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Commensal Effect of Pectate Lyases Secreted from *Dickeya dadantii* on Proliferation of *Escherichia coli* O157:H7 EDL933 on Lettuce Leaves[▽]

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The outbreaks caused by enterohemorrhagic *Escherichia coli* O157:H7 on leafy greens have raised serious and immediate food safety concerns. It has been suggested that several phytopathogens aid in the persistence and proliferation of the human enteropathogens in the phyllosphere. In this work, we examined the influence of virulence mechanisms of *Dickeya dadantii* 3937, a broad-host-range phytopathogen, on the proliferation of the human pathogen *E. coli* O157:H7 EDL933 (EDL933) on postharvest lettuce by coinoculation of EDL933 with *D. dadantii* 3937 derivatives that have mutations in virulence-related genes. A type II secretion system (T2SS)-deficient mutant of *D. dadantii* 3937, A1919 (Δ*outC*), lost the capability to promote the multiplication of EDL933, whereas Ech159 (Δ*rpoS*), a stress-responsive σ factor RpoS-deficient mutant, increased EDL933 proliferation on lettuce leaves. A spectrophotometric enzyme activity assay revealed that A1919 (Δ*outC*) was completely deficient in the secretion of pectate lyases (Pels), which play a major role in plant tissue maceration. In contrast to A1919 (Δ*outC*), Ech159 (Δ*rpoS*) showed more than 2-fold-greater Pel activity than the wild-type *D. dadantii* 3937. Increased expression of *pelD* (encodes an endo-pectate lyase) was observed in Ech159 (Δ*rpoS*) *in planta*. These results suggest that the pectinolytic activity of *D. dadantii* 3937 is the dominant determinant of enhanced EDL933 proliferation on the lettuce leaves. In addition, RpoS, the general stress response σ factor involved in cell survival in suboptimal conditions, plays a role in EDL933 proliferation by controlling the production of pectate lyases in *D. dadantii* 3937.

Strains of enterohemorrhagic *Escherichia coli* (EHEC) belonging to the serotype O157:H7 are known to be associated with severe human diseases (12). Human EHEC infections progress in three stages: (i) intimate attachment of bacteria to host cells, (ii) actin condensation and microvillus effacement (hallmark attaching and effacing lesions), and (iii) production and delivery of Shiga toxin. The progression of the EHEC infection through these stages can lead to hemorrhagic colitis and hemolytic-uremic syndrome in humans (41). In recent years, a great majority of *E. coli* O157:H7 outbreaks have been traced to consumption of contaminated meats and leafy greens (12). Healthy cattle are the principal and natural reservoir of *E. coli* O157:H7 (58). The potential mechanisms of *E. coli* O157:H7 contamination include soil amendments and water and airborne deposition from off-farm activities, such as cattle/dairy and manure/composting operations (12, 22). Thus, the contamination of leafy greens by *E. coli* O157:H7 is generally thought to occur during the growth and harvesting of crops. Although the soil and phyllosphere of plants present an inhospitable environment for survival of enteric human pathogens, field crops have been implicated in many outbreaks of foodborne illnesses (52). Specifically, several severe *E. coli* O157:H7 outbreaks have been traced back to vegetables, such as alfalfa (18), spinach (21), and lettuce (19). *E. coli* O157:H7 has been shown to persist in the soil of crop fields for an extended period of time, over 5 months (26). Processing of leafy green vegetables has also proven to be inefficient in eliminating pathogens. After harvesting, lettuce is generally washed with a primary chlorine rinse, normally 200 ppm, and stored at a low temperature (0 to 2°C) (9). However, even after sanitization and cold storage, the threat of *E. coli* O157:H7 contamination on lettuce leaves remains, owing to the fact that the penetration depth of *E. coli* O157:H7 into lettuce tissues from cut edges increases as storage temperature decreases (49).

