Integrated downstream regulation by the quorum-sensing controlled transcription factors LrhA and RcsA impacts phenotypic outputs associated with virulence in the phytopathogen *Pantoea stewartii* subsp. *stewartii*

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**ABSTRACT**

*Pantoea stewartii* subsp. *stewartii* is a Gram-negative proteobacterium that causes leaf blight and Stewart’s wilt disease in corn. Quorum sensing (QS) controls bacterial exopolysaccharide production that blocks water transport in the plant xylem at high bacterial densities during the later stage of the infection, resulting in wilt. At low cell density the key master QS regulator in *P. stewartii*, EsaR, directly represses *rcsA*, encoding an activator of capsule biosynthesis genes, but activates *lrhA*, encoding a transcription factor that regulates surface motility. Both RcsA and LrhA have been shown to play a role in plant virulence. In this study, additional information about the downstream targets of LrhA and its interaction with RcsA was determined. A transcriptional fusion assay revealed autorepression of LrhA in *P. stewartii* and electrophoretic mobility shift assays (EMSA) using purified LrhA confirmed that LrhA binds to its own promoter. In addition, LrhA binds to the promoter for the RcsA gene, as well as those for putative fimbrial subunits and biosurfactant production enzymes in *P. stewartii*, but not to the *flhDC* promoter, which is the main direct target of LrhA in *Escherichia coli*. This work led to a reexamination of the physiological function of RcsA in *P. stewartii* and the discovery that it also plays a role in surface motility. These findings are broadening our understanding of the coordinated regulatory cascades utilized in the phytopathogen *P. stewartii*.

**Subjects** Agricultural Science, Genetics, Microbiology, Molecular Biology, Plant Science

**Keywords** LrhA, *Pantoea stewartii* subsp. *stewartii*, Phytopathogen, Quorum sensing, RcsA, Transcription factor

**INTRODUCTION**

*Pantoea stewartii* subsp. *stewartii*, a Gram-negative rod-shaped, gamma-proteobacterium, is the causal agent of leaf blight and Stewart’s wilt in susceptible varieties of *Zea mays*. It is primarily transmitted to the plant by the corn flea beetle, *Chaetocnema pulicaria* (*Esker & Nutter, 2002*). After being deposited through excrement into wounds generated during insect feeding, the pathogen gains access to the leaf apoplast and causes water-soaked...
lesions through the Hrp-type III secretion system (Ham et al., 2006; Roper, 2011). In a second phase of the disease, the bacteria then also migrate to the xylem, where they grow to high cell density and form a biofilm that blocks water flow within the plant. This results in wilt disease and even death, if the plants were infected at the seedling phase (Braun, 1982). Quorum sensing (QS), a mechanism of bacterial cell density-dependent communication, controls the virulence, capsule production and surface motility of this pathogen (Roper, 2011; Von Bodman, Bauer & Coplin, 2003).

During QS, P. stewartii produces N-acyl homoserine lactone (AHL) signals due to the activity of Esal, a LuxI-type protein (Beck von Bodman & Farrand, 1995). The AHL signal then interacts with the master QS regulatory protein EsaR, a LuxR homologue, when the cell density reaches a critical threshold. EsaR is a dual-level transcriptional regulator that binds to DNA at its recognition sites to either activate or repress its downstream targets at low cell density (Beck von Bodman & Farrand, 1995; Von Bodman et al., 2003; Von Bodman, Majerczak & Coplin, 1998). When EsaR and AHL interact at high cell density, the EsaR-AHL complex is unable to bind to the DNA resulting in transcriptional deactivation or derepression of its target genes (Schu et al., 2009; Shong et al., 2013). Multiple approaches have been used to identify several direct targets of EsaR, including classic genetic (Minogue et al., 2005), proteome-level (Ramachandran & Stevens, 2013) and transcriptome-level (Ramachandran et al., 2014) analysis. Two of these direct targets, rcsA and lrhA, are involved in plant virulence and control capsule production and surface motility, respectively (Kernell Burke et al., 2015).

