcriptional regulators, of which more than 130 SlyA protein homologues have been identified in bacteria and archaea (9, 23, 29, 31). SlyA/MarR regulators function as negative regulators of an array of genes involved in antibiotic resistance and virulence (1, 30). In *Salmonella enterica* serovar Typhimurium, SlyA regulates the expression of genes encoding the T3SS located on *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2) by directly controlling transcription of the TCS sensor kinase, SsrA (17, 23). In *Pectobacterium carotovorum* subsp. *carotovorum*, *slyA* was originally identified as a gene required for the production of pigment and the antibiotic carbapenem, whose function is similar to regulation of antibiotic and pigment (Rap) of *Serratia marcescens* (28). Recently, it was shown that SlyA contributes to the extracellular enzyme production and pellicle formation in soft-rot bacteria *P. carotovorum* subsp. *carotovorum* SCC3193 and *D. dadantii* (12, 26). In *P. carotovorum* subsp. *carotovorum* SCC3193, the expression of *slyA* is dependent on the quorum-sensing (QS) signal molecule *N*-acylhomoserine lactone (AHSL) synthesized by an AHSL

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Reference or source
Strains		
Escherichia. coli		
EC100D$^+$		Epicentre Technologies
S17-1λ *pir*/pMiniHimar RB1	pMiniHimar RB1 transposon vector in S17-1λ *pir*; Km^r	2
Dickeya dadantii		
3937	Wild type, *Saintpaulia* (African violet) isolate	N. Hugouvieux-Cotte-Pattat
WPP92	*hrpY*::Kan; 3937 derivative; Km^r	40
WPP67	*hrpX:aadA*; 3937 derivative; Sp^r Sm^r	40
Ech3049	*slyA*::Kan; Himar RB1 transposon mutant; Km^r	This work
Ech4120	*slyA*::Kan; Himar RB1 transposon mutant; Km^r	This work
Ech4380	*slyA*::Kan; Himar RB1 transposon mutant; Km^r	This work
Ech4396	*slyA*::Kan; Himar RB1 transposon mutant; Km^r	This work
Ech163	*slyA*::Kan; deletion mutant; 3937 derivative; Km^r	This work
Ech164	*expI*::Kan; deletion mutant; 3937 derivative; Km^r	This work
Ech165	*expR*::Kan; deletion mutant; 3937 derivative; Km^r	This work
Ech166	Chromosomal insertion of *lacY-slyA-cm-prt* in Ech163; Km^r Cm^r	This work
Ech188	*hrpS*::Kan; deletion mutant; 3937 derivative; Km^r	This work
Plasmids		
pPROBE-AT	Promoter-GFP reporter plasmid; Ap^r	21
pKD4	Template plasmid carrying Kan cassette; Km^r	8
pGEM-T easy	Cloning vector; *lacZ*; Ap^r	Promega Corp.
pPhrpS	*hrpS* promoter region cloned in pPROBE-AT; Ap^r	34
pPhrpL	*hrpL* promoter region cloned in pPROBE-AT; Ap^r	34
pPhrpA	*hrpA* promoter region cloned in pPROBE-AT; Ap^r	This work
pPhrpN	*hrpN* promoter region cloned in pPROBE-AT; Ap^r	This work
pPslyA	*slyA* promoter region cloned in pPROBE-AT; Ap^r	This work
pPhrpX	*hrpX* promoter region cloned in pPROBE-AT; Ap^r	This work
pPhrpY	*hrpY* promoter region cloned in pPROBE-AT; Ap^r	This work
pML123	RSF1010-derived expression and *lac*-fusion broad-host-range vector, Gm^r	16
pWM91	Suicide vector; *oriR6K mobRP4 lacZa* (of pBluescript II) *sacB*; Suc^r Ap^r	20
pWM91*slyA*::Kan	3.7-kb fragment containing *slyA*::Kan in pWM91	This work
pWM91*expI*::Kan	3.0-kb fragment containing *expI*::Kan in pWM91	This work
pWM91*expR*::Kan	3.2-kb fragment containing *expR*::Kan in pWM91	This work
pMLslyA	1.5-kb *slyA* with native promoter region cloned in pML123; Gm^r	This work
pMLhrpL	1.6-kb *hrpL* with native promoter region cloned in pML123; Gm^r	This work
pTCLS-Cm	6.4-kb *lacY-cm-prt* region cloned in pGEM-T Easy	15
pTCLSslyA-Cm	1.5-kb *slyA* with its promoter region cloned in pTCLS-Cm; Ap^r Cm^r	This work

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Gm^r, gentamicin resistance; Sp^r, streptomycin resistance.

synthase, ExpI (26). In the absence of AHSL, ExpR1 and ExpR2, two QS regulators, activate the expression of *rsmA*, which negatively regulates the expression of *slyA* (26).