Upon contact with plants, *E. coli* O157:H7 attaches to and colonizes damaged areas of the plant to obtain nutrients and water essential for long-term survival in the phyllosphere (49). Interestingly, recent reports suggest that the survival of *E. coli* O157:H7 on plants can be extended and strengthened by the presence of phytopathogenic bacteria (5, 10). The phytopathogen *Xanthomonas campestris* pv. vitians has been reported to improve persistence of *E. coli* O157:H7 on lettuce plants (5). Recently, *Dickeya dadantii* 3937 (synonymous with *Erwinia chrysanthemi* 3937) was reported to promote the multiplication of *E. coli* O157:H7 on postharvest lettuce (10). *D. dadantii* is a causative agent of soft-rot disease in an array of economically important crops (34). In this bacterium, several virulence determinants mutually cooperate to cause plant disease. Initially, *D. dadantii* senses the plant hormone jasmonate, which is synthesized in wounded plant tissues, and moves toward the wound openings by chemotaxis and motility mechanisms (3, 4). Entry of the bacteria into the plant host induces expression of the type III secretion system (T3SS), which transfers several type III (T3) effectors directly into host cells to suppress host

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli O157:H7 EDL933	Enterohemorrhagic *E. coli* wild type, Rif^r	Lab stock
D. dadantii		
3937	Wild type	N. Hugouvieux-Cotte-Pattat, France
A1919	*lmrT*(Con) *lacZ outC*::*uidA*-Km	14a
Ech159	Δ*rpoS*::Km^r	32
Ech166	Δ*fliA*::Km^r	Lab stock
Ech169	Δ*hrpA*::Km^r	Lab stock
A1919C	A1919 complemented by single-copy chromosomal integration of *outC*	This work
Ech159C	Ech159 complemented by single-copy chromosomal integration of *rpoS*	This work
Plasmids		
pPROBE-AT	Promoterless *gfp* containing the broad-host-range vector, Ap^r	38
pAT-NPTII	pPROBE-AT carrying Km^r and its native promoter, Ap^r and Km^r	This work
pPROBE-AT::P*pelD*	pPROBE-AT carrying a 0.6-kb DNA fragment containing the *pelD* promoter, Ap^r	56
pTCLSCm	Cm^r in between the *lacY* and protease coding gene, Cm^r and Ap^r	57
pTCLS-outC	pTCLSCm carrying *outC* and its native promoter, Cm^r	This work
pTCLS-rpoS	pTCLSCm carrying *rpoS* and its native promoter, Cm^r	This work

[a] Rif^r, resistant to rifampin; Ap^r, resistant to ampicillin; *uidA*-Km, *uidA* linked to a kanamycin resistance cassette; Km^r, resistant to kanamycin; Cm^r, resistant to chloramphenicol.

defenses and facilitate proliferation (1, 54). Finally, when *D. dadantii* reaches a certain cell density, it produces a large number of cell wall-degrading enzymes (CWDEs) and causes soft-rot symptoms (8, 25, 29, 51).

There is a growing number of reports on the occurrence of novel interactions between phytopathogenic and human enteropathogenic bacteria on plants (5, 10). However, the mechanisms by which phytopathogenic bacteria facilitate persistence and proliferation of human pathogenic bacteria in leafy greens in agricultural fields and during postharvest treatment remain largely unknown. *D. dadantii* 3937 has been reported to promote the multiplication of *E. coli* O157:H7 on postharvest lettuce leaves (10); however, information that describes how this plant pathogen aids the growth and/or survival of *E. coli* O157:H7 is limited. The objective of this study is to elucidate mechanisms by which *D. dadantii* 3937 influences the multiplication of *E. coli* O157:H7 on postharvest lettuce leaves.

MATERIALS AND METHODS

Plant material, bacterial strains, and growth conditions. Romaine lettuce leaves were purchased at a local supermarket in Milwaukee, WI. Bacterial strains and plasmids used in this study are listed in Table 1. Wild-type *D. dadantii* 3937 and its derivatives were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 6.8) or minimal medium (MM) at 28°C (55). When required, antibiotics were added as follows: 100 μg/ml of ampicillin (Ap), 50 μg/ml of kanamycin (Km), 30 μg/ml of chloramphenicol (Cm), and 150 μg/ml of rifampin (Rif).

Recombinant DNA techniques. Preparation of genomic or plasmid DNA, PCR, restriction digestion, ligation, DNA electrophoresis, and electroporation were performed as described by Ausubel and associates (7).

