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SlyA, a MarR Family Transcriptional Regulator, Is Essential for Virulence in *Dickeya dadantii* 3937[▽]

M. Manjurul Haque,[1]§† M. Shahinur Kabir,[2]§ Luqman Qurata Aini,[3]
Hisae Hirata,[1] and Shinji Tsuyumu[1,2,3]*

Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan[1]; Institute for Genetic Research and Biotechnology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan[2]; and Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan[3]

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SlyA, a MarR family transcriptional regulator, controls an assortment of biological functions in several animal-pathogenic bacteria. In order to elucidate the functions of SlyA in the phytopathogen *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) 3937, a *slyA* gene deletion mutant (denoted Δ*slyA*) was constructed. The mutant exhibited increased sensitivity to sodium hypochlorite, the cationic antimicrobial peptide polymyxin B, and oxidative stress. The mutant showed reduced production of pectate lyase and exopolysaccharide and an inability to form a pellicle. The mutant lacking a functional *slyA* gene showed a significantly reduced ability to cause maceration of potato tubers. Accordingly, the mutant exhibited significantly reduced bacterial growth and failed to hyperinduce pectate lyase production in planta. Introduction of a plasmid containing *slyA* into the Δ*slyA* mutant caused all of these phenotypes to recover to wild-type levels. These results suggest that SlyA plays an important role in virulence to plants by positively regulating the expression of multiple pathogenicity-related traits of *D. dadantii* 3937.

The MarR (*m*ultiple *a*ntibiotic *r*esistance *r*egulator) proteins are prototypical members of the MarR family of transcriptional regulators that are widely distributed in bacteria and archaea (2, 13). Members of the MarR family regulate a wide variety of cellular processes, including resistance to multiple antibiotics, organic solvents, household disinfectants, and oxidative-stress agents, which are collectively termed the multiple antibiotic resistance phenotypes (1, 2). They also regulate the synthesis of virulence factors in microbes that infect humans or plants, e.g., *slyA* in *Salmonella enterica* serovar Typhimurium (10, 30, 31a), *rovA* in *Yersinia pseudotuberculosis* (36), *aphA* in *Vibrio cholerae* (27), *mgrA* in *Staphylococcus aureus* (22), *pecS* in *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) 3937 (46), *hor* in *Pectobacterium carotovorum* subsp. *carotovorum* (formerly *Erwinia carotovora* subsp. *carotovora*) (58), and *hpaR* in *Xanthomonas campestris* pv. campestris (62). It has been demonstrated that the role of MarR in controlling the expression of virulence-associated genes varies among pathogens (7, 22, 27, 29, 30, 34, 44, 45, 62). In *Y. pseudotuberculosis*, *rovA* transcription is primarily controlled by RovA itself (13, 13a, 28) and by H-NS (28), a histone-like protein that is important for the proper nucleoid packaging of the bacterial chromosome (3), in a temperature-dependent manner. Conversely, the expression of *slyA* in *Escherichia coli* is regulated by temperature but in an H-NS-independent manner (9). In *S. enterica* serovar Typhimurium, the expression of *slyA* is regulated by its own product, SlyA (57), and is induced during the stationary phase, as well as during the infection of macrophages (7). Moreover, *slyA* expression in *S. enterica* serovar Typhimurium is positively controlled by the PhoP-PhoQ two-component regulatory system in responding to magnesium starvation (40, 54).

D. dadantii 3937 is a phytopathogenic enterobacterium that attacks a wide range of economically important plant species. A variety of factors have been shown to influence the ability of *D. dadantii* 3937 to attack plant tissue, but pectinolytic enzymes are considered to be the major determinant of pathogenicity (41, 41a, 59a, 61). Additional factors that contribute to its pathogenicity are exopolysaccharide (8), lipopolysaccharide (53), siderophore-mediated iron transport systems (14), the type III secretion system encoded by the *hrp* gene cluster (5), antimicrobial peptides (18), motility (12), and proteins involved in resistance against plant defense mechanisms (12, 33). The expression of these functions is regulated in a coordinated manner in response to different stimuli, such as the presence of pectic substances, acidic pH, iron, magnesium limitation, plant extract, and the presence of antimicrobial molecules. Different regulatory proteins, such as KdgR, PecS, H-NS, PecT, Pir, CRP, Fur, and PhoP-PhoQ (8, 18, 36a, 39, 45, 46, 47), have been shown to be involved in such complex regulation. To gain further insight into the regulation of pathogenicity factors in *D. dadantii* 3937, we investigated the role of the MarR family transcriptional regulator SlyA in this process. Here, we show that SlyA is an important virulence factor that regulates numerous pathogenicity-related traits in *D. dadantii* 3937.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth media, and chemicals. Bacterial strains and plasmids and their relative characteristics are listed in Table 1. *E. coli* strains were cultivated in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract,

* Corresponding author. Mailing address: Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka Shi 422-8529, Japan. Phone and fax: 81-54-238-4823. E-mail: tsuyumu@agr.shizuoka.ac.jp.

§ M.M.H. and M.S.K. contributed equally to this work.

† Present address: Department of Bioenvironmental Science, Faculty of Agriculture, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Salna, Gazipur-1706, Bangladesh.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
D. dadantii		
3937	Wild type; Nal^r	Laboratory collection
Δ*slyA*	Δ*slyA* mutant; Nal^r	This study
3937Δ*slyA*	pDEL-SlyA into *D. dadantii* 3937; Nal^r Ap^r Stm^r	This study
Δ*slyA*(pSlyA)	pSlyA into Δ*slyA* mutant; Nal^r Ap^r	This study
MH70	Km-inserted *phoP* mutant; Nal^r Km^r	18
MH72	Km-inserted *phoQ* mutant; Nal^r Km^r	18
E. coli		
DH5α	λ$^-$ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1* (note the roman M15 after *lacZ*Δ)	TaKaRa, Japan
CC118	Δ(*ara*, *leu*) *araD* Δ*lacX*74 *galE galK phoA*20 *thi-1 rps rpoB argE*(Am) *recA*1 Sp^r	19
CC118 (λ*pir*)	CC118 lysogenized with λ*pir* phage; Sp^r	19
S17-1	*recA thi pro hsdR*-M+RP4:2-Tc:Mu:km Tn*7* λ*pir* Sm^r	Biomedal
BL21(DE3)	F$^-$ *ompT hsdS$_B$*(r_B^- m_B^-) *gal dcm* (DE3)	Nippon gene
Plasmids		
pGEM-T Easy	A-T cloning vector; Ap^r	Promega
pMRS101	R6K origin *sacB* marker exchange vector; *mob*$^+$, Ap^r Stm^r	51
pSlyX	2.34-kb ApaI-SacI fragment cloned into same site of pGEM-T Easy; Nal^r Ap^r	This study
pSlyY	0.911-kb ApaI-SmaI fragment upstream from *slyA* ORF cloned into same site of pGEM-T Easy, Nal^r, Ap^r	This study
pSlyZ	0.999-kb SmaI-SacI fragment downstream from *slyA* ORF cloned into same site of pGEM-T Easy; Nal^r Ap^r	This study
pSlyYZ	1.91-kb ApaI-SacI fragment contained deletion of *slyA* ORF cloned into same site of pGEM-T Easy; Nal^r Ap^r	This study
pDEL-SlyA	1.75-kb ApaI-SpeI fragment containing deletion of *slyA* ORF cloned into same site of pMRS101, Nal^r, Ap^r, Stm^r	This study
pSlyA	1.29-kb fragment containing entire *slyA* gene with its promoter cloned into pGEM-T Easy; Nal^r Ap^r	This study
pET28a(+)	T7 expression vector; Km^r	Novagen
pETSlyA	0.44-kb fragment containing entire *slyA* gene in pET28a(+)	This study
pGEM-T	A-T cloning vector; Ap^r	Promega
pGEMphoP	0.29-kb *phoP* promoter fragment in pGEM-T	Promega