EsaR directly represses the P. stewartii rcsA gene at low cell density, insuring precise control over the timing of capsule synthesis (Carlier & Von Bodman, 2006; Minogue et al., 2005; Von Bodman, Majerczak & Coplin, 1998). At high cell density, gene activation by RcsA leads to production of stewartan, a polymer of galactose, glucose and glucuronic acid in a 3:3:1 ratio, which is the main component of the exopolysaccharide (EPS) (Nimtz et al., 1996). Stewartan is a primary virulence factor of P. stewartii (Carlier, Burbank & Von Bodman, 2009; Minogue et al., 2005; Roper, 2011). Previous work has shown that the lrhA gene is directly activated by EsaR at low cell density and a P. stewartii LrhA deletion mutant exhibits decreased surface motility and intermediate virulence levels in comparison to the wild type (Kernell Burke et al., 2015). However, little is known about the precise role of LrhA and its targets with regard to surface motility and virulence in P. stewartii.

In Escherichia coli, the function of LrhA is better understood. It is the key regulator controlling the expression of flagella, motility and chemotaxis by regulating the synthesis of FlhD2C2, the master regulator of flagella and chemotaxis gene expression (Lehnen et al., 2002). In E. coli, LrhA directly activates its own expression and represses the expression of flhD/flhC, thereby suppressing motility and chemotaxis (Lehnen et al., 2002). LrhA also controls fimA expression (Blumer et al., 2005) and regulates RpoS translation (Gibson & Silhavy, 1999; Peterson et al., 2006), but its binding site is not well defined (Lehnen et al., 2002).

In contrast to E. coli, P. stewartii is only capable of swarming rather than swimming motility. The bacterium’s swarming motility is controlled by QS and contributes to its pathogenicity (Herrera et al., 2008). The surface motility is flagellar-dependent since
deletion of fliC renders the bacterium aflagellar and incapable of swarming (Herrera et al., 2008). There is no evidence demonstrating that EsaR plays a direct role in regulating flagella synthesis. However, EsaR does directly regulate lrhA in P. stewartii and thereby indirectly regulates surface motility and plant virulence (Kernell Burke et al., 2015) through unknown mechanisms. A transcriptome-level analysis of the LrhA regulon in P. stewartii showed that LrhA activates three genes and represses 23 genes four-fold or more (Kernell Burke et al., 2015). In the present study, CKS_0458 and CKS_5211, genes putatively encoding a fimbrial subunit and biosurfactant production enzyme, respectively, have now been confirmed to be direct targets of LrhA. In addition, LrhA has also been demonstrated to repress its own gene and that of RcsA. Follow-up studies led to the finding that RcsA also plays a role in surface motility. This work has helped further reveal how the QS regulatory cascade in P. stewartii coordinate controls genes important for interactions with the plant host.

MATERIALS AND METHODS

Strains and growth conditions
Table 1 lists strains and plasmids used in this study. E. coli strains were grown in Luria-Bertani (LB) (10 g/l tryptone, 5 g/l yeast extract, and 5 g/l NaCl) broth or plates with 1.5% agar at 37 °C. P. stewartii strains were grown in either LB or Rich Minimal (RM) medium (1 × M9 salts, 2% casamino acids, 1 mM MgCl2, and 0.4% glucose) at 30 °C. Growth medium was supplemented with the following antibiotics: ampicillin (Ap, 100 µg/ml), chloramphenicol (Cm, 35 µg/ml), kanamycin (Kn, 50 µg/ml), nalidixic acid (Nal, 30 µg/ml), or streptomycin (Str, 100 µg/ml) as required (see Table 1).