Single-copy complementation. An *outC*-deficient mutant, A1919, was complemented by site-directed insertion using pTCLSCm that carries the *lacY-prt* locus of *D. dadantii* 3937, a chloramphenicol resistance cassette, and an engineered multicloning site (57). A 1,140-bp fragment containing the *outC* open reading frame (ORF) and its native promoter was PCR amplified using primers outC_comp_F (5′-ctcgagCGGGAAACAGGATGCCTGT-3′) and outC_comp_R (5′-ctcgagTTACTCGTCTCCCCCAAAT-3′) (lowercase nucleotides represent the XhoI recognition site). This fragment was digested with XhoI, gel purified, and cloned into pTCLSCm. The resulting plasmid, pTCLS-outC, was introduced into A1919 (Δ*outC*) by electroporation. The transformants were grown in low-phosphate buffer medium [100 mM Tris base, 4 mM $MgSO_4$, 7.57 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate, 250 μM potassium phosphate buffer (pH 7.0), 0.2% (wt/vol) glycerol, 0.1% glucose] for 48 h at 28°C, and double-crossover strains were selected by replica plating on LB plates in the presence or absence of Ap. To complement an *rpoS*-deficient mutant, Ech159, a 2,592-bp fragment containing the *rpoS* ORF and its native promoter was PCR amplified using primers rpoS_comp_F (5′-ctcgagTACCGTTGCCAGTCTCGGTA-3′) and rpoS_comp_R (5′-ctcgagTTATTCGCGGAACAGCTCTTCG-3′) and cloned into the XhoI site of pTCLSCm to create pTCLS-rpoS. Double-crossover strains were selected by the same method used for *outC* complementation.

Leaf inoculation and measurement of bacterial populations on lettuce leaves. The *E. coli* O157:H7 strain EDL933 (EDL933) wild type and the *D. dadantii* 3937 wild type and derivative strains were grown in LB medium supplemented with the appropriate antibiotics at 37°C and 28°C, respectively. Coinoculation of lettuce leaves with EDL933 and *D. dadantii* and measurement of bacterial populations on inoculated leaves were performed as previously described (10) with slight modifications. A 2.5-g sample of middle-aged leaves cut crosswise into 2-cm-wide pieces was placed into a sterile hybridization bag and coinoculated with 4 ml of a bacterial suspension containing EDL933 and *D. dadantii*, each at 1×10^6 CFU/ml in 0.5 mM potassium phosphate buffer. Each bag was heat sealed and incubated at 28°C. At each sampling time, 36 ml of potassium phosphate buffer (10 mM) was added to each sample, and inoculated leaves were ground with a pestle. The resulting suspensions were plated onto LB agar and MG agar (1% mannitol, 0.2% glutamic acid, 0.05% KH_2PO_4, 0.02% NaCl, 0.02% $MgSO_4$, pH 7.2) containing the appropriate antibiotics for the measurement of the EDL933 and *D. dadantii* population sizes, respectively. Three independent experiments were performed, and three replicate samples were used in each experiment.

Pectate lyase (Pel) activity assay. Bacteria were grown in MM or MM supplemented with 1% polygalacturonic acid (PGA) and subjected to the spectrophotometric assay. The cell density of an overnight culture grown in MM was measured at an optical density of 600 nm (OD_{600}), and the supernatant was obtained by centrifugation at 15,000 rpm for 2 min. For bacteria cultured in MM supplemented with PGA, cells were separated from the supernatant by centrifugation at 3,000 rpm for 10 min and resuspended in 0.5 mM potassium phosphate buffer to measure cell density at OD_{600}. Pectate lyase (Pel)-specific activity was measured at OD_{230} by using the culture supernatants and normalized by cell density as previously described (36).

Promoter activity assay for *pelD*. Promoter activity of *pelD* was measured by flow cytometry (BD Biosciences, San Jose, CA) as previously described (44). To measure *pelD* promoter activity *in vitro*, bacterial strains carrying the *pelD* promoter-*gfp* transcriptional fusion were grown overnight at 28°C in LB medium supplemented with the appropriate antibiotics. Cells were transferred to MM with or without 1% PGA and incubated at 28°C for 20 h. To measure *pelD*

promoter activity *in planta*, lettuce leaves were inoculated with bacterial suspension by using the same method described for the measurement of bacterial population on leaves. A constitutive expressing promoter for *nptII* was used to drive *gfp* in order to demonstrate that the mutation in *rpoS* had no significant effect on the green fluorescent protein (GFP) itself. The GFP intensity was measured using flow cytometry as previously described (44).

Statistical analysis. Means and standard deviations were calculated using Excel (Microsoft, Redmond, WA), and the statistical analysis was performed using R version 2.8.1 (http://www.r-project.org/).