Along with the known regulators reported in the HrpX-HrpY-HrpS-HrpL and GacS-GacA-*rsmB*-RsmA-HrpL pathways, we hypothesized that other unidentified regulators may play an essential role in T3SS regulation. In this study, *slyA* was identified as a T3SS regulator in a transposon mutagenesis screening. An *slyA* deletion mutant of *D. dadantii* 3937 was then constructed, and the regulatory effect of SlyA on T3SS gene expression was evaluated. We showed that in *D. dadantii* 3937, the regulation of SlyA on T3SS is through two different pathways: SlyA negatively regulates the expression of *hrpL* while it positively regulates the expression of HrpL regulon genes, *hrpA* and *hrpN*, in parallel with HrpL. Additionally, in contrast to *P. carotovorum* subsp. *carotovorum*, the regulation of *slyA* expression in *D. dadantii* 3937 is independent of the ExpI QS synthase. Finally, the effect of SlyA on pellicle formation and swimming motility was also investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *D. dadantii* strains were cultured in Luria-Bertani (LB) medium, mannitol-glutamate (MG) medium (33), or minimal medium (MM) (38) at 28°C. SOBG medium was used to evaluate pellicle formation (12, 40). *Escherichia coli* strains were cultured in LB medium at 37°C. Antibiotics were added at the following concentrations when needed: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; gentamicin, 10 μg/ml; and chloramphenicol, 50 μg/ml. Reporter plasmids pPhrpA, pPhrpN, pPhrpX, pPhrpY, and pPslyA (Table 1) were constructed by transcriptional fusion of the *hrpA*, *hrpN*, *hrpX*, *hrpY*, or *slyA* promoter regions to the gene encoding green fluorescent protein (*gfp*) in the promoter-probe vector, pPROBE-AT. The GFP reporter plasmids pPhrpS and pPhrpL were con-

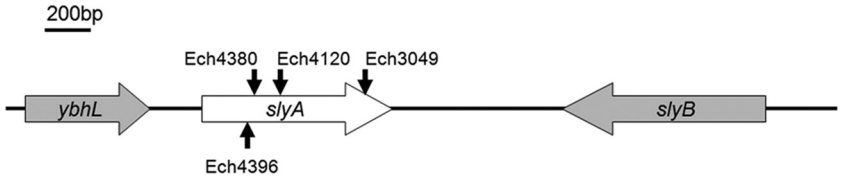

FIG 1 The *slyA* locus of *D. dadantii* 3937. The MiniHimar RB1 of four transposon mutants was inserted at bp 120 (Ech4396), 122 (Ech4380), 177 (Ech4120), and 392 (Ech3049) of the 441-bp nucleotide in the *slyA* ORF.

structed previously (35). Primers used in this study are listed in Table S1 in the supplemental material. The overexpression plasmid pMLhrpL was constructed by cloning a 1.6-kb fragment containing *hrpL* into pML123; the *nptII* promoter in the plasmid permits constitutive expression of *hrpL*.

Transposon mutagenesis and fluorescence-activated cell sorting (FACS) screening. To identify genes involved in regulation of *hrpS* in *D. dadantii* 3937, we performed a mutagenesis with the MiniHimar RB1 transposon (2). The transposon was mobilized into *D. dadantii* 3937 harboring GFP reporter plasmid pPhrpS by conjugation with *E. coli* S17-1 *pir*+ carrying pMiniHimar RB1. Transconjugants were selected on MG plates containing kanamycin and ampicillin. The resulting transposon mutants were grown in LB containing kanamycin and ampicillin at 28°C overnight, and cells were transferred to MM and grown for an additional 24 h. Bacterial cells were harvested by centrifugation, washed, and resuspended in phosphate-buffered saline (PBS) as previously described (35). The expression of *hrpS* was determined by measuring the GFP intensity using a Calibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA), and the data were analyzed with Cell Quest software (BD Biosciences, San Jose, CA). To identify the genes disrupted by the transposon, the chromosomal DNA of the mutants was digested with BamHI, self-ligated, and transformed into *E. coli* EC100D$^+$. Plasmid was isolated from kanamycin-resistant colonies and sequenced using the primers himar1 and 615 (2). Among the 11 mutants screened, four (Ech3049, Ech4120, Ech4380, and Ech4396) were found to have transposon insertions at different sites in the *slyA* gene.

Construction of mutants and functional complementation. To construct the *slyA* mutant, fragments flanking the *slyA* gene were amplified from *D. dadantii* genomic DNA by PCR. A kanamycin cassette was also amplified using the plasmid pKD4 as a template (8). Primers for amplification of flanking regions and the kanamycin cassette were designed to incorporate restriction sites to facilitate cloning. DNA fragments slyA1 (flanks the 5′ end) and slyA2 (flanks the 3′ end) were digested with BamHI/EcoRI and HindIII/XhoI, respectively. The kanamycin cassette was digested with EcoRI and HindIII. The slyA1 and slyA2 fragments were ligated to the kanamycin cassette at EcoRI and HindIII sites, and this recombinant fragment was cloned into pWM91 at BamHI and XhoI sites to generate plasmid pWM91*slyA*::*kan*. The *slyA* mutant of *D. dadantii* was generated by biparental mating between the *E. coli* S17-1λ *pir* and *D. dadantii* 3937 as described previously (20). In this study, the *expI* and *expR* mutants were also constructed in a manner similar to that of the *slyA* mutant. These mutants were confirmed by PCR and DNA sequencing.