Green fluorescent protein fusion (GFP) construction and testing
A transcriptional fusion between the lrhA promoter (903 bp) and the gene for GFP was created through traditional molecular techniques as described previously (Kernell Burke et al., 2015). PCR primers (Table S1) with the restriction sites EcoRI and KpnI added to the 5′ and 3′ ends of the promoter sequence, respectively, were used to facilitate subcloning into the pPROBE'-GFP [tagless] vector (Miller, Leveau & Lindow, 2000). E. coli S17-1 was transformed with this plasmid construct containing P\textasciitilde{lrhA}-gfp, which was then moved into the wild-type P. stewartii DC283, ∆lrhA and ∆lrhA/lrhA\textasciitilde strains (Table 1) via conjugation. The transconjugates were grown in RM medium supplemented with Kn and Nal overnight, diluted in fresh medium to an OD_{600} of 0.05 at 30 °C with shaking at 250 rpm to an OD_{600} of 0.2–0.5, diluted a second time in fresh medium to an OD_{600} of 0.025 and grown to an OD_{600} of 0.5. GFP measurements were done as previously described (Kernell Burke et al., 2015) with average relative fluorescence/OD_{600} from three experiments of triplicate samples, standard errors, and two-tailed homoscedastic Student’s t-test values calculated for each strain.

Overexpression of LrhA
The lrhA coding sequence was amplified using primers with BamHI and HindIII sites (Table S1), cloned into pGEM-T (Promega, Madison, WI, USA), and sequenced. After double digestion with BamHI and HindIII, the construct was ligated into pET28a
Table 1  Strains and plasmids used in the study.

| Strains | Genotype and notes | References |
|---------|--------------------|------------|
| **Pantoea stewartii strains** | | |
| DC283 | Wild-type strain; Nal' | Dolph, Majerczak & Coplin (1988) |
| ΔlrhA | Unmarked deletion of lrhA coding sequence from DC283; Nal' | Kernell Burke et al. (2015) |
| ΔlrhA/lrhA + | ΔlrhA with chromosomal complementation of lrhA and its promoter downstream of glmS; Nal' Cm' | Kernell Burke et al. (2015) |
| ΔrcsA-2015 | Unmarked deletion of rcsA coding sequence from DC283; Nal' | Kernell Burke et al. (2015) |
| ΔrcsA/rcsA +-2015 | ΔrcsA with chromosomal complementation of rcsA and its promoter downstream of glmS; Nal' Cm'; missing 66-kb region | This study |
| ΔrcsA-2017 | Unmarked deletion of rcsA coding sequence from DC283; Nal' | This study |
| ΔrcsA/rcsA +-2017 | ΔrcsA with chromosomal complementation of rcsA and its promoter downstream of glmS; Nal' Cm'; missing 66-kb region | This study |
| ΔCKS_0458-CKS_0459 | Unmarked deletion of both CKS_0458 and CKS_0459 coding sequence from DC283; Nal' | This study |
| ΔCKS_0458-CKS_0459 /CKS_0459* | ΔCKS_0458-CKS_0459 with chromosomal complementation of CKS_0458 and its promoter downstream of glmS; Nal' Cm' | This study |
| ΔCKS_0458-CKS_0459 /CKS_0459* | ΔCKS_0458-CKS_0459 with chromosomal complementation of CKS_0458-CKS_0459 and their promoter downstream of glmS; Nal' Cm' | This study |
| ΔCKS_5208 | Unmarked deletion of CKS_5208 coding sequence from DC283; Nal' | This study |
| ΔCKS_5208/CKS_5208* | ΔCKS_5208 with chromosomal complementation of CKS_5208 and its promoter downstream of glmS; Nal' Cm' | This study |
| ΔCKS_5211 | Unmarked deletion of CKS_5211 coding sequence from DC283; Nal' | This study |
| ΔCKS_5211/CKS_5211* | ΔCKS_5211 with chromosomal complementation of CKS_5211 and its promoter downstream of glmS; Nal' Cm' | This study |
| ΔCKS_5211/ΔCKS_5208 | Unmarked deletion of both CKS_5211 and CKS_5208 coding sequences from DC283; Nal' | This study |
| **Escherichia coli strains** | | |
| Top 10 | F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZ ΔM15 ΔlacX 74 deoR recA1 araD139 Δ ara-leu)7697 galU galK rpsL (Str') endA1 nupG | Grant et al. (1990) |
| DH5 α λpir | F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80lacZ ΔM15 Δ(lacZYA-argF)U169 hsdR17(rK- mK+ ) λpir | Kvitko et al. (2012) |
| S17-1 | recA pro hsdR RP4-2-Tc::Mu-Km::Tn7 | Simon, Priefer & Pühler (1983) |
| S17-1 λpir | recA pro hsdR RP4-2-Tc::Mu-Km::Tn7 λpir | Labes, Pühler & Simon (1990) |
| BL21-DE3 | fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhisD λ DE3 = λ sbamH10 ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5 | Studier & Moffatt (1986) |
| **Plasmids** | | |
| pGEM-T | Cloning vector, Ap' | Promega |
| pET28a | Expression vector, Kn' | Novagen |
| pDONR201 | Entry vector in the Gateway system, Kn' | Life Technologies |