RESULTS AND DISCUSSION

Effect of *D. dadantii* 3937 virulence factors on EDL933 proliferation on lettuce leaves. In a previous report, the phytopathogen *D. dadantii* 3937 promoted the multiplication of O157:H7 on postharvest lettuce leaves (10). We hypothesized that the virulence determinants of *D. dadantii* 3937 may play a role in facilitating the growth of the animal pathogen O157:H7 on plant leaves. Bacterial motility, T3SS, and CWDEs are major virulence determinants in *D. dadantii* 3937 (3, 13, 54). In order to determine whether these virulence factors of *D. dadantii* 3937 may affect EDL933 proliferation on postharvest lettuce, leaves were coinoculated with EDL933 and either wild-type *D. dadantii* or its mutant derivatives Ech166 (Δ*fliA*), Ech169 (Δ*hrpA*), A1919 (Δ*outC*), or Ech159 (Δ*rpoS*) (Table 1), and the population dynamics of these strains were initially surveyed. The *fliA*, *hrpA*, *outC*, and *rpoS* genes of *D. dadantii* 3937 encode σ^{28}, the T3SS pilus, the type II secretion system (T2SS) membrane component, and σ^{38}, respectively. FliA regulates flagellar biosynthesis and bacterial motility (47). HrpA is one of the T3SS components and forms filament-like extracellular structures (48). A mutation in *hrpA* disables the injection of T3 effectors into plant cells (28). CWDEs, such as pectate lyases (Pels), polygalacturonases, and cellulases, also play a crucial role in *D. dadantii* pathogenicity (8, 29). These enzymes are secreted from the bacterial cells to the extracellular space via the T2SS (25, 51), and an *outC* mutant of *D. dadantii* is completely deficient in the secretion of CWDEs (17, 43). RpoS is an RNA polymerase σ factor that plays a central role in the regulation of gene expression in stationary phase (16, 50). Several major virulence-related factors, including bacterial motility, T3SS, and production of CWDEs, are under the control of RpoS (2, 27).

Similar population sizes of EDL933 were observed in lettuce leaves at 24 h when coinoculated with either wild-type *D. dadantii* 3937 or A1919 (Δ*outC*). However, A1919 was reduced in its ability to enhance EDL933 proliferation at 48 h (Fig. 1A). In contrast to *outC*, a mutation in *rpoS* of *D. dadantii* 3937 was found to have a positive effect on the *E. coli* population. When EDL933 was coinoculated with Ech159 (Δ*rpoS*), the *E. coli* population size at 48 h postinoculation was 3-fold higher than that with the wild-type *D. dadantii* coinoculum (Fig. 1A), but when coinoculated with either Ech166 (Δ*fliA*) or Ech169 (Δ*hrpA*), the population sizes of EDL933 at 24 and 48 h were comparable to that when coinoculated with the *D. dadantii* wild type (Fig. 1A). The effect of the *D. dadantii* 3937 *rpoS* or *outC* mutation on the EDL933 population size on lettuce leaves was restored to the wild-type level by single-copy chromosomal complementation of *rpoS* or *outC*, respectively (Fig. 2A). A reduction in the bacterial population of A1919 (Δ*outC*) was observed in lettuce at 24 and 48 h after coinoculation with

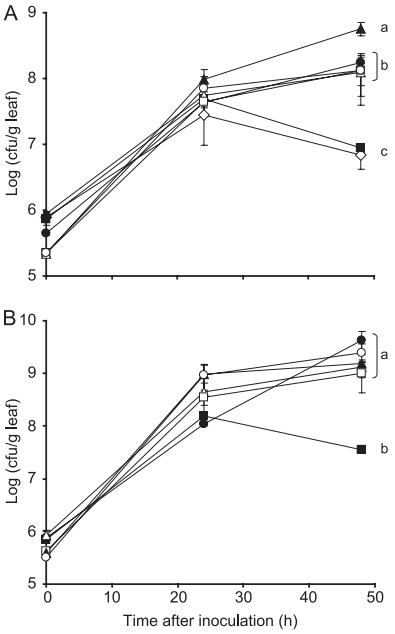

FIG. 1. Population dynamics of *E. coli* O157:H7 EDL933 (A) and the *D. dadantii* wild type and its derivatives (B). The population sizes of EDL933 and *D. dadantii* on middle-aged leaves of romaine lettuce were counted at 24 and 48 h after inoculation with EDL933 alone (open diamond) or coinoculation with EDL933 and either the *D. dadantii* wild type (closed circle), A1919 (Δ*outC*) (closed square), Ech159 (Δ*rpoS*) (closed triangle), Ech166 (Δ*fliA*) (open square), or Ech169 (Δ*hrpA*) (open triangle). Each datum point represents the mean of results from three individual experiments, each with three replicate samples. Bars represent standard deviations of the means. Datum points labeled with different letters represent significantly different means as revealed by the Tukey-Kramer method at P values of <0.05.