To complement the *slyA* mutant, a fragment containing the complete coding sequence for the *slyA* gene along with its native promoter was cloned to pTCLS-Cm between the *lacY* and *cm* loci at the NruI and XhoI sites. *slyA* was inserted into the chromosome of the *D. dadantii* strain at an intergenic region by allelic exchange as described previously (15).

RNA isolation and Northern blot analysis. Cultures of *D. dadantii* strains grown overnight in LB broth were diluted 1:100 in MM and grown for 12 h at 28°C to induce expression of T3SS genes. Total RNA was isolated by TRI reagent (Sigma-Aldrich, St. Louis, MO) (34), and each sample was treated with 2 U of Turbo DNA-free DNase (Ambion, Austin, TX) for 1 h at 37°C. The concentration of RNA samples was determined using a spectrophotometer (ND-1000). For each sample, 10 to 15 μg of total RNA was electrophoresed on a 1.0% denaturing agarose gel. Hybridization probes of *rsmB*, *hrpA*, and *rsmA* were amplified by PCR using the primers listed in Table S1 in the supplemental material. Probes were labeled using a BrightStar psoralen-biotin kit (Ambion, Austin, TX). Hybridization and detection were performed by using the NorthernMax kit and the BrightStar BioDetect kit (Ambion, Austin, TX) according to the manufacturer's instructions. 16S rRNA was used as an internal control.

Real-time RT-PCR analysis. Strains were grown overnight in LB broth. Cultures were diluted 1:100 in MM and grown for 12 h at 28°C. Total RNA was isolated from 2 ml of each sample using an RNeasy minikit (Qiagen Sciences, MD) and treated with a Turbo DNA-free DNase kit (Ambion, Austin, TX). cDNA was synthesized with 0.5 μg of treated RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The Real Master Mix (5 PRIME, Gaithersburg, MD) was used to quantify the cDNA level of target genes. Real-time reverse transcription (RT)-PCR data were collected by the Opticon 2 system (Bio-Rad, Hercules, CA) as described previously (35) and analyzed using the relative expression software tool (25). The *rplU* was used as the endogenous control for data analysis (18, 43). Primers used for real-time PCR are listed in Table S1 in the supplemental material. Primer efficiencies were tested for all primers, all of which are within the 1.95-to-2.05 range.

Swimming motility and pellicle formation. In the swimming motility assay, 10 μl of an overnight bacterial suspension (optical density at 600 nm [OD_{600}] of 0.1) was inoculated in the center of an MG plate containing 0.3% agar (42). The plates were incubated at 28°C, and the diameter of radial growth was measured after 24 h. The experiment was repeated three times with three biological replicates in each experiment.

The pellicle-forming ability of bacterial strains was evaluated in SOBG medium as described before (12, 40). Briefly, overnight bacterial cultures in LB broth were inoculated to SOBG broth at a concentration of 10^6 CFU/ml. Cultures were incubated in glass test tubes without shaking at 28°C for 2 days to observe formation of the pellicle.

RESULTS

SlyA downregulates the expression of *hrpS*. To identify novel T3SS regulators other than the previously identified TCS HrpX and HrpY that act upstream of *hrpS*, we employed a random transposon mutagenesis by introducing a MiniHimar RB1 transposon into *D. dadantii* 3937 carrying a plasmid-borne reporter, pPhrpS. pPhrpS contains an *hrpS* promoter-*gfp* transcriptional fusion which was used to identify transposon mutants with altered *hrpS* expression that can be detected using a flow cytometer to measure green fluorescent protein levels. Four mutants, Ech3049, Ech4120, Ech4380, and Ech4396, each with increased *hrpS-gfp* expression, had a transposon insertion in a 441-bp open reading frame (ORF) that encodes an SlyA protein (ASAP identifier [ID] 15312). Sequence analysis revealed that the transposons were inserted at bp position 392 (Ech3049), 177 (Ech4120), 122 (Ech4380), and 120 (Ech4396) of *slyA* (Fig. 1). The SlyA protein of *D. dadantii* 3937 showed 97% identity (99% similarity) to the SlyA protein of *Dickeya zeae* EC1 (NCBI accession no. ACC62398), 85% identity (94% similarity) to the SlyA protein of *P. carotovorum* subsp. *carotovorum* SCC3193 (NCBI accession no. ABY91292), and 75% identity (85% similarity) to the SlyA protein of *Salmonella* Typhimurium LT2 (NCBI accession no. NP460467). To confirm the increase in *hrpS*

TABLE 2 Promoter activity of *hrpS* in wild-type *D. dadantii* 3937 and *slyA* mutant Ech163 in minimal medium

Gene promoter[a]	Mean fluorescence intensity[b]	
	12 h	24 h
3937(pPhrpS)	81.1 ± 3.2	82.9 ± 5.4
Ech163(pPhrpS)	185.7 ± 4.6*	158.7 ± 5.0*
Ech166(pPhrpS)	58.7 ± 1.1*	66.3 ± 0.8
3937(pPhrpL)	13.9 ± 1.1	14.6 ± 1.6
Ech163(pPhrpL)	24.2 ± 1.2*	20.5 ± 2.0*
Ech166(pPhrpL)	6.5 ± 0.1*	9.5 ± 0.1*
3937(pPhrpA)	71.2 ± 8.3	88.1 ± 8.0
Ech163(pPhrpA)	24.3 ± 2.6*	23.2 ± 6.3*
3937(pPhrpN)	44.5 ± 8.2	46.2 ± 8.0
Ech163(pPhrpN)	18.4 ± 3.8*	12.1 ± 3.1*
3937(pPROBE-AT)	3.2 ± 0.3	3.4 ± 0.2

[a] Ech163 is a deletion mutant of *slyA* in the *D. dadantii* 3937 background. Ech166 contains the *slyA* gene with its native promoter integrated into a neutral chromosomal locus, *lacY-prt*, of Ech163.