(continued on next page)
Table 1 (continued)

| Strains            | Genotype and notes | References                           |
|--------------------|--------------------|--------------------------------------|
| pAUC40             | Suicide vector pKNG101::attR-ccdB-Cm\(^R\); Cm\(^R\), Str\(^R\), sacB | Carlier, Burbank & Von Bodman (2009) |
| pEVS104            | Conjugative helper plasmid, \(tra\, trb\); Kn\(^R\) | Stabb & Ruby (2002)                  |
| pUC18R6K-mini-Tn7-cat | Tn7 vector for chromosomal integration into the intergenic region downstream of \(glmS\); Cm\(^R\), Ap\(^R\) | Choi et al. (2005)                   |
| pPROBE\(^{-}\)GFP[\text{tagless}] \(P\)\(_{lrhA}\) | pPROBE\(^{-}\)GFP[\text{tagless}] vector with the promoter of \(lrhA\); Kn\(^R\) | This study                           |

Notes.
\(^a\) Ap\(^R\), ampicillin resistance; Nal\(^R\), nalidixic acid resistance; Kn\(^R\), kanamycin resistance; Cm\(^R\), chloramphenicol resistance; Str\(^R\), streptomycin resistance.

(Novagen, Madison, WI, USA) and transformed into \(E. coli\) (BL21-DE3) (Studier & Moffatt, 1986) to express LrhA with a His\(_6\) tag at the N-terminus (37 kDa). Induction of protein expression with 0.1 M isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG) was performed at an OD\(_{600}\) of 0.5–0.8, 19 °C, overnight, shaking at 250 rpm. Cells were pelleted by centrifugation at 5,000 rpm in a JA-10 rotor (Beckman Coulter, Brea, CA, USA) for 20 min at 4 °C, snap-frozen with liquid nitrogen and stored at −75 °C. The cell pellet was then resuspended in Ni-NTA wash buffer (50 mM Tris–HCl, 300 mM NaCl, 50 mM imidazole) and sonicated to release proteins. Ultracentrifugation at 40,000 rpm in a Beckman Ti70 rotor for 1 h at 4 °C was used to subsequently remove the cell debris. The protein was purified using a Ni-NTA column (HisTrap HP, GE Healthcare) with Ni-NTA elution buffer (50 mM Tris–HCl, 300 mM NaCl, 500 mM imidazole). The protein purity was observed through standard SDS-PAGE electrophoresis.