EDL933 (Fig. 1B), but no significant difference was seen in the *D. dadantii* population size among the wild type and *fliA*, *hrpA*, and *rpoS* mutants (Fig. 1B). The attrition of the A1919 (Δ*outC*) and A1919-coinoculated EDL933 populations at 24 and 48 h after inoculation was recovered by *outC* complementation (Fig. 2B). Although there was no significant difference between the population size of wild-type *D. dadantii* and that of Ech159 (Δ*rpoS*) after 48 h, the population size of EDL933 coinoculated with Ech159 (Δ*rpoS*) was larger than that when coinoculated with wild-type *D. dadantii* (Fig. 1), possibly because Ech159 (Δ*rpoS*) causes more severe symptoms on lettuce leaves than the wild-type *D. dadantii*. Finally, it is important to note that similar levels of EDL933 cell densities were observed at 24 h, when leaves were inoculated with either EDL933 or EDL933 combined with the *D. dadantii* wild type or its derivatives (Fig. 1A). This seems to be indicative of the pathogenicity cycle of *D. dadantii*; under our test conditions, it takes at least 24 h postinoculation before CWDEs are secreted into the extracellular space. This result also indicates that the leaf strips provide nutrients to sustain growth of EDL933 at least 24 h after inoculation. The same phenomenon has been observed in a previous report (10).

Alteration of Pel activity in *outC* and *rpoS* mutants of *D. dadantii*. An increase or reduction in EDL933 population sizes was observed in lettuce leaves when EDL933 was coinoculated with either Ech159 (Δ*rpoS*) or A1919 (Δ*outC*), respectively, in

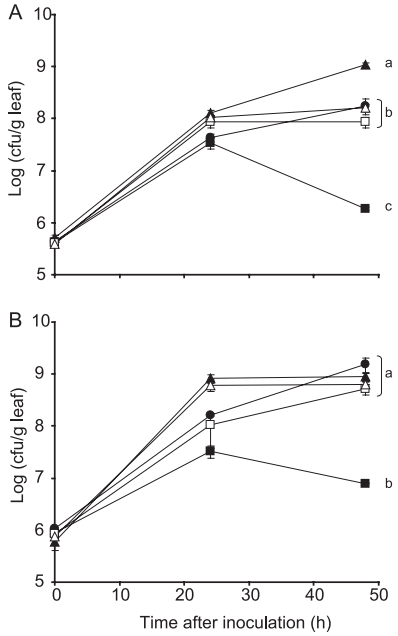

FIG. 2. Population dynamics of EDL933 (A) and *D. dadantii* mutants and their complemented strains (B). Lettuce leaves were inoculated with EDL933 and either the *D. dadantii* wild type (closed circle), A1919 (Δ*outC*) (closed square), Ech159 (Δ*rpoS*) (closed triangle), A1919C (*outC*$^+$) (open square), or Ech159C (*rpoS*$^+$) (open triangle). Each datum point represents the mean of results from three individual experiments, each with three replicate samples. Bars represent standard deviations of the means. Datum points labeled with different letters represent significantly different means as revealed by the Tukey-Kramer method at P values of <0.05.

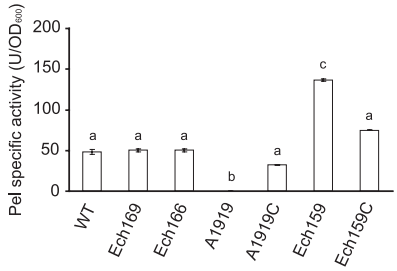

FIG. 3. Pectate lyase (Pel) activity of the *D. dadantii* wild type, mutants Ech166 (Δ*fliA*), Ech169 (Δ*hrpA*), A1919 (Δ*outC*), Ech159 (Δ*rpoS*), and complemented strains A1919C (*outC*$^+$) and Ech159C (*rpoS*$^+$). Cells were cultured in minimal medium supplemented with polygalacturonic acid (PGA) to induce expression of *pel* genes. One unit of Pel activity is equivalent to an increase in optical density at 230 nm of 1×10^{-3} in 1 min. Each value represents the mean of results from triplicates. Bars represent standard deviations of the means. Bars labeled with different letters represent significantly different means as revealed by the Tukey-Kramer method at P values of <0.01.