[b] GFP intensity was determined on gated populations of bacterial cells by flow cytometry. Values of mean fluorescence intensity (MFI) are an average GFP fluorescence intensity of total bacterial cells with standard deviations (SD). Similar results have been observed in two individual experiments, and the results of one experiment have been shown here. Three replicates were used in this experiment. Asterisks indicate statistically significant differences in GFP MFI between the wild-type strain (3937) and the *slyA* mutant or the *slyA* complemented strain ($P < 0.01$, Student's *t* test).

expression observed in the *slyA* transposon mutants, an *slyA* deletion mutant (Ech163) was constructed. Similar to the transposon mutants, dramatic increases of both promoter activity and mRNA level of *hrpS* were detected in Ech163 compared to those of the wild type (Table 2, Fig. 2A). The enhanced *hrpS* expression of Ech163 could be partially restored to the wild-type levels in Ech166, which contains an *slyA* gene integrated into a neutral chromosomal locus *lacY-prt* of Ech163 and expressed from its native promoter (Table 2).

SlyA and HrpXY regulate the expression of *hrpS* independently. The expression of *hrpS* in *D. dadantii* 3937 is dependent on the TCS HrpX/HrpY, with HrpY directly activating expression of *hrpS* (40, 41). In this study, we showed that the expression of *hrpS* is also controlled by SlyA. To determine whether the HrpX/HrpY signaling cascade regulates the expression of *hrpS* through SlyA, the promoter activity of *slyA* was measured in *D. dadantii* 3937, an *hrpX* mutant (WPP67), and an *hrpY* mutant (WPP92). Similar expression levels of *slyA* were observed among the wild type, WPP67, and WPP92 (Table 3). This result suggests that the expression of *slyA* is not influenced by HrpX and HrpY. SlyA regulates T3SS expression by controlling the transcription of the TCS kinase SsrA in *S.* Typhimurium (17, 23). We also investigated the effect of SlyA on expression of the *hrpX* and *hrpY* genes. The GFP intensity of Ech163 carrying the reporters pPhrpX and pPhrpY, which contain transcriptional fusions of *hrpX* and *hrpY* promoters with the *gfp* gene, respectively, was measured by flow cytometry. Compared with the wild-type strain, the *slyA* mutant exhibited no difference in either *hrpX* or *hrpY* expression (Table 3). This indicates that SlyA does not regulate the expression of *hrpX* and *hrpY*.

SlyA negatively regulates *hrpL* at transcriptional and post-transcriptional levels. In *D. dadantii* 3937, HrpS initiates the transcription of *hrpL* (40). As discovered from the transposon mutagenesis and the deletion of *slyA*, SlyA downregulates *hrpS* expression. To determine whether SlyA also regulates *hrpL*, the expression level of *hrpL* was evaluated in the wild type and *slyA* mutant Ech163 by promoter activity assay and quantitative RT-PCR (qRT-PCR). Compared with that of the wild type, an approximate 2-fold increase in *hrpL* promoter activity was observed in Ech163 (Table 2). The increased *hrpL* expression observed in Ech163 could be partially restored to the wild-type level in an *slyA* complementation strain, Ech166 (Table 2). In addition, analysis by qRT-PCR showed a 3-fold increase in the *hrpL* mRNA in Ech163 ($P \leq 0.01$) compared to the wild type (Fig. 2A).

In *D. dadantii* 3937, in addition to the transcriptional regulation by HrpS, the *hrpL* mRNA level is also controlled through the GacS-GacA-*rsmB*-RsmA-HrpL regulatory pathway at the post-transcriptional level. To further explore the role of this pathway, the expression levels of *rsmA* and *rsmB* in wild type and Ech163 were analyzed by Northern blot analysis. Compared to the wild type, a reduction in *rsmA* mRNA was detected in Ech163 and was restored to near-wild-type levels in the Ech166 complemented strain (Fig. 3A). Similar amounts of *rsmB* RNA were observed between the wild type and Ech163 (Fig. 3B). RsmA is a small-RNA-binding protein that acts by reducing the half-life of the *hrpL* mRNA. These results suggest that the increased *hrpL* mRNA level observed in Ech163 is derived not only from the increased *hrpS*