0.5% NaCl, pH 7.0) at 37°C. *D. dadantii* strains were grown in yeast extract-peptone (YP) medium (1% peptone, 0.5% yeast extract, pH 6.8) or M63 glycerol minimal medium [2.5 g of NaCl, 3 g of KH_2PO_4, 7 g of K_2HPO_4, 2 g of $(NH_4)_2SO_4$, 0.5 mg of $FeSO_4$, 1 g of $MgSO_4$, 2 g of thiamine hydrochloride in 1 liter] and 0.2% (wt/vol) glycerol (4) at 27°C. The plant extract (potato tuber), a crude juice obtained from potato tubers and sterilized by filtration through a 0.45-μm Millipore filter, was used at a final concentration of 1% (vol/vol). The optical density (OD) of the bacterial culture was measured using a Bactomonitor BACT-500 (Intertech, Tokyo, Japan) at 660 nm. When required, antibiotics were added at the following final concentrations: ampicillin at 50 μg/ml, nalidixic acid at 50 μg/ml, kanamycin at 50 μg/ml, streptomycin at 25 μg/ml, and spectinomycin at 70 μg/ml.

Recombinant DNA techniques. Preparation of chromosomal and plasmid DNAs, PCR, gel electrophoresis, restriction endonuclease digestion, DNA ligation, electroporation, and Southern blot hybridization were carried out as described previously (50). Nucleotide sequence analysis was done using a DNA autosequencer (Model 4000; Li-cor, Lincoln, NE). Restriction and modifying enzymes were purchased from Nippon Gene (Tokyo, Japan) and New England Biolabs (Beverly, MA).

Cloning of a *slyA* homologue from *D. dadantii* 3937. The *slyA* gene of *D. dadantii* 3937 is located between *slyB*, an outer membrane lipoprotein gene, and a gene for iron-sulfur (Fe-S) protein (http://asap.ahabs.wisc.edu/annotation/php/ASAP1.htm). To clone the *slyA* gene, a 2.34-kb fragment containing the *slyA* gene, together with 911 bp upstream from the start codon and 999 bp downstream from the stop codon of the *slyA* gene, was amplified by PCR from genomic DNA of *D. dadantii* 3937 using the primer pair PS1 (5′-GGGCCCCCCAGTGCGATATGACCGAA-3′) and PS4 (5′-GAGCTCTGGTGCGTTCGACACACCA-3′). The PCR primers were designed based on the sequence of the *D. dadantii* 3937 *slyA* gene (http://www.genome.wisc.edu/tools/asap.htm). The PCR was carried out for 33 cycles under the following conditions: 94°C for 30 s for denaturation, 60°C for 1 min for annealing, and 72°C for 2 min for extension. The PCR-amplified fragment of the *slyA* homologue was cloned into pGEM-T Easy to generate pSlyX and confirmed by PCR.

Construction of the *slyA* deletion mutant. To construct the *slyA* deletion mutant, the primer pair PS1 (see above) and PS2 (5′-GGGCCCGGGTTCTCCTTATAGTTAGCATACTAAGC-3′) was used to amplify a 911-bp fragment containing the upstream region of *slyA*, while the primer pair PS3 (5′-CCCGGGTTAATAGAAAAAGTAAATGTCTTGGCGGGC-3′) and PS4 (see above) was used to amply a 999-bp fragment containing the downstream region of *slyA* from pSlyX. These two fragments were purified from an agarose gel after gel electrophoresis using a QIAEX II gel extraction kit (Qiagen, Hilden, Germany), ligated, and cloned into a pGEM-T Easy plasmid to create pSlyY and pSlyZ, respectively. The plasmids pSlyY and pSlyZ were digested using ApaI and SmaI, or SmaI and SacI, respectively. The resultant fragments were subcloned into pGEM-T Easy to generate pSlyYZ. Thus, a fragment was constructed with a 438-bp deletion of the entire *slyA* open reading frame (ORF). To construct pDEL-SlyA, the primer pair PS1 (see above) and PS5 (5′-GGCACTAGTGGAGACGTTACAGTGCATCCGAA-3′) was used to PCR amplify a 1.75-kb fragment from pDEL-SlyYZ. After ApaI-SpeI digestion, the product was ligated into the suicide vector pMRS101 (51). This plasmid was used to create a deletion in the *slyA* homologue in the *D. dadantii* 3937 chromosome by double homologous recombination using the sucrose selection marker (*sacB*) as described previously (24).

Complementation of the Δ*slyA* mutant. For complementation of the Δ*slyA* mutant, a 1.29-kb DNA fragment containing the entire *slyA* gene, together with 340 bp upstream from the start codon and 512 bp downstream from the stop codon, was PCR amplified from total genomic DNA of *D. dadantii* 3937 as the template using the primer pair PC1 (5′-AAGCTTTGTTTTCGCAGCCGCAGCGAGCGTTTTAAT-3′) and PC2 (5′-AAGCTTGATACCAAATTCCACGCCGGACAACGTGTG-3′). The resulting fragment was cloned into the multicopy vector pGEM-T Easy to generate pSlyA. This plasmid was then electroporated into the Δ*slyA* mutant using a Cell-Porator (set at 9.4 kV/cm, 160 μF, and 4 Ω;

Bethesda Research Laboratories, Bethesda, MD). The Δ*slyA* mutant carrying pSlyA was then screened on a YP agar plate containing nalidixic acid and ampicillin. A confirmed representative was named Δ*slyA*(pSlyA) and chosen for further study.

Susceptibility to antimicrobial peptides. Susceptibility to antimicrobial peptides was assayed as previously described (18) with a few modifications. Polymyxin B, a cationic, cyclic, amphipathic lipopeptide antibiotic, and salmon protamine, a linear antibiotic, were purchased from Sigma and dissolved in sterilized water to a final concentration of 1 mg/ml as a stock solution. Bacterial strains were grown in M63 glycerol minimal medium to stationary phase, and then 1 ml of the culture was harvested and centrifuged. The cell pellet was resuspended in sterile distilled water. A 100-μl aliquot of the serially diluted bacterial suspension (1×10^7 CFU/ml) was inoculated into M63 glycerol minimal medium containing different concentrations of polymyxin B and salmon protamine and was incubated at 27°C for 4 h with shaking. A portion of each sample was then serially diluted and plated on YP agar containing the appropriate antibiotic. The numbers of CFU with or without treatment were compared after overnight incubation, and the sensitivity was expressed as a percentage by setting the sensitivity of the strains without polymyxin B and salmon protamine to 100%. The experiments were performed in triplicate.

Susceptibility to oxidative stress. For testing susceptibility to oxidative stress, H_2O_2 at 10 mM, which corresponds to a lethal concentration (45), was added to stationary-phase cells grown in M63 glycerol minimal medium, and the cell suspensions were incubated for a further 2 h at 27°C. The number of CFU was determined by serial dilution and plating onto YP agar just prior to incubation and then every 30 min over a 2-h incubation in H_2O_2. The survival of the H_2O_2-treated cells was normalized to the number of CFU at the beginning of the challenge. Similarly, determination of resistance to osmotic challenge was carried out after incubating the stationary-phase cells in the presence of 1 M NaCl. All the experiments were performed in triplicate.