comparison to coinoculation with wild-type *D. dadantii* 3937. OutC is an inner membrane component of the T2SS and crucial for T2 protein secretion (14, 46). A mutation in *outC* shuts down the secretion of CWDEs of *D. dadantii* 3937 (17, 43). Among the CWDEs secreted by *D. dadantii*, pectate lyases are the most important enzymes for degrading plant cell walls and macerating plant tissues, since purified Pels are able to mimic symptoms of the bacterial infection (13). Given that RpoS negatively regulates the production of Pels in *Erwinia carotovora* subsp. *carotovora* and *D. dadantii* 3937 (32, 40), we speculated that RpoS of *D. dadantii* 3937 affects proliferation of EDL933 on lettuce leaves through pectinolytic enzymes. To examine this possibility, Pel activity was assessed in wild-type *D. dadantii* and its derivatives. Bacterial cells were grown in MM broth supplemented with 1% polygalacturonic acid (PGA) to induce bacterial Pel production. Our results showed that there was almost no Pel activity in A1919 (Δ*outC*); meanwhile, Ech159 (Δ*rpoS*) showed more than 2-fold-greater Pel activity than wild-type *D. dadantii* 3937 (Fig. 3). In addition, Pel activity was comparable among wild-type *D. dadantii* 3937, Ech166 (Δ*fliA*), and Ech169 (Δ*hrpA*) (Fig. 3). Pel activity in A1919 (Δ*outC*) and Ech159 (Δ*rpoS*) was restored to the wild-type level by single-copy chromosomal complementation with *outC* and *rpoS*, respectively (Fig. 3).

RpoS regulates *pel* expression at the transcriptional level. We further tested whether the negative regulation of RpoS on Pel activity is due to the effect of RpoS on *pel* gene expression. The *D. dadantii* 3937 genome encodes nine *pel* genes (*pelA* to *pelE*, *pelI*, *pelL*, *pelX*, and *pelZ*) (A Systematic Annotation Package for Community Analysis of Genomes [ASAP], http://asap.ahabs.wisc.edu/asap/home.php). Among them, the *pelD* gene product (an endo-pectate lyase) has been known to have dominant effects on both tissue maceration and symptom development (25). Hence, we examined the effect of RpoS on *pelD* promoter activity in *D. dadantii* 3937. The *pelD* promoter activity and Pel-specific activity were measured in the *D. dadantii* wild type, Ech159 (Δ*rpoS*), and Ech159C (*rpoS*$^+$), with each one of these bacterial strains carrying the GFP reporter plasmid pPROBE-AT::P*pelD* (56). When grown in MM, a basal level of Pel enzymatic activity and *pelD* promoter activity was observed in the wild type and Ech159 (Δ*rpoS*), whereas both activities were highly induced in MM supplemented with PGA (Fig. 4A and B). Although an increase in Pel-specific activity and *pelD* promoter activity was observed in both Ech159 (Δ*rpoS*) and wild-type 3937 when PGA was added to the culture medium, both basal and PGA-induced levels of Pel activity and *pelD* promoter activity were greater in Ech159 (Δ*rpoS*) (Fig. 4A and B). Additionally, the activity of Pels and the *pelD* promoter of Ech159 (Δ*rpoS*) was restored to near wild-type levels by chromosomal single-copy complementation with *rpoS* (Fig. 4A and B). The mutation in *rpoS* of *D. dadantii* 3937 had no significant influence on GFP itself when grown in MM or MM plus PGA, as evidenced by a lack of significant difference in the GFP intensity emitted by the wild-type strain and Ech159 (Δ*rpoS*) carrying pAT-NPTII, on which *gfp* is constitutively expressed from the *nptII* promoter (Fig. 4C). In summary, these results suggest that RpoS controls the Pel activity of *D. dadantii* 3937 by regulating *pelD* and possibly other *pel* genes at the transcriptional level (Fig. 4A and B).