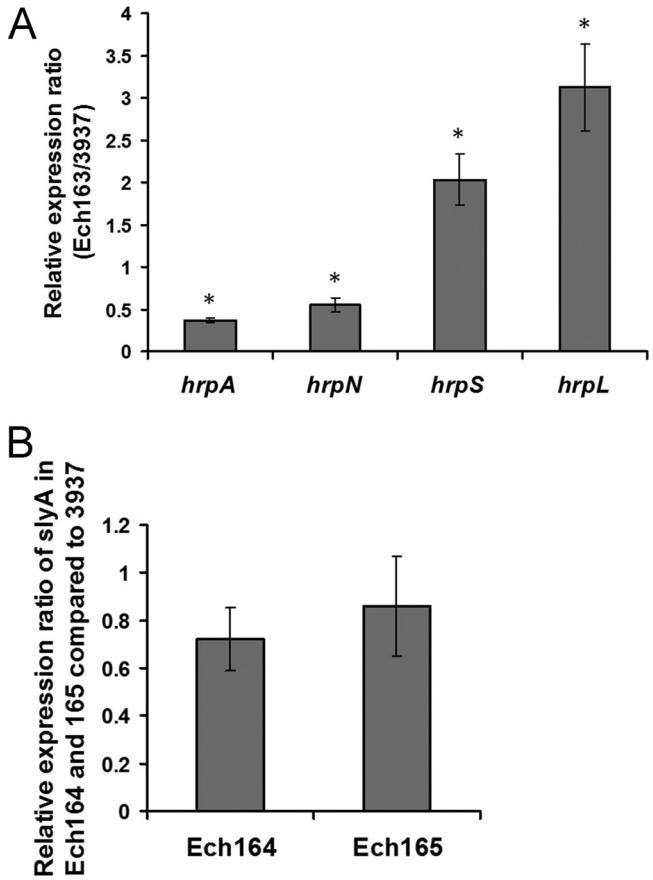

FIG 2 (A) Expression ratios of *hrpA*, *hrpN*, *hrpS*, and *hrpL* mRNAs in *slyA* mutant Ech163 compared to those of *D. dadantii* 3937. (B) The relative mRNA levels of *slyA* in the *expI* mutant Ech164 and the *expR* mutant Ech165 compared to that of *D. dadantii* 3937. Bacterial strains were grown in minimal medium for 12 h. Total RNA was isolated, and the mRNA of target genes was measured by real-time RT-PCR. Asterisks indicate statistically significant differences in mRNA levels of the mutants compared to that of the wild type ($P < 0.05$). Similar results were observed in two independent experiments.

TABLE 3 Promoter activities of *hrpX* and *hrpY* in *D. dadantii* 3937 and Ech163 and of *slyA* in 3937, WPP67, and WPP92

Gene promoter[a]	Mean fluorescence intensity[b]		
	6 h	12 h	24 h
3937(pPslyA)	119.5 ± 7.3	131.7 ± 3.3	128.1 ± 2.7
WPP67(pPslyA)	119.1 ± 2.4	133.7 ± 0.8	140.7 ± 0.8
WPP92(pPslyA)	127.5 ± 5.4	128.5 ± 0.5	134.3 ± 5.3
3937(pPROBE-AT)	2.6 ± 0.5	2.1 ± 0.1	3.4 ± 0.6
3937(pPhrpX)	10.8 ± 0.5	14.3 ± 0.8	14.8 ± 0.7
Ech163(pPhrpX)	11.0 ± 0.2	12.0 ± 0.4	13.5 ± 0.9
3937(pPhrpY)	25.4 ± 1.0	35.2 ± 1.1	34.3 ± 0.9
Ech163(pPhrpY)	24.8 ± 0.6	29.5 ± 0.8	28.6 ± 0.6
3937(pPROBE-AT)	2.3 ± 0.1	2.6 ± 0.3	4.4 ± 0.6

[a] WPP67 and WPP92 are *hrpX* and *hrpY* mutants of *D. dadantii* 3937, respectively. Ech163 is the *slyA* mutant of *D. dadantii* 3937.

[b] Similar results have been observed in two individual experiments with three biological replicates, and the results of one experiment have been shown here. No significant difference in *slyA* promoter activity was observed among the wild-type strain (3937), WPP67, WPP92, and Ech163 ($P > 0.05$, Student's *t* test).

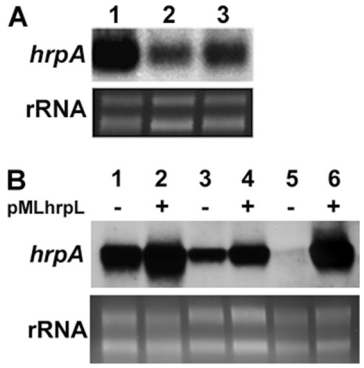

FIG 4 (A) Northern blot analysis of *hrpA* in wild-type *D. dadantii* 3937 (lane 1), *slyA* mutant Ech163 (lane 2), and Ech166, the chromosomal complemented strain of Ech163 (lane 3). (B) Northern blot analysis of *hrpA* mRNA in *D. dadantii* 3937 carrying empty vector pML123 (lane 1), *D. dadantii* 3937 carrying *hrpL* overexpression plasmid pMLhrpL (lane 2), *slyA* mutant Ech163 carrying pML123 (lane3), Ech163 carrying pMLhrpL (lane 4), *hrpS* mutant Ech188 carrying pML123 (lane 5), and Ech188 carrying pMLhrpL (lane 6). Similar results were observed in two independent experiments.

expression but also from the decreased *rsmA* expression. Overall, SlyA negatively regulates *hrpL* by downregulating *hrpS* and upregulating *rsmA*.