Enzyme assays. Plate assays for major extracellular enzymes, such as pectate lyases, polygalacturonases, cellulases, and proteases, were carried out as described previously (35). A spectrophotometric assay for pectate lyase activity was done as described by Haque and Tsuyumu (18). In brief, bacterial strains were grown in M63 glycerol minimal medium until an OD at 660 nm (OD_{660}) of 1.0 was reached. One milliliter of culture was then sonicated twice for 20 s each time (Ultrasonic Disrupter UD-200; Tomy, Tokyo, Japan) on ice and centrifuged at $20{,}400 \times g$ for 5 min to remove the cell debris. Pectate lyase activity in planta was determined for a homogenate of macerated tissues that were collected using a sterile spatula. The debris was removed by centrifugation ($20{,}400 \times g$ for 5 min), and the supernatant was used for assaying pectate lyase activity. Ten microliters of the supernatant was added to 990 μl of the reaction buffer (0.05% polygalacturonic acid [PGA], 0.1 M Tris-HCl, pH 8.5, 0.1 mM $CaCl_2$ prewarmed to 30°C). After the solution was mixed, the increase in the OD_{230} was measured every 3 min (Ultrospec 3000; Pharmacia Biotech, Cambridge, England). One unit of pectate lyase activity was defined as the amount of enzyme that produced a change in absorbance of 0.001 at 230 nm over 1 min, and the specific activity was expressed as units per milliliter (U/OD_{660} unit).

Quantification of exopolysaccharide. To estimate exopolysaccharide production, bacterial strains were cultured in 5 ml of YP or M63 glycerol minimal medium at 27°C with shaking at 180 rpm until an OD_{660} of 1.0 was reached. One milliliter of culture was then centrifuged at $1{,}500 \times g$ for 10 min at 4°C. The resultant supernatant was mixed with 3 volumes of chilled 95% ethanol and centrifuged at $9{,}100 \times g$ for 20 min at 4°C to precipitate the exopolysaccharide from the culture supernatant. The supernatant was discarded, and after drying, the pellet was redissolved in distilled water. To quantify the purified exopolysaccharide, a phenol-sulfuric acid method described by Hodge and Hofreiter (20) was used. In brief, 1 ml of exopolysaccharide sample was mixed with 1 ml of 5% phenol. Five milliliters of H_2SO_4 was then added and mixed carefully, and the mixture was incubated at room temperature for 20 min. The concentration was measured with a spectrophotometer at an absorbance of 488 nm. The amount of exopolysaccharide was determined using a standard curve for glucose solution.

Pellicle assays. In order to study pellicle formation, bacterial strains were precultured in YP broth to an OD_{660} of 0.8. About 10^6 CFU/ml bacteria was inoculated (1:100) into salt-optimized broth plus 2% glycerol (SOBG) medium (per liter, 20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 2.4 g of $MgSO_4$, 0.186 g of KCl, and 50 ml of 40% glycerol) (64) and incubated at 27°C without shaking for 48 h.

Virulence assays. Potato tubers were purchased from a local supermarket, surface sterilized by submersion in 1% sodium hypochlorite for 10 min, and dried on a clean bench. Single colonies of each bacterial strain were inoculated into M63 glycerol minimal medium and grown to an OD_{660} of 0.2 before being centrifuged and resuspended in sterile distilled water. The potato tubers were inoculated with 20 μl of a suspension containing 1×10^4 CFU using a sterile micropipette tip. Each potato tuber was inoculated with the wild-type and mutant strains separately. The tubers were incubated in a moist chamber at 27°C for 36 h. Due to the small size of the potato tubers, a second experiment was carried out in which only the complemented Δ*slyA*(pSlyA) strain was inoculated. Twenty tubers were inoculated for each experiment. The potato tubers were then sliced in half, and the rotten tissue generated by each strain was scraped out and weighed. The masses of rot generated by each strain were compared, and the differences between the strains were assessed for statistical significance using a two-tailed *t* test. To estimate bacterial populations, the whole of the macerated tissue generated by the wild type and the Δ*slyA* and Δ*slyA*(pSlyA) was collected separately, and the number of bacteria per inoculation site was determined by dilution plating.

TABLE 2. Primers used in this study

Primer set	Sequence (5′→3′)
pelA-F	AACATTCCGGCTAACACC
pelA-R	GGTAACGGTATACCGGATCT
pelB-F	ACCAAAGGCATCACCATC
pelB-R	GTCACGTTGCTGTACAGG
pelC-F	CAACGGTTCTTCCGCTAAC
pelC-R	GACGTACGTTCAGACCAGA
pelD-F	TCAGCGTTCCGTCCAACA
pelD-R	TGCCAAGACCGAAGCTGT
pelE-F	AGCATTCCGTCCAACACC
pelE-R	CCGATACCGAAGCTGTACTG
pelI-F	GTCGTCAGCGAAAATCAGG
pelI-R	GCCACAATTTCCCGTGTTC
pelL-F	GTACAGGCGTCTTATGGG
pelL-R	ATGTTTCTGCCCTGACTG
pelX-F	CAAACCGTCGGCTCCATTC
pelX-R	GCTGTAATCGCCGTCCATC
pelZ-F	ACCACTGCAGCTTTGCCT
pelZ-R	GCCAGATTGTCGCTCATCCA
phoP-F	CCAAACCGTTCCACATCGAG
phoP-R	AGCGACTCCTTGCTGACTAC
phoQ-F	GGGTTGGTTCGTGTATGG
phoQ-R	TATTTCGCTGCGTGTCTG
16S rRNA-F	AGAGGATGACCACCCACACT
16S rRNA-R	CGCATTACACCGCTACACACCT

Quantitative reverse transcription-PCR. The wild type and the Δ*slyA* mutant were grown in M63 glycerol minimal medium, M63 glycerol minimal medium plus 0.4% PGA, and M63 glycerol minimal medium plus 0.4% PGA plus 1% potato tuber extract to early stationary phase, and total RNA was then isolated using a Qiagen RNeasy RNA isolation kit as described by the manufacturer (Qiagen, Hilden, Germany). The RNA was quantified using a NanoDroP ND-100 spectrophotometer (NanoDroP Technologies, Wilmington, DE). After random-decamer-primed first-strand cDNA was synthesized using the Omniscript RT kit (Qiagen, Japan), real-time PCR was performed in an MX3000p Multiplex quantitative-PCR system (Stratagene) using the SYBR Premix Ex*Taq* RT-PCR kit (TaKaRa, Japan). Primers were designed based on *D. dadantii* 3937 DNA sequences obtained from ASAP (http://www.genome.wisc.edu/tools/asap.htm) (14a) using Primer Quest software (http://test.idtdna.com/Scitools/Applications/Primerquest/), and the sequences are shown in Table 2. Primer specificity was assessed by using the dissociation curve protocol on the MX3000p Multiplex quantitative-PCR system (Stratagene). The efficiencies of all primer pairs were verified. The PCR amplification conditions were as follows: denaturing at 95°C for 30 s, annealing at 50°C for 60 s, and extension at 72°C for 30 s for 50 cycles. All PCR experiments were performed in triplicate, and standard deviations were calculated. The fluorescence intensity of SYBR green at each point of the annealing phase was detected, and the cycle threshold (C_T) of each sample was calculated. The calculated C_T data were used for quantitative analysis by the comparative C_T method. For each amplification run, the calculated C_T for each gene amplification was normalized to the C_T of the 16S rRNA gene amplified from the corresponding sample before the difference between the wild type and the mutant was calculated using the following formula: change = $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ for gene $j = (C_{T,j} - C_{T,\text{16S rRNA}})_{\text{mutant}} - (C_{T,j} - C_{T,\text{16S rRNA}})_{\text{wild type}}$.