RpoS downregulates *pelD* expression of *D. dadantii in planta*. We found that RpoS negatively regulates *pelD* promoter activity when *D. dadantii* 3937 was grown in minimal medium (Fig. 4A and B). To investigate the effect of RpoS on *pelD* expression *in planta*, lettuce leaves were inoculated with the pPROBE-AT::P*pelD*-transformed *D. dadantii* wild type, Ech159 (Δ*rpoS*), and Ech159C (*rpoS*$^+$), and the GFP intensities of the bacterial cells were measured. The *pelD* promoter

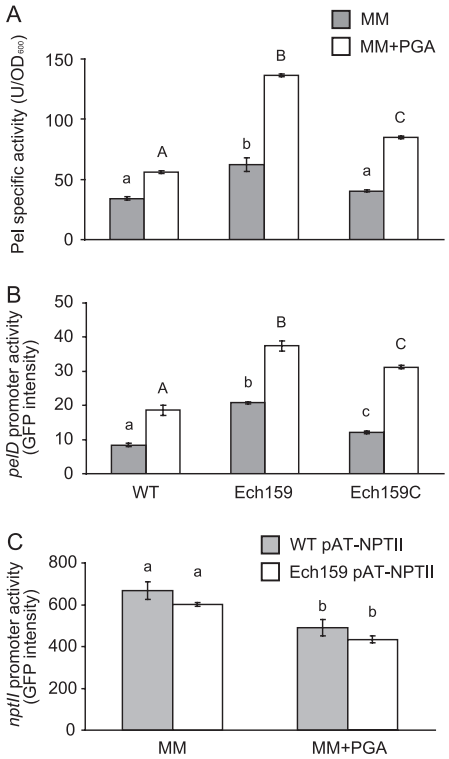

FIG. 4. Pel-specific activity and *pelD* promoter activity of *D. dadantii*. Cells were cultured in minimal medium, and the expression of *pel* genes was induced by the addition of PGA. Each value represents the means of results from triplicates. Bars represent standard deviations of the means. Bars labeled with different letters represent significantly different means as revealed by the Tukey-Kramer method at P values of <0.01. Statistical analysis on the data set for MM or MM plus PGA was performed separately. (A) Pel-specific activity was measured spectrophotometrically. One unit of Pel activity is equivalent to an increase in optical density at 230 nm of 1×10^{-3} in 1 min. (B) *In vitro* promoter activity of *pelD* in the *D. dadantii* wild type, Ech159 (Δ*rpoS*), and Ech159C (*rpoS*$^+$) was measured by flow cytometry. (C) The effect of the mutation in *rpoS* of *D. dadantii* on the green fluorescent protein (GFP) was examined *in vitro*. Cells were cultured in MM and MM plus PGA, and the GFP intensity of the *D. dadantii* wild type or Ech159 (Δ*rpoS*) cells carrying pAT-NPTII on which *gfp* is constitutively expressed from the *nptII* promoter was measured by flow cytometry.