**Electrophoretic mobility shift assays (EMSA)**
Promoter regions of genes of interest were amplified with FAM-labeled primers (Table S1) and extracted from a 1% agarose gel to examine the specific binding with purified His\(_6\)-LrhA over a range of concentrations. Twenty \(\mu\)l reactions with purified His\(_6\)-LrhA, 5 nM FAM-DNA in 1X EMSA buffer (10% glycerol, 1 mM MgCl\(_2\), 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl, 50 \(\mu\)g/ml poly (dl-dC) and 150 \(\mu\)g/ml BSA) were incubated at room temperature for 1 h before loading on to 1 × TBE (10.8 g/l Tris–HCl, 5.5 g/l boric acid, 2 mM EDTA, pH 8.0) 4%, 5%, or 6% acrylamide native gels followed by electrophoresis at 80 V for 2–3 h. Images were visualized on a Typhoon Trio Scanner (GE Healthcare). Experiments were done in duplicate.

**Construction of unmarked deletion mutant strains**
Chromosomal deletions of \(CKS\_0458/CKS\_0459\), \(CKS\_5208\), \(CKS\_5211\), and \(CKS\_5211/CKS\_5208\) were constructed based on the Gateway system (Life Technologies) and suicide vectors as described previously (Kernell Burke et al., 2015), but with primers listed in Table S1. In addition, another chromosomal deletion of \(rcsA\) was constructed using the same approach as described in a previous study (Kernell Burke et al., 2015), due to a deletion of \(\sim 66\)-kilobases (kb) in the chromosome of the original construct.

**Construction of chromosomal complementation strains**
Complementation strains were constructed by generating a chromosomal insertion of the promoter and coding regions of the target gene into the neutral region downstream of \(glmS\).
on the *P. stewartii* chromosome using the pUC18R6K-mini-Tn7-cat vector system (*Choi et al., 2005*) as previously described (*Kernell Burke et al., 2015*), but with primers listed in Table S1.

**Phenotypic surface motility assay**
Swarming motility for the wild-type, deletion and complementation strains was investigated under strict conditions to ensure a reproducible phenotype as previously described (*Kernell Burke et al., 2015*). Briefly, five µl of cell culture at an OD$_{600}$ of 0.5 were spotted directly on the agar surface of LB 0.4% agar quadrant plates supplemented with 0.4% glucose (*Herrera et al., 2008*). Plates were incubated in a closed plastic box at 30 °C for 2 days prior to observation.

**Phenotypic capsule production assay**
Bacterial strains were grown overnight in LB broth supplemented with the appropriate antibiotics at 30 °C with shaking. The overnight cultures were subcultured in fresh LB medium to an OD$_{600}$ of 0.05 and grown to an OD$_{600}$ of 0.5 at 30 °C with shaking. The strains were then cross-streaked with sterilized wooden sticks on 1.5% agar plates containing 0.1% casamino acids, 1% peptone and 1% glucose (CPG) (*Kernell Burke et al., 2015; Von Bodman, Majerczak & Coplin, 1998*). Plates were incubated at 30 °C, lid-up for 2 days to observe the capsule production and visualized using the Bio-Rad Gel Doc imager system.

**Plant virulence assay**
Virulence assays with *P. stewartii* strains in *Zea mays* seedlings were adapted from established methods (*Von Bodman, Majerczak & Coplin, 1998; Kernell Burke et al., 2015*) with some modifications. In this study, *Zea mays* cv. Jubilee, 2B seeds were planted in Sunshine mix #1 or Promix soil for seven or six days, respectively, in a 28 °C growth chamber with ~100–200 µE m$^{-2}$ s$^{-1}$ light intensity, 16 h light/8 h dark and ~80% relative humidity (Percival Scientific, Inc.). Fifteen seedlings between 6 and 10 cm of height with two separated leaves were inoculated with five µl (~3 × 10$^5$ CFU) of bacterial culture grown to an OD$_{600}$ of 0.2 in LB broth (~6 × 10$^7$ CFU/ml). Prior to plant inoculation, the bacterial cells were washed and resuspended in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$ and 2 mM KH$_2$PO$_4$, pH 7.4). Wild-type strain and PBS controls were included in each trial, then, accumulated numbers of control-inoculated plants across all experiments were analyzed. A sterile needle (26G 5/8, 15.9 mm, SUB-Q Becton, Dickinson and Company) was used to make an ~1 cm incision in the stem ~1 cm above the soil line and the bacteria were added to the plant by slowly pipetting the inoculum while moving across the wound five times. The plants were observed on day 12 post-infection to assess the virulence by two independent observers. Symptom severity was scored based on a five-point scale with 0 = no symptoms; 1 = few scattered lesions; 2 = scattered water soaking symptoms; 3 = numerous lesions and slight wilting; 4 = moderately severe wilt; 5 = death. The data for each treatment were averaged together and used to calculate the mean and standard error. A Student’s $t$-test was used to calculate the $p$-value for experimental treatments compared to the wild-type treatment.
RESULTS