Purification of the recombinant SlyA. The *slyA* gene was amplified by PCR using primers SlyA_F (5′-CCGCATATGGAATTGCCGTTAGGTTCT-3′) and SlyA_R (5′-GCGGTCGACTTAAGACTGACTCTCATG-3′), which have NdeI

and SalI recognition sites, respectively, at their 5′ ends, and *D. dadantii* 3937 genomic DNA as a template. The amplified fragment was ligated into a pGEM-T Easy vector (Promega) to construct plasmid pGEMSlyA and then subcloned into the NdeI and SalI sites of a pET28a(+) vector (Novagen, Madison, WI) to generate the expression construct pETSlyA. The construct was then transformed into *E. coli* strain BL21(DE3) (Nippon Gene, Japan). A culture of the recombinant *E. coli* BL21(DE3) strain carrying pETSlyA was grown aerobically at 37°C in LB medium with 50 μg/ml kanamycin until mid-log phase (OD_{600} = 0.5 to 0.6) before the addition of inducer (1 mM IPTG [isopropyl-β-D-thiogalactopyranoside]). The culture was further incubated at 37°C for 3 h, and cells were then collected by centrifugation. The harvested cells were resuspended in buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, and 8 M urea, pH 7.6) and sonicated on ice using an Ultrasonic Disruptor UD-200 (Tomy Inc., Tokyo, Japan) with duty cycle 60 and input 4 for 5 min with 3- to 5-min intervals. The cell lysate was collected by centrifugation. The crude lysate was then filtered through a Steradisc 25 (pore size, 0.45 μm; Kurabo, Osaka, Japan) and loaded on a 1-ml HisTrap column from a HisTrap kit (GE Healthcare Biosciences AB, Uppsala, Sweden). The purification of His_6-SlyA was performed according to the manufacturer's instructions with minor modifications. In brief, the bound His_6-SlyA was eluted with elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, and 8 M urea, pH 7.5). The eluted fractions were dialyzed against dialysis buffer (20 mM Tris-Cl, pH 7.4, 500 mM sodium chloride, and 20% glycerol). The partially purified His_6-SlyA protein was then subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane in a Trans-Blot SD semidry transfer cell (Bio-Rad). The His_6-SlyA protein spot was excised from the membrane and subjected to N-terminal amino acid sequencing with a protein sequencer (PPSQ-21A; Shimadzu, Japan).

Gel mobility shift assay. The electrophoretic mobility shift assay (EMSA) was performed as described previously (4) with minor modifications. The 290-bp region (278 bp upstream and the 12-bp coding region of *phoP*) was amplified by PCR with phoP_F (5′-TCTAAGCTTGGCGGCATTCTCTCG-3′) and phoP_R (5′-CAGGGATCCAAGAATGCGCATGAC-3′) primers, and the amplified fragment was ligated to a pGEM-T vector (Promega) to generate plasmid pGEMphoP. After the orientation of the fragment in pGEMphoP was confirmed, the probe was prepared by PCR using primers phoP_F and T7 (5′-CCATGGCCGCGGGAT-3′) labeled with fluorescent rhodamine dye (Fasmac, Atsugi, Japan) with pGEMphoP as the template. The labeled *phoP* promoter fragment was purified from the agarose gel after electrophoresis using an Illustra GFX PCR DNA and Gel Purification Kit (GE Healthcare, Buckinghamshire, United Kingdom). For EMSA, 25 ng of labeled promoter fragment and an appropriate concentration of partially purified protein (50 nM, 100 nM, 200 nM, and 400 nM) were mixed and incubated in a binding buffer [4 mM Tris-Cl, pH 8.0, 0.1 mM EDTA, 50 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg poly(dI-dC) · poly(dI-dC), 2 μg bovine serum albumin, and 10% glycerol]. After incubation for 30 min at 27°C, the reaction mixture was loaded onto a 6% polyacrylamide nondenaturing gel in high-ionic-strength buffer (1× Tris-borate-EDTA), and electrophoresis was carried out in the same buffer for about 2 h. The gel was then visualized with a Pharos FX Plus Molecular Imager using Quantity One one-dimensional gel analysis software (Bio-Rad, Hercules, CA).

RESULTS

Generation of the Δ*slyA* mutant. The *slyA* gene of *D. dadantii* 3937 was cloned into the pGEM-T Easy vector, and its nucleotide sequence was confirmed (data not shown). The sequence data showed that the 438-bp ORF of *slyA* encodes a protein of 145 amino acids. Homology searches revealed that the *slyA* ORF of *D. dadantii* 3937 had 85, 74, and 75% similarity to the translated products of Hor from *P. carotovorum* subsp. *carotovorum* (ID-AAD50820), SlyA of *E. coli* K-12 (ID-ABE-005495) and *S. enterica* serovar Typhimurium (ID-ABS-0083026), and RovA of *Y. pseudotuberculosis* (ID-ACZ-0002372), respectively, at the amino acid level. A *slyA* gene deletion mutant (denoted Δ*slyA*) was constructed as described in Materials and Methods. A mutation in the *slyA* gene was confirmed by PCR and DNA sequencing (data not shown) following the selection of five independent colonies exhibiting the same phenotypes, e.g., pectate lyase production and pellicle formation. When the growth rate of the Δ*slyA* mutant was compared with that of the wild-type strain in YP and M63 glycerol minimal media, no significant difference was observed (data not shown).

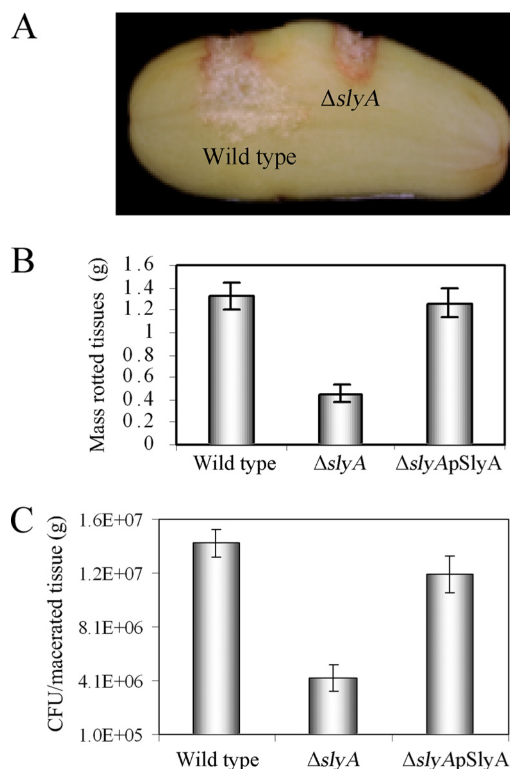

FIG. 1. Inactivation of *slyA* attenuates the virulence of *D. dadantii* 3937. The bacterial inoculum of 20 μl contained 10^4 CFU/ml of the wild type (*D. dadantii* 3937), the Δ*slyA* mutant, and the complementation strain [Δ*slyA*(pSlyA)]. The disease symptoms (A), mass of the rotted tissues (B), and bacterial populations in macerated tissue (C) were documented after 36 h of incubation at 27°C with high relative humidity. The error bars indicate standard deviations.

Reduced virulence of the Δ*slyA* mutant. The impact of the Δ*slyA* mutant on the virulence of *D. dadantii* 3937 was evaluated in potato tubers by measuring the extent of macerated tissue 36 h postinoculation. The Δ*slyA* mutant was significantly reduced (threefold [$P < 0.0001$]) in virulence compare to the wild-type strain (Fig. 1A). When the plasmid pSlyA (with a *slyA* gene on a multicopy vector, pGEM-T Easy) was introduced into Δ*slyA*, virulence recovered to the wild-type level (Fig. 1B) ($P > 0.42$). Bacterial populations of the Δ*slyA* mutant in potatoes were found to be reduced significantly (1 order of magnitude; $P < 0.0001$) compared to the wild-type strain (Fig. 1C). Furthermore, when pectate lyase production levels by the wild type and the Δ*slyA* mutant in macerated potato tubers were compared, the specific enzymatic activity of the Δ*slyA* mutant was reduced to 7.12% (a 14-fold reduction) with respect to that of the wild-type strain.