SlyA upregulates the transcription of *hrp* genes in the HrpL regulon in spite of its downregulation of *hrpL*. To further investigate whether SlyA affects the expression of genes in the HrpL regulon, the expression of *hrpA* and *hrpN* was measured in *D. dadantii* 3937 and Ech163 by measuring promoter activity. Surprisingly, a reduction in *hrpA* and *hrpN* expression was observed in Ech163 (Table 2). A decrease in the mRNA level of *hrpA* and *hrpN* was also confirmed in Ech163 in comparison to that of the wild-type strain by qRT-PCR (Fig. 2A). Finally, the expression of *hrpA* was partially complemented in Ech166 (Fig. 4A). These results suggest that SlyA exerts two different modes of regulation on the T3SS in *D. dadantii* 3937: it negatively regulates the expression of *hrpS* and *hrpL*, whereas it positively regulates the expression of HrpL regulon genes, such as *hrpA* and *hrpN*.

SlyA controls the HrpL regulon genes in parallel with the T3SS master regulator HrpL. In the *slyA* mutant, the expression of *hrpA* and *hrpN* is reduced even though *hrpL* expression is induced, which suggests that SlyA regulates HrpL regulon genes independent of HrpL. To test this hypothesis, the *hrpA* mRNA levels were compared in *D. dadantii* 3937, the *slyA* mutant Ech163, and the *hrpS* mutant Ech188 in the presence and absence of an *hrpL* overexpressing plasmid pMLhrpL (Fig. 4B). When *hrpL* is overexpressed in *D. dadantii* 3937, a dramatic induction of *hrpA* expression was observed compared to that of the wild type carrying the empty vector pML123 (Fig. 4B, lanes 1 and 2). HrpS controls *hrpA* expression through its regulation of *hrpL*. As expected, the *hrpS* mutant Ech188 was deficient in *hrpA* expression (Fig. 4B, lane 5). By introducing pMLhrpL into Ech188, the expression of *hrpA* was restored to a level similar to that of the wild type carrying pMLhrpL (Fig. 4B, lane 6). The *hrpA* expression was greatly reduced in the *slyA* mutant, which indicates that SlyA positively regulates *hrpA* (Fig. 4B, lane 3). However, in the *slyA* mutant Ech163 with pMLhrpL, *hrpA* expression was unable to be restored to the same level as that in Ech188 with pMLhrpL (Fig. 4B, lanes 4 and 6). In fact, the *hrpA* expression in Ech163 carrying pMLhrpL (lane 4) was slightly lower than that of *D. dadantii* 3937 with the empty vector (lane 1). Similar results were observed by Northern blot analysis using an *hrpN* probe (data not shown). These results suggest that the regulation of SlyA on the HrpL regulon genes, such as *hrpA* and *hrpN*, is in parallel with the T3SS master regulator HrpL.

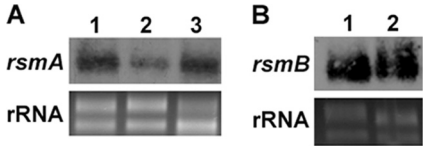

FIG 3 (A) Northern blot analysis of *rsmA* in *D. dadantii* 3937 (lane 1), *slyA* mutant Ech163 (lane 2), and *slyA* chromosomal complementation strain Ech166 (lane 3). (B) Northern blot analysis of *rsmB* mRNA in *D. dadantii* 3937 (lane 1) and Ech163 (lane 2). Similar results were observed in two independent experiments.

***slyA* is not controlled by ExpR or ExpI in *D. dadantii* 3937.** In *P. carotovorum* subsp. *carotovorum* SCC3193, the expression of *slyA* is dependent on the concentration of AHSL, a QS signal molecule (26). A mutation in *expI*, a gene encoding the AHSL synthase, leads to a reduction in *slyA* expression. This is due to the fact that in the absence of AHSL, ExpR1 and ExpR2 activate the expression of *rsmA*, which in turn negatively regulates *slyA* expression. However, in the presence of the AHSL, the expression of *rsmA* in *P. carotovorum* subsp. *carotovorum* SCC3193 is fully repressed (26). The *D. dadantii* 3937 genome contains *expI* (ASAP ID 19415) and *expR* (ASAP ID 19414), encoding an AHL synthase and an AHSL responsive regulator, respectively (10, 14). The *D. dadantii* genome also contains a putative LuxR regulator (ASAP ID 15900) with low similarity (23% identity) to ExpR. Besides *expI*, no other homologue of the AHSL synthase was found in the *D. dadantii* 3937 genome. To determine whether QS controls the expression of T3SS by regulating *slyA* in *D. dadantii* 3937, deletion mutants of *expI* (Ech164) and *expR* (Ech165) were generated, and the expression of *rsmA*, *slyA*, and *hrpA* was measured in *D. dadantii* 3937, Ech164, and Ech165. In contrast to that in *P. carotovorum* subsp. *carotovorum* SCC3193, similar levels of *rsmA* RNA were observed among the wild type, the *expI* mutant (Ech164),

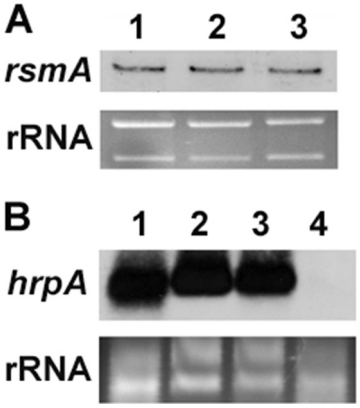

FIG 5 (A) Northern blot analysis of *rsmA* mRNA in *D. dadantii* 3937 (lane 1), the *expI* mutant Ech164 (lane 2), and the *expR* mutant Ech165 (lane 3). (B) Northern blot analysis of *hrpA* in *D. dadantii* 3937 (lane 1), *expI* mutant Ech164 (lane 2), *expR* mutant Ech165 (lane3), and *hrpY* mutant WPP92 (lane 4). Similar results were observed in two independent experiments.