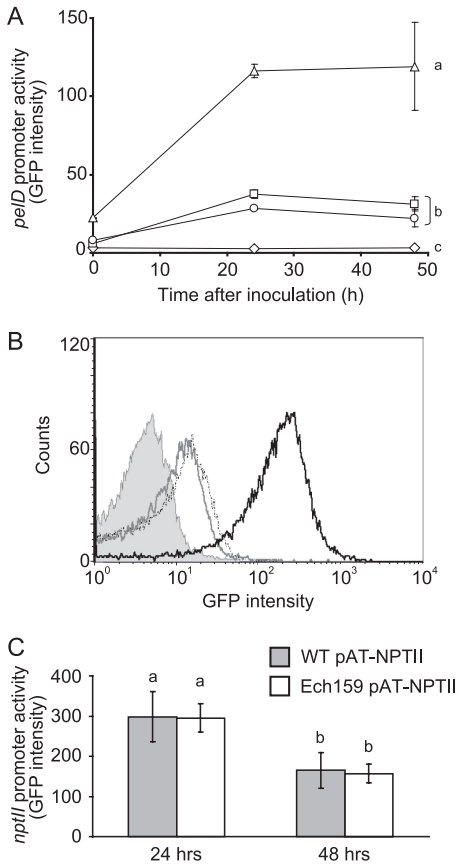

FIG. 5. *In planta* promoter activity of *pelD*. (A) Lettuce leaves were inoculated with the *D. dadantii* wild type carrying pPROBE-AT (open diamond), the wild type carrying pPROBE-AT::P*pelD* (open square), Ech159 (Δ*rpoS*) carrying pPROBE-AT::P*pelD* (open triangle), or Ech159C (*rpoS*$^+$) carrying pPROBE-AT::P*pelD* (open circle). Cells were collected at 0, 24, and 48 h after inoculation to measure the *pelD* promoter activity as GFP intensity. Each datum point represents the mean of results from three individual experiments, each with three replicate samples. Bars represent standard deviations of the means. Datum points labeled with different letters represent significantly different means as revealed by the Tukey-Kramer method at P values of <0.05. (B) Distribution of cells expressing P_{pelD}-*gfp* at 48 h after inoculation obtained by flow cytometry. Samples: *D. dadantii* wild type carrying pPROBE-AT (gray line filled with gray), the wild type carrying pPROBE-AT::P*pelD* (gray line), Ech159 (Δ*rpoS*) carrying pPROBE-AT::P*pelD* (black line), and Ech159C (*rpoS*$^+$) carrying pPROBE-AT::P*pelD* (black dotted line). (C) The effect of the mutation in *rpoS* of *D. dadantii* on GFP was examined *in planta*. Lettuce leaves were inoculated with either the *D. dadantii* wild type or Ech159 (Δ*rpoS*) cells carrying pAT-NPTII on which *gfp* is constitutively expressed from the *nptII* promoter. GFP intensity was measured by flow cytometry.

activity in Ech159 (Δ*rpoS*) was higher than that of the wild type after 24 h, and this tendency continued up to 48 h postinoculation (Fig. 5A). The chromosomal single-copy complementation restored *pelD* expression in Ech159 (Δ*rpoS*) to the wild-type level (Fig. 5A and B). No significant difference was observed in the GFP intensities of the *D. dadantii* 3937 wild type and Ech159 (Δ*rpoS*) when the strains were transformed with pAT-NPTII, on which *gfp* is constitutively expressed from the *nptII* promoter (Fig. 5C).

In the pathogenicity of *D. dadantii* 3937, T3SS, motility, and CWDEs are considered major virulence factors (3, 13, 54), which are partially regulated by the general stress response σ factor RpoS (2, 27, 32). In addition, among T2SS-dependent CWDEs, Pels are known to play a central role in tissue maceration to cause soft-rot symptoms on host plants (13). Considering these reports, our data strongly suggest that RpoS of *D. dadantii* 3937 has a negative effect on EDL933 proliferation on lettuce leaves mainly through negative regulation of pectate lyases, even though our approaches are indirect, owing to the redundancy of *pel* genes and the intricate regulatory mechanisms which control their expression (8, 14, 25). Since RpoS has a global effect on gene regulation (16, 50), it also seems possible that other RpoS-regulated factors besides pectate lyases may affect the EDL933 proliferation on lettuce leaves.

In *E. coli* and other Gram-negative bacteria, RpoS is a key element in the cell's response to changing environmental conditions (23). The expression of RpoS has shown to be regulated at transcriptional, translational, and posttranslational levels by

a number of environmental stimuli (11, 24, 31, 45). For example, the expression of *rpoS* is affected at the transcriptional level by cell growth rate and energy-limiting conditions (15, 42). In addition, the RpoS protein is controlled at the translational level by cell density, temperature, osmolarity, and pH shift (6, 37, 53). Furthermore, carbon/phosphate starvation and heat shock downregulate RpoS proteolysis (24, 27, 32, 35, 39). In this manner, a broad array of environmental factors intricately controls the expression and stability of RpoS (23). Our findings in this study indicate that environmental changes in the phyllosphere may affect the survival, persistence, and proliferation of pathogenic *E. coli* via an RpoS-Pel regulatory cascade in *D. dadantii* 3937. For example, during postharvest, temperature change and/or starvation may affect the production and secretion of Pels through RpoS in *D. dadantii*, which further influences the *E. coli* population on leafy greens. In addition, cultivation practices in the field, i.e., fertilization and irrigation, may also alter the environmental stresses toward bacterial pathogens and/or the severity of plant disease, which may further affect the persistence and proliferation of pathogenic *E. coli* on field crops. Recent reports have proposed relationships between pathogenic *E. coli* and the phyllosphere/rhizosphere bacterial community which supports the survival and persistence of the pathogen on plants (20, 33). Moreover, expression of subsets of genes related to pathogenicity (T3), oxidative stress tolerance, and antimicrobial resistance is reported to be induced by exposure of EDL933 cells to lettuce lysates (30). Clarification of the interaction between human pathogens and plant pathogens in the phyllosphere/rhizosphere is becoming more important in terms of food safety. Our findings may provide additional insight into mechanisms which promote or inhibit these interactions.

In conclusion, our data showed that the pectinolytic activity of *D. dadantii* 3937 is an important determinant of EDL933 proliferation on lettuce. This study suggests that the global stress responsive σ factor RpoS is one of the key factors affecting *E. coli* O157:H7 proliferation on the leaf surface by negatively regulating the expression of pectinolytic enzymes.

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