LrhA autorepresses its own gene expression in *P. stewartii*

A GFP reporter was used to measure levels of transcription from the *lrhA* promoter in the wild-type, ΔlrhA and ΔlrhA/lrhA+ strains of *P. stewartii* DC283 (Table 1). Expression levels of GFP in the ΔlrhA strain were significantly higher than the wild-type strain (Fig. 1, \( p = 0.00001 \)) indicating that LrhA normally represses its own expression in the wild-type strain. The expression level of the *lrhA* promoter in the complementation ΔlrhA/lrhA+ strain was restored to levels closer to those of the wild-type strain, and was also significantly different than the deletion strain (Fig. 1, \( p = 0.00002 \)).

Identification of LrhA direct targets through EMSAs

To determine if the observed *lrhA* autorepression occurred directly or indirectly, electrophoretic mobility shift assays (EMSAs) were performed. First, direct binding of LrhA to the promoter of its own gene was demonstrated by EMSA analysis (Fig. 2A). Next, the ability of LrhA to directly regulate additional gene targets was explored, using the *lrhA* promoter as a positive control for the His$_6$-LrhA activity and unlabeled P$_{lrhA}$ DNA to prove the specificity of the binding. In *E. coli*, LrhA is known as a repressor of motility by direct interaction with the promoter region of *flhD/flhC*, whose products promote the expression of flagellar gene synthesis (*Lehnen et al., 2002*). However, RNA-Seq data of expression levels of *flhD/flhC* in *P. stewartii* showed less than a two-fold difference between wild-type and...
Figure 2  Examination of binding of LrhA to select target promoters via EMSA. FAM-DNA probes were incubated with increasing concentrations of His$_6$-LrhA (LrhA) from left to right, corresponding to the slope of the triangles, to investigate the mobility shift upon specific binding to the protein. The competition reaction (indicated by the asterisk, *) was conducted with 25 nM unlabeled DNA of P$_{lrhA}$ to prove the specificity of the interaction. Autoregulation of LrhA was confirmed with the direct binding between purified LrhA to its promoter (A). Shifted bands were also observed with P$_{flhDC}$ (B), P$_{cks_0458}$ (D), and P$_{cks_5211}$ (E). There were no shifted bands observed for P$_{flhDC}$ (B) and P$_{cks_5208}$ (F), while the positive controls for LrhA activity showed a shift (−: reaction with P$_{lrhA}$ probe in the absence of LrhA, +: reaction with P$_{lrhA}$ probe in the presence of 200 nM LrhA). Concentrations of LrhA tested for P$_{lrhA}$ (A) are 0, 25, 50, 100, 200, 400, and 800 nM. Concentrations of LrhA tested for P$_{flhDC}$ (B) are 0, 400, 600, 800, and 1,000 nM. Concentrations of LrhA tested for P$_{cks_0458}$ (D), P$_{cks_5211}$ (E) and P$_{cks_5208}$ (F) are 0, 200, 400, 600, 800, and 1,000 nM. Grey arrows highlight unbound DNA probes. White arrows indicate unbound DNA generated during PCR reactions that do not interact specifically with LrhA. Black arrows point to the lane with specific binding at the highest concentration of LrhA.

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