Failure of hyperinduction of pectate lyase in the Δ*slyA* mutant. When bacteria were grown in M63 glycerol minimal medium (noninduced conditions), the basal level of pectate lyase production by the wild type was higher than that by the Δ*slyA*

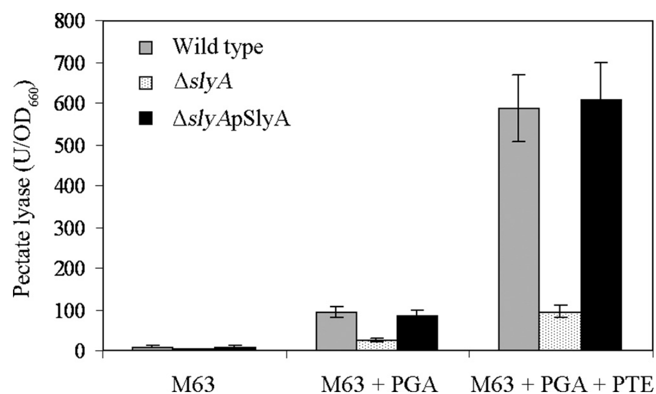

FIG. 2. Pectate lyase specific activity. Strains were grown in M63 glycerol minimal medium (noninduced), M63 glycerol minimal medium plus 0.4% PGA (induced), and M63 glycerol minimal medium plus 0.4% PGA plus 1% potato tuber extract (PTE) (hyperinduced) to an OD_{660} of 1.0 before the total pectate lyase specific activity was determined with a spectrophotometer. The mean of pectate lyase activities from five independent experiments was expressed as the specific activity (U/OD_{660}). The error bars indicate the standard deviations.

mutant (Fig. 2). Pectate lyase production was greatly induced (9.7-fold) in the wild type compared to the Δ*slyA* mutant when the M63 glycerol minimal medium was supplemented with 0.4% PGA (Fig. 2). Furthermore, when bacterial strains were grown in M63 glycerol minimal medium with 0.4% PGA and 1% potato tuber extract, pectate lyase synthesis was hyperinduced (61-fold) in the wild type but not in the Δ*slyA* mutant (Fig. 2). The Δ*slyA* mutant transformed with pSlyA restored pectate lyase production to the wild-type level (Fig. 2). However, the synthesis of polygalacturonases, cellulases, and proteases was not affected under the same conditions (data not shown). Thus, the SlyA homologue of *D. dadantii* 3937 appears to play an important role specifically in the regulation of pectate lyase, but not of the other extracellular enzymes tested.

Expression of *pel* genes under various growth conditions. *D. dadantii* 3937 produces numerous pectate lyase isozymes encoded by *pel* genes. In order to identify which *pel* genes are controlled by *slyA*, the transcriptional profile of a *D. dadantii* 3937 mutant strain lacking a functional *slyA* gene was compared with that of the wild-type strain under noninduced, induced, and hyperinduced conditions by quantitative reverse transcription-PCR (Table 3). The transcription levels of *pelA*, *pelB*, *pelC*, *pelD*, *pelE*, *pelI*, and *pelL* were lower in the Δ*slyA* mutant than in the wild-type strain under noninducing conditions. However, the addition of PGA and PGA plus potato tuber extract showed different effects on the individual *pel* genes. The transcription levels of *pelA*, *pelD*, *pelE*, *pelI*, *pelL*, and *pelZ* showed greater induction in the presence of PGA than in M63 glycerol minimal medium (noninduced) only in the wild-type background. However, in the presence of PGA plus potato tuber extract, the transcription levels of *pelD*, *pelI*, and *pelL* showed hyperinduction in the wild-type but not in the Δ*slyA* background. Thus, the *slyA* homologue of *D. dadantii* 3937 appears to be involved specifically in the product induction (60) of all isozymes of pectate lyase and in the hyperinduction of one major and two minor *pel* genes.

Stress response of the Δ*slyA* mutant. Members of the MarR family in animal-pathogenic bacteria have shown resistance to multiple antibiotics, household disinfectants, and oxidative stress (1, 2, 7). When the sensitivities of the wild-type and Δ*slyA* mutant strains to various antibiotics, including tetracycline (0.1 μg/ml), chloramphenicol (0.1 μg/ml), erythromycin (0.01 μg/ml), and rifampin (rifampicin) (0.1 μg/ml), were compared, no significant differences were observed (data not shown). However, after 15 min of exposure to 1% sodium hypochlorite, there was an 80% reduction in the survival of the Δ*slyA* mutant compared to the wild-type strain (data not shown).

When stationary-phase cultures of the wild type and the Δ*slyA* mutant were diluted to 10^7 CFU/ml in M63 glycerol minimal medium and exposed to 10 mM H_2O_2 and the number of viable cells was determined as a function of time, the survival of the Δ*slyA* mutant was significantly lower than that of the wild-type strain (Fig. 3A). However, when plasmid pSlyA was transformed into the Δ*slyA* mutant, sensitivity to H_2O_2 returned to the wild-type level.

A *Salmonella* mutant lacking a functional *slyA* gene was reported to be highly sensitive to polymyxin B due to alteration of cell surface properties, mainly modification of lipopolysaccharide (54). The sensitivities of *D. dadantii* 3937 wild type and Δ*slyA* to the *c*ationic *a*nti*m*icrobial *p*eptide (CAMP) polymyxin B and a linear antimicrobial peptide, salmon protamine, were compared. The Δ*slyA* mutant showed increased sensitivity to polymyxin B compared to the wild-type strain (Fig. 3B), but no difference was found in the sensitivities to protamine (data not shown). Resistance to polymyxin B in the Δ*slyA* mutant was completely restored by complementation with the *slyA* gene and its promoter on a multicopy vector, pGEM-T Easy, confirming that SlyA is essential for the resistance exhibited to polymyxin B in *D. dadantii* 3937.

SlyA homologues of *S. enterica* serovar Typhimurium, *E. coli*, and *Y. pseudotuberculosis* are regulated by various environmental cues, such as temperature, pH, and osmolarity (9, 36). However, sensitivity to high temperature (37°C), acidic pH (4.0 to 5.5), and high osmolarity (1 M NaCl) were indistinguishable between the wild type and the Δ*slyA* mutant in *D. dadantii* 3937, suggesting that SlyA of *D. dadantii* 3937 may not

TABLE 3. Differential expression of *pel* genes under various growth conditions

Gene name	Fold change compared to the Δ*slyA* mutant[a]		
	M63 glycerol minimal medium	M63 glycerol minimal medium + 0.4% PGA	M63 glycerol minimal medium + 0.4% PGA + 1% potato tuber extract
pelA	0.9 ± 0.2	12.8 ± 1.3	16.5 ± 2.7
pelB	0.4 ± 0.03	0.7 ± 0.04	3.3 ± 0.7
pelC	0.6 ± 0.06	0.7 ± 0.04	2.9 ± 0.8
pelD	0.9 ± 0.08	13.5 ± 1.4	1052.7 ± 94
pelE	0.5 ± 0.02	1.3 ± 0.09	9.7 ± 0.9
pelI	0.6 ± 0.02	11.9 ± 1.8	1031.3 ± 83
pelL	0.3 ± 0.06	42.8 ± 3.3	826.7 ± 79
pelZ	0.56 ± 0.1	4.9 ± 0.7	14.3 ± 3.6

[a] Expressed as the ratio of the specific gene expression level in the wild type compared to that in the Δ*slyA* mutant normalized to the level of expression of the 16S rRNA gene.

