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 (ΔoutC), lost the capability to promote the multiplication of EDL933, whereas Ech159 (ΔrpoS), a stress-responsive σ factor RpoS-deficient mutant, increased EDL933 proliferation on lettuce leaves. A spectrophotometric enzyme activity assay revealed that A1919 (ΔoutC) was completely deficient in the secretion of pectate lyases (Pel), which play a major role in plant tissue maceration. In contrast to A1919 (ΔoutC), Ech159 (ΔrpoS) 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 (ΔrpoS) 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 (hallmark attaching and effacing lesions), and (iii) production of pectate lyases in *Escherichia coli* O157:H7 (58). The potential 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 (ΔoutC), lost the capability to promote the multiplication of EDL933, whereas Ech159 (ΔrpoS), a stress-responsive σ factor RpoS-deficient mutant, increased EDL933 proliferation on lettuce leaves. A spectrophotometric enzyme activity assay revealed that A1919 (ΔoutC) was completely deficient in the secretion of pectate lyases (Pel), which play a major role in plant tissue maceration. In contrast to A1919 (ΔoutC), Ech159 (ΔrpoS) 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 (ΔrpoS) 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.

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defenses and facilitate proliferation (1, 54). Finally, when \textit{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. \textit{D. dadantii 3937} has been reported to promote the multiplication of \textit{E. coli O157:H7} on postharvest lettuce leaves (10); however, information that describes how this plant pathogen aids the growth and/or survival of \textit{E. coli O157:H7} is limited. The objective of this study is to elucidate mechanisms by which \textit{D. dadantii 3937} influences the multiplication of \textit{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 \textit{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 \(\mu\)g/ml of ampicillin (Ap), 50 \(\mu\)g/ml of kanamycin (Km), 30 \(\mu\)g/ml of chloramphenicol (Cm), and 150 \(\mu\)g/ml of rifampicin (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 \textit{outC}-deficient mutant, A1919, was complemented by site-directed insertion using \textit{pTCLScm} that carries the lacY-protector of \textit{D. dadantii 3937}, a chloramphenicol resistance cassette, and an engineered multicloning site (57). A 1,140-bp fragment containing the \textit{outC} open reading frame (ORF) and its native promoter was PCR amplified using primers \textit{outC_comp_F} (5'-ctcgagGGGAAAACAGGATGGATCT-3') and \textit{outC_comp_R} (5'-ctcgagTATCTGCTTCCCAATAT-3') (lowercase nucleotides represent the XhoI recognition site). This fragment was digested with XhoI, gel purified, and cloned into \textit{pTCLScm}. The resulting plasmid, \textit{pTCLScm-outC}, was introduced into A1919 (\textit{ΔoutC}) by electroporation. The transformants were grown in low-phosphate buffer medium [100 mM Tris base, 4 mM MgSO\(_4\), 7.57 mM (NH\(_4\))\(_2\)SO\(_4\), 1.7 mM sodium citrate, 250 mM 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 \textit{rpoS}-deficient mutant, Ech159, a 2,592-bp fragment containing the \textit{rpoS} ORF and its native promoter was PCR amplified using primers \textit{rpoS_comp_F} (5'-ctcgagTACCTGTCGCGGAGATTGCT-3') and \textit{rpoS_comp_R} (5'-ctcgagTTATCCGCGGAAAGACGTTCCTTG-3') and cloned into the XhoI site of \textit{pTCLScm} to create \textit{pTCLScm-rpoS}. Double-crossover strains were selected by the same method used for \textit{outC} complementation.

**Leaf inoculation and measurement of bacterial populations on lettuce leaves.** The \textit{E. coli O157:H7} strain EDL933 (EDL933) wild type and the \textit{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 \textit{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 coincubated with 4 ml of a bacterial suspension containing EDL933 and \textit{D. dadantii}, each at 1 \(\times\) 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\(_2\)PO\(_4\), 0.02% NaCl, 0.02% MgSO\(_4\), pH 7.2) containing the appropriate antibiotics for the measurement of the EDL933 and \textit{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\(_{412}\) by using the culture supernatants and normalized by cell density as previously described (36).

**Promoter activity assay for \textit{pelD}.** Promoter activity of \textit{pelD} was measured by flow cytometry (BD Biosciences, San Jose, CA) as previously described (44). To measure \textit{pelD} promoter activity in vitro, bacterial strains carrying the \textit{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 \textit{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).

**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 co inoculated with EDL933 and either wild-type _D. dadantii_ or its mutant derivatives Ech166 (ΔfliA), Ech169 (ΔhrpA), A1919 (ΔoutC), or Ech159 (ΔrpoS) (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 co inoculated with either wild-type _D. dadantii_ 3937 or A1919 (ΔoutC). 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 co inoculated with Ech159 (ΔrpoS), the _E. coli_ population size at 48 h postinoculation was 3-fold higher than that with the wild-type _D. dadantii_ co inoculant (Fig. 1A), but when co inoculated with either Ech166 (ΔfliA) or Ech169 (ΔhrpA), the population sizes of EDL933 at 24 and 48 h were comparable to that when co inoculated 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 (ΔoutC) was observed in lettuce at 24 and 48 h after co inoculation with 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 attirion of the A1919 (ΔoutC) and A1919-co inoculated 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 (ΔrpoS) after 48 h, the population size of EDL933 co inoculated with Ech159 (ΔrpoS) was larger than that when co inoculated with wild-type _D. dadantii_ (Fig. 1), possibly because Ech159 (ΔrpoS) 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 co inoculated with either Ech159 (ΔrpoS) or A1919 (ΔoutC), respectively, in
EFFECT OF Pels ON E. coli O157:H7 EDL933 PROLIFERATION

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, pelF, 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 endopectate 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 (ΔropS), and Ech159C (ropS⁺), with each one of these bacterial strains carrying the GFP reporter plasmid pPROBE-AT::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 (ΔropS), 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 (ΔropS) 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 (ΔropS) (Fig. 4A and B). Additionally, the activity of Pels and the pelD promoter of Ech159 (ΔropS) was restored to near wild-type levels by chromosomal single-copy complementation with ropS (Fig. 4A and B). The mutation in ropS 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 (ΔropS) 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::pelD-transformed D. dadantii wild type, Ech159 (ΔropS), and Ech159C (ropS⁺), and the GFP intensities of the bacterial cells were measured. The pelD promoter

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 (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 (ΔoutC); meanwhile, Ech159 (ΔropS) 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 (ΔhrpA), and Ech169 (ΔhrpA) (Fig. 3). Pel activity in A1919 (ΔoutC) and Ech159 (ΔropS) was restored to the wild-type level by single-copy chromosomal complementation with outC and ropS, 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.
which are partially regulated by the general stress response CWDEs are considered major virulence factors (3, 13, 54).

...sidering these reports, our data strongly suggest that RpoS of...
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 intrinsically 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. ACKNOWLEDGMENTS This work is dedicated to Noel T. Keen. We thank Guy Condemine for providing the bacterial strain A1919 and Eulandria Biddle for critical discussions and reading of the manuscript. This project is supported by grants from the CSRES NIFA (agreement no. 2008-35201-18709) and the Research Growth Initiative of the University of Wisconsin-Milwaukee.

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