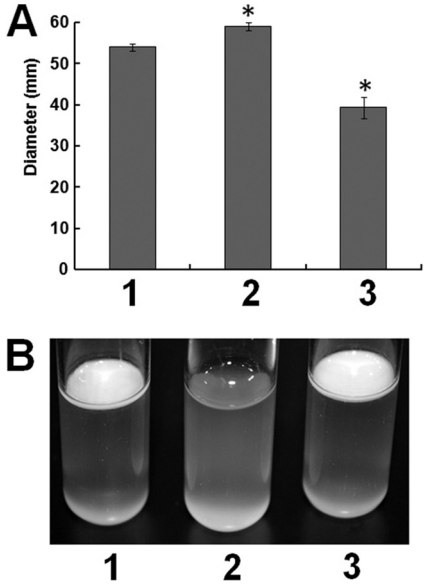

FIG 6 (A) Swimming motility of *D. dadantii* 3937 (lane 1), *slyA* mutant Ech163 (lane 2), and Ech166 (lane 3), a *slyA* chromosomal complementation strain of Ech163. (B) Pellicle formation of *D. dadantii* 3937 (lane 1), Ech163 (lane 2), and Ech166 (lane 3). Similar results were observed in two independent experiments with two biological replicates in each experiment.

and the *expR* mutant (Ech165) (Fig. 5A). qRT-PCR analysis showed no significant differences in *slyA* mRNA levels in Ech164 ($P = 0.55$) or Ech165 ($P = 0.983$) compared to that in the wild-type strain (Fig. 2B). In addition, similar levels of *hrpA* mRNA were observed in the *expI* and *expR* mutants and the wild type when examined by Northern blot analysis (Fig. 5B). Given that HrpY is essential for the expression of *hrpA*, an *hrpY* mutant (WPP92) was included as a control (Fig. 5B). These results indicate that unlike *P. carotovorum* subsp. *carotovorum* SCC3193, ExpI and ExpR are not involved in regulating the expression of *slyA* or the T3SS genes in *D. dadantii* 3937.

SlyA controls bacterial swimming motility and pellicle formation. Motility contributes to bacterial virulence, whereas biofilm and pellicle formation aids bacterial survival in a variety of environments. In *D. dadantii* 3937, the T3SS is required for pellicle formation (12, 40). Compared to wild-type *D. dadantii* 3937, increased swimming motility was observed in Ech163, and this phenotype could be complemented in the complementation strain Ech166 (Fig. 6A). Additionally, pellicle formation was completely suppressed in Ech163 compared to that in the wild type, and the mutant phenotype could be restored to the wild-type level in Ech166 (Fig. 6B).

DISCUSSION

In this study, a novel regulator, SlyA, which displays a complex and intricate regulation of the T3SS in *D. dadantii* 3937 (Fig. 7), was identified and characterized. Previous reports have described that the T3SS structural and effector genes are regulated by two major regulatory pathways, HrpX/HrpY-HrpS-HrpL and GacS/GacA-rsmB-RsmA pathways. Our work demonstrates that, in contrast to HrpX/HrpY, SlyA regulates *hrpS* negatively (Table 3). In addition, SlyA positively controls RsmA, a posttranscriptional regulator of *hrpL* (Fig. 3A). The negative regulation on *hrpS* and positive regulation on *rsmA* leads to a decrease in *hrpL* expression (Fig. 2A and Table 2). Interestingly, although SlyA represses the *hrpL* expression, the deletion of *slyA* leads to reduced expressions of two T3SS genes in the HrpL regulon, *hrpA* and *hrpN* (Fig. 2A and Table 2). These results suggest that SlyA plays an opposing role in T3SS regulation: it acts as a negative regulator of *hrpS* and *hrpL* but serves as a positive regulator of HrpL regulon genes, such as *hrpA* and *hrpN*. This also implies that along with the well-studied HrpX-HrpY-HrpS-HrpL and GacA-GacS-RsmA-*rsmB*-HrpL pathways, an additional regulatory pathway, SlyA-HrpA/HrpN, regulates T3SS in *D. dadantii* 3937 (Fig. 7). Finally, *hrpA* expression was completely shut down when HrpL was absent in an *hrpS* mutant, but a reduced level of *hrpA* expression was observed in the *slyA* mutant (Fig. 4B, lanes 3 and 5). This suggests that HrpL plays a greater role in controlling the HrpL regulon genes than SlyA. In this study, the expression of *hrpS*, *hrpL*, and *hrpA* was partially complemented when the *slyA* gene was inserted into a neutral chromosomal site of Ech163. The partially complemented phenotype in the mutant may be due to temporal and spatial differences in the placement of the complemented copy of *slyA* in the intergenic region.

HrpL is the master regulator of the T3SS genes encoding the basic structural and functional components of the T3SS. By binding to a consensus sequence called the *hrp* box, HrpL initiates the transcription of the HrpL regulon genes. Our data suggest that SlyA regulates HrpL regulon genes in parallel with HrpL. Reports have been published describing novel T3SS regulators of the group I T3SS; however, the influence of these regulators on the HrpL regulon genes are mediated through the regulation of HrpL, either transcriptionally or posttranscriptionally (22, 42, 43). To our knowledge, this is the first report describing a transcriptional regulator that controls HrpL regulon genes in an HrpL-independent manner (Fig. 7).

Quorum sensing (QS) is a common mechanism of gene regulation among Gram-negative bacteria in which individual cells produce and respond to special AHSL signal molecules (11). The SlyA homologue has recently been described as a QS target in *P. carotovorum* subsp. *carotovorum* SCC3193 (26). An increase in *slyA* expression was observed in an *expI* mutant of *P. carotovorum* subsp. *carotovorum* SCC3193 (26). In this regulatory pathway,

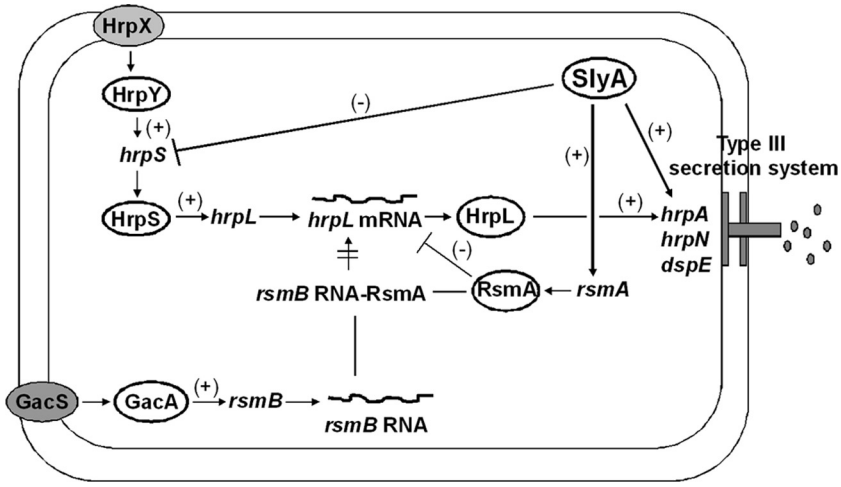

FIG 7 The regulation of SlyA on the T3SS expression and extracellular enzyme production. SlyA negatively regulates the transcription of *hrpS*, through an HrpX/HrpY-independent pathway. It also upregulates the expression of *rsmA*. The influence of SlyA on *hrpS* and *rsmA* leads to a downregulation of *hrpL*. However, SlyA upregulates the expression of the HrpL regulon genes, such as *hrpA* and *hrpN*, in parallel with HrpL. The lines with bars and arrows represent negative and positive regulations, respectively.

RsmA negatively regulates the expression of *slyA*, and *rsmA* expression is positively regulated by two QS regulators, ExpR1 and ExpR2, in the absence of AHSL (26). The SlyA protein of *D. dadantii* 3937 was annotated based on high similarity to SlyA of *P. carotovorum*. However, our result showed that inactivation of *expI* or *expR* of *D. dadantii* 3937 had little effect on the expression of *slyA*. This suggests that the expression of *slyA* may be controlled dissimilarly among different bacteria. For example, SlyA is controlled in an AHSL-independent manner in *P. carotovorum* subsp. *carotovorum* strain ATTn10 (19). A previous report demonstrated that the expression of *hrpN* of *D. dadantii* 3937 is independent of the QS signal *N*-(3-oxo-hexanoyl)-L-homoserine lactone (OHHL) (22). Similarly, our results show that in *D. dadantii* 3937, the expression of *hrpA* and *hrpN* is not under the control of ExpI and ExpR.

Cell-cell aggregation is an important process for bacteria switching from a single-cell mode to a multicellular community (39). In *D. dadantii* 3937, the T3SS genes are required for pellicle formation (12, 40). Our study demonstrated that SlyA is required for the pellicle formation, which may be due to the regulatory effect of SlyA on *hrpA* and *hrpN* expression.

SlyA homologues represent a growing family of novel bacterial regulatory proteins which play important roles in the global regulation of diverse physiological processes in animal and plant pathogens (28). In soft-rot bacteria, a reduction in extracellular enzymes and pellicle formation was observed in an *slyA* mutant of *P. carotovorum* subsp. *carotovorum* Scc3139 and *D. dadantii* (12, 26). In this study, SlyA also shows a global regulatory effect to control other biological characteristics, such as T3SS, motility, and pellicle formation. Our results indicate that SlyA upregulates the expression of *hrpA* and *hrpN*, which is independent of the known HrpS-HrpL and posttranscriptional Rsm-mediated regulatory pathways.

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