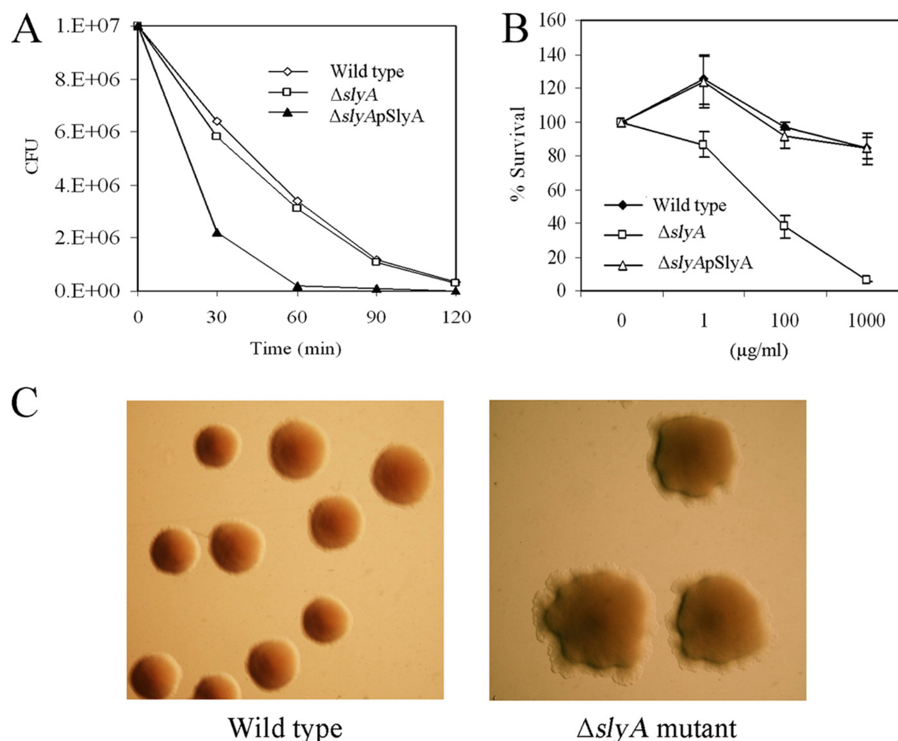

FIG. 3. Sensitivity tests. (A) Sensitivities of the different bacterial strains to H_2O_2. The data represent one of three separate experiments, which gave similar results. (B) Sensitivity to polymyxin B, showing the mean and standard deviation of five separate experiments. (C) Colony morphologies of the wild type and the Δ*slyA* mutant after exposure to hydrogen peroxide. The photographs represent one of three separate experiments, which gave similar results.

be involved in these forms of regulation. Although the wild type and the Δ*slyA* mutant had similar appearances when grown in YP and on minimal medium agar plates without treatment, Δ*slyA* colonies did respond to hydrogen peroxide (Fig. 3C), sodium hypochlorite, polymyxin B, high temperature, acidic pH, and high osmolarity (data not shown) by showing irregular or wrinkled colonies compared to the normal smooth and round colonies of the wild type (Fig. 3C). This result suggested that SlyA mediates alteration of the cell surface properties of *D. dadantii* 3937, as reported for *Salmonella* (54).

Reduced exopolysaccharide production. Synthesis of exopolysaccharide is an important pathogenicity factor for many plant-pathogenic bacteria, including *D. dadantii* 3937 (8, 11, 29). When the wild-type and Δ*slyA* mutant strains were grown in M63 glycerol minimal medium until early stationary phase (OD_{660} = 1.0), production of exopolysaccharide by the Δ*slyA* mutant (0.31 ± 0.04 mg/ml) was reduced to 50% of the wild-type strain production (0.59 ± 0.07 mg/ml). Similar results were also found when the bacterial strains were grown in YP medium (data not shown). Thus, SlyA appears to be required for exopolysaccharide production in *D. dadantii* 3937.

It has been reported that exopolysaccharide production and motility are coregulated in many plant-associated bacteria (6, 38, 65). When the wild-type and Δ*slyA* mutant strains were grown overnight in YP or M63 glycerol minimal medium and the cultures were examined for motility under a phase-contrast microscope, both the wild type and the Δ*slyA* mutant were motile (data not shown). Conversely, the Δ*slyA* mutant (22 ± 0.67 mm) migrated slightly faster than the wild-type strain (19 ± 0.8 mm) through 0.3% YP soft-agar plates. However, when purified flagellin proteins from the wild type and the Δ*slyA* mutant were analyzed by SDS-PAGE, the amounts of flagellin protein were indistinguishable between the wild type and the Δ*slyA* mutant (data not shown). Thus, we are currently unable to correlate exopolysaccharide production and motility, which is considered to be one of the important virulence factors in many soft-rotting bacteria. Similarly, *cytR*, encoding a transcriptional repressor, was shown to negatively regulate motility by controlling the expression of genes required for flagellum biosynthesis without affecting exopolysaccharide production in *P. carotovorum* subsp. *carotovorum* (35).

Inability to form pellicles. After 48 h of incubation at 27°C, the wild type and the Δ*slyA*(pSlyA) strain formed thick and rigid pellicles (a bacterial network at the air-liquid interface that blocks the surface of the standing culture) on SOBG medium, whereas the Δ*slyA* mutant was unable to form a pellicle (Fig. 4A). In the enterobacterial animal and plant pathogens *S. enterica* serovar Typhimurium, *E. coli*, and *D. dadantii* 3937, it has been reported that cellulose contributes to pellicle formation (48, 55, 64). To determine whether cellulose is also a component of the pellicle formed by *D. dadantii* 3937, cells at a density of 10^5 CFU/ml were placed on M63 glycerol minimal medium agar plates containing the cellulose binding dye calcofluor (200 μg/ml) and incubated at 27°C for 48 h before being checked under UV light (366 nm). The wild-type and Δ*slyA*(pSlyA) strains induced bright fluorescence, while the Δ*slyA* mutant did not (Fig. 4B). Thus, the SlyA homologue

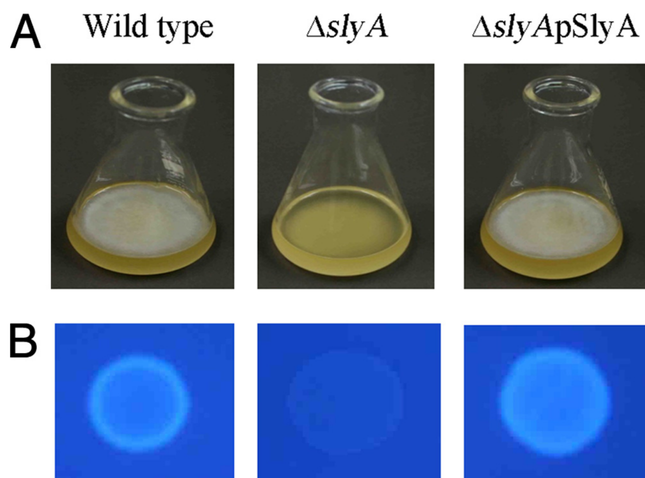

FIG. 4. (A) Pellicle formation following growth in SOBG medium without shaking at 27°C for 48 h. (B) Strains were grown in M63 glycerol minimal medium agar plates with calcofluor at 27°C for 48 h and then exposed to UV light. The photographs represent one of three separate experiments, which gave similar results.

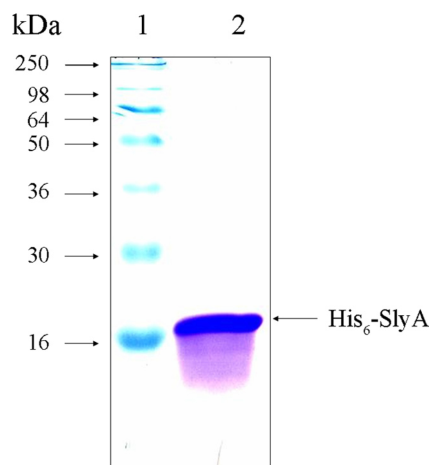

FIG. 6. SDS-PAGE of recombinant His_6-SlyA. *E. coli* BL21(DE3) carrying pETSlyA was induced by IPTG and purified as described in Materials and Methods. The purified protein (lane 2) was subjected to 12% SDS-PAGE. Molecular mass standards were loaded in lane 1.

may positively regulate pellicle formation, and those pellicles have been confirmed to contain cellulose in *D. dadantii* 3937.

SlyA regulates the expression of the *phoP-phoQ* operon. The Δ*slyA* mutant of *D. dadantii* 3937 showed phenotypes similar to those of *phoP* and *phoQ* mutants, including reduced virulence, hyperinduction of pectate lyase, and sensitivity to CAMP (18). To see whether SlyA has an effect on the transcription of *phoP* and *phoQ*, we determined the expression of these genes following growth of the wild type and the Δ*slyA* mutant in M63 glycerol minimal medium supplemented with low (10 μM) or high (10 mM) concentrations of magnesium. The *phoP* and *phoQ* expression levels were then analyzed by quantitative reverse transcription-PCR. The expression levels of *phoP* and *phoQ* were indistinguishable in the wild type and the Δ*slyA* mutant at high magnesium concentrations (data not shown). However, at low magnesium concentrations, both *phoP* and *phoQ* showed increased levels of transcription in the wild type compared to that in the Δ*slyA* mutant (Fig. 5). The reduced expression levels of *phoP* and *phoQ* in the Δ*slyA* mutant were recovered by introducing plasmid pSlyA into the Δ*slyA* mutant (data not shown), indicating that SlyA may play a role in regulating the transcription of the *phoP-phoQ* operon in *D. dadantii* 3937.

The SlyA protein binds to the promoter region of the *phoP* gene. Inspection of the *phoP* promoter region revealed that a putative SlyA binding site (ATAGTTCACTAA; the consensus is TTAGCAAGCTAA) is present in the promoter region (76 to 65 bp upstream of the translational start site). We speculated that SlyA possibly binds to this site and regulates *phoP* expression. In order to determine whether SlyA physically binds or associates with the *phoP* promoter region, polyhistidine-tagged SlyA (His-SlyA) was purified (Fig. 6) and EMSA analysis was performed with the protein. It was revealed that His-SlyA was able to bind to a *phoP* promoter fragment in a concentration-dependent manner (Fig. 7). Binding was observed at a His-SlyA concentration of 200 nM. At a His-SlyA

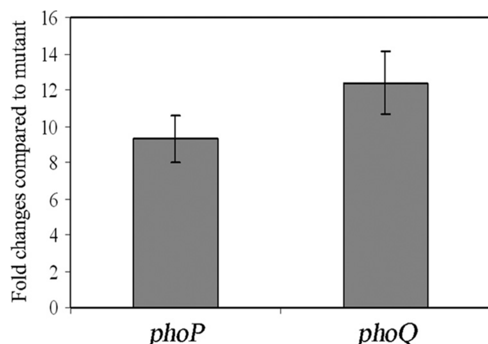

FIG. 5. Effect of *slyA* on *phoP-phoQ* expression. Bacterial strains were grown in M63 glycerol minimal medium containing a low concentration (10 μM) of magnesium plus 0.4% PGA plus 1% potato tuber extract to an OD_{660} of 1.0 before total RNA was harvested. The expression levels of *phoP* and *phoQ* in a Δ*slyA* background were compared to those in the wild type by quantitative reverse transcription-PCR and normalized to the level of expression of the 16S rRNA gene. The results show the means of three replicates. The error bars indicate the standard deviations.

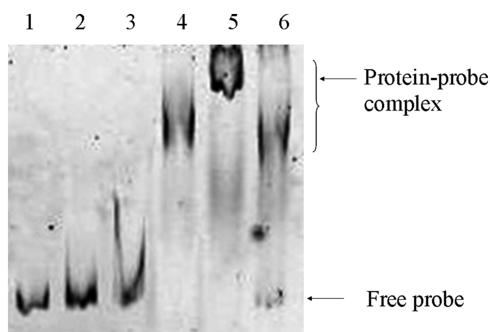

FIG. 7. EMSA using the promoter region of *phoP* and purified His-SlyA protein. Labeled probe (25 ng) was incubated in the absence (lane 1) or presence of increasing amounts of protein (50, 100, 200, and 400 nM) (lanes 2 to 5, respectively) or in the presence of labeled probe (25 ng), protein (400 nM), and a 50-fold excess of unlabeled probe as a specific competitor (lane 6).

concentration of 400 nM, a binding complex with a higher molecular weight (Fig. 7, lane 5) was observed, with apparently complete binding of the probe. At 200 nM, the protein possibly formed a dimer, and at a higher concentration (400 nM), it possibly formed a tetramer. When a 50-fold excess of unlabeled promoter fragment was used, unbound labeled probe was detected at the bottom of the gel, indicating that SlyA specifically binds to the *phoP* promoter region (Fig. 7, lane 6).

DISCUSSION

In this study, we demonstrated that a mutant strain lacking a functional *slyA* gene in *D. dadantii* 3937 had pleiotropic effects: (i) diminished virulence in potato tubers; (ii) decreased survival ability in its host, potato; (iii) increased sensitivity to the CAMP polymyxin B, sodium hypochlorite, and oxidative stress; (iv) reduced exopolysaccharide production; (v) inability to form pellicles; and (vi) failure of hyperinduction of pectate lyase production while normal levels of polygalacturonases, cellulases, and proteases were observed. Changes in these phenotypes in the Δ*slyA* mutant were restored by introducing the *slyA* homologue of *D. dadantii* 3937 on a multicopy vector, pGEM-T Easy [Δ*slyA*(pSlyA)]. Thus, SlyA is a global transcriptional regulator involved in the regulation of the synthesis of a large group of virulence-associated factors in *D. dadantii* 3937.

SlyA was originally identified as an *S. enterica* serovar Typhimurium gene product by screening for cytolysin on blood agar plates and was first thought to encode a cytolysin, a cytotoxic protein that forms stable, cation-selective transmembrane pores. It was later shown to be a regulator, activating the expression of a cryptic cytolysin in *E. coli* K-12 (13, 34). It has been reported that *Salmonella slyA* mutant strains are reduced in virulence and are unable to colonize the Peyer's patches, mesenteric lymph nodes, liver, and spleen (7, 13, 30). It has also been reported that the reduced virulence of the *slyA* mutant of *S. enterica* serovar Typhimurium is, in part, due to hypersusceptibility to reactive oxygen species (7). The Δ*slyA* mutant of *D. dadantii* 3937 also had high sensitivity to oxidative stress. Thus, the increased sensitivity of the Δ*slyA* mutant to hydrogen peroxide might have reduced its population in planta, which ultimately resulted in lower levels of disease than with the wild type. RovA, whose amino acid sequence is 75% identical to that of *D. dadantii* 3937 SlyA, also plays an important role in regulating the invasion of mammalian cells by *Yersinia* and mediates the regulation of the invasion response to environmental signals (36).

The Hor regulator, a SlyA homologue reported by Thomson et al. (58) in the phytopathogenic bacteria *P. carotovorum* subsp. *carotovorum*, has some common features with the SlyA protein of *D. dadantii* 3937, as the corresponding mutant is reduced in pectate lyase production and virulence. Sensitivity to antimicrobial peptides, susceptibility to hydrogen peroxide and sodium hypochlorite, pellicle formation, and exopolysaccharide production have been described for *P. carotovorum* subsp. *carotovorum*, too (58). However, it seems that there are differences between the *P. carotovorum* subsp. *carotovorum* and *D. dadantii* mutants, as polygalacturonase and cellulase production is affected in the former but not in the latter.

PecS, a MarR family transcriptional regulator, is required for pathogenesis in *D. dadantii* 3937 (46). It is interesting that both PecS and SlyA are involved in both pectate lyase production and sensitivity to oxidative stress. Here, we observed that the Δ*slyA* mutant was sensitive to oxidative stress and reduced pectate lyase production, whereas the *pecS* mutant was more resistant to oxidative stress (45) and increased pectate lyase production (45). In addition, PecS negatively affects the expression of cellulase and flagellar biosynthesis (42, 46, 49) and also positively regulates the expression of polygalacturonase (37). However, the expression of cellulase, polygalacturonase, and flagellar biosynthesis was not regulated by SlyA of *D. dadantii* 3937 (data not shown). These differences in the production of plant cell-wall-degrading enzymes, sensitivity to oxidative stress, and flagellar biosynthesis may be distinguishable between PecS and SlyA.

Increased sensitivity to polymyxin B, as in the Δ*slyA* mutant of *D. dadantii* 3937, is also reported in the *slyA* mutant of *S. enterica* serovar Typhimurium. In *S. enterica* serovar Typhimurium, the increased sensitivity to the CAMP is due to the effects of genes regulated by the PhoP-PhoQ system, namely, *pmrA-pmrB* (15, 16), *pagP* (17), and *ugtL* (54). In all these cases, the gene product modifies the cell surface properties of the bacterium by changing the structure of lipid A in the bacterial lipopolysaccharide. *pmrA-pmrB* are responsible for adding 1 unit of 4-aminoarabinose, and *pagP* adds one extra acyl group to lipopolysaccharide. Such modification may lead to altered resistance to the CAMP polymyxin B (16). We speculate that a similar mechanism operates in *D. dadantii* 3937, as genes with high homology to *pmrA* (60%), *pmrB* (51%), and *pagP* (70%) are found in the *D. dadantii* 3937 genome database (http://asap.ahabs.wisc.edu/annotation/php/ASAP1.html). It should be noted that lipopolysaccharide plays an important role in the pathogenesis of *D. dadantii* 3937 and *Ralstonia solanacearum* (53, 59).

Exopolysaccharides are considered to be essential for bacterial growth, survival under environmental stress conditions, and virulence in many plant-pathogenic bacteria (8, 31, 32). In the case of *Pseudomonas syringae*, it has been reported that the exopolysaccharide alginate contributes to virulence and epiphytic fitness by facilitating colonization and/or dissemination of the bacterium in planta (65). The mutants of *P. syringae* pv. syringae with defective exopolysaccharide production, such as *algT*, *aefR*, and the double mutant *ahlI-ahlR*, exhibited increased sensitivity to hydrogen peroxide and heat shock (25, 43). In the case of *D. dadantii* 3937, a *pecT* mutant was shown to have reduced exopolysaccharide production and virulence on saintpaulia (8). We observed that the Δ*slyA* mutant had significantly reduced exopolysaccharide production and virulence on its host, potato, suggesting that exopolysaccharide is an important virulence factor in *D. dadantii* 3937. Recently, Corbett and associates (9) reported that SlyA is involved in the regulation of capsular polysaccharide (K5) in *E. coli*.

In contrast, pellicles are essential for the survival of bacteria under various environmental stress conditions. For examples, *S. enterica* serovar Typhimurium cells within pellicles showed resistance to chlorine treatment (52). Solano et al. (55) also demonstrated that cellulose is a component of the pellicle and that cellulose is required for chlorine resistance by *Salmonella*, since cellulose-negative mutants did not survive under low concentrations of NaOCl. However, cellulose deficiency does not affect *Salmonella* virulence (55). In our present study, the

Δ*slyA* mutant was unable to form pellicles and did not produce cellulose (Fig. 4). Thus, formation of a pellicle by SlyA may play an important part in the survival of *D. dadantii* cells under unfavorable environmental stress conditions.

Salmonella slyA mutants exhibit phenotypes similar to those displayed by *phoP* and *phoQ* mutants (30, 54). The PhoP-PhoQ two-component regulatory system is required for transcription from one of the promoters of the *slyA* gene in *Salmonella* (38), suggesting that the phenotypes displayed by *phoP* and *phoQ* mutants could be caused by their inability to express the SlyA protein and implying that SlyA participates in the transcription of a subset of PhoP-regulated genes. It has been suggested that the PhoP protein regulates the *slyA* gene indirectly, because a PhoP box could not be identified in the *slyA* promoter (41). However, Shi et al. (54) argued that PhoP controls *slyA* transcription directly. Furthermore, Song and associates (56) demonstrated that SlyA fine tunes the cellular level of the PhoP-PhoQ system and participates in a positive feedback loop, which facilitates transcription of the *phoPQ* loci, in turn stimulating transcription of the PhoP regulon. We observed that SlyA regulates expression of *phoP* and *phoQ* in response to magnesium (Fig. 5). Furthermore, the transcription levels of genes encoding the pectate lyases PelA, PelB, PelC, PelD, and PelE, previously identified as being controlled by PhoQ (61), were found to be regulated by SlyA of *D. dadantii* 3937 (Table 3). These findings prompted us to speculate that SlyA may regulate the expression of the *phoP* gene. EMSA clearly demonstrated the specific binding of the SlyA protein to the *phoP* promoter region. Thus, SlyA possibly regulates the pathogenicity-related phenotypes by utilizing the PhoP regulator. Therefore, it may provide evidence for an intimate link between PhoP-PhoQ and SlyA in *D. dadantii* 3937.

The ability of *D. dadantii* 3937 to macerate plant tissue results from the actions of at least five major and five minor *pel* genes. A basal level of *pel* gene expression was observed in *D. dadantii* 3937 in the absence of induction substrates (21). In the presence of pectin and its derivatives from host plants, pectinase synthesis is further induced, leading to symptom development. It has been reported that during plant infection, *pelD*, *pelE*, and *pelI* are highly expressed in potato tubers, whereas *pelA*, *pelB*, *pelC*, *pelL*, *pelZ*, and *pemA* are moderately expressed (47). Using a Gus reporter, Jafra and associates (23) showed that *pelI* and *pelL* of *D. dadantii* 3937 were highly (10-fold) induced in potato tubers. Recently, Peng et al. (41) discovered that a significant increase in *pelD* expression occurred in potato tubers 24 h postinoculation. Minor *pel* genes have been shown to be induced only in plant tissue or in the presence of plant extract (26). In this study, we showed that pectate lyase production is hyperinduced in planta. Accordingly, when bacteria were grown in M63 glycerol minimal medium, along with 0.4% PGA and 1% potato tuber, transcript levels of *pelD*, *pelI*, and *pelL* were increased (Table 3). The roles of the *pelD*, *pelI*, and *pelL* genes in pathogenicity have been reported previously (23). Thus, SlyA may contribute to virulence in terms of hyperexpression of these *pel* genes in *D. dadantii* 3937.

In conclusion, our results show that the SlyA homologue of *D. dadantii* 3937 supports a wide variety of cellular functions, including synthesis of multiple virulence factors and stress tolerance. In many bacteria, the production of virulence factors is regulated by more than one mechanism to facilitate coordinated regulation. SlyA may operate as part of the multiple regulatory mechanism that governs virulence in *D. dadantii* 3937. Also, these results imply that SlyA has become adapted to counteract different but related stresses in plant- and animal-pathogenic bacteria. To gain a better understanding of the mechanisms of pathogenicity in *D. dadantii* 3937, a SlyA microarray analysis is under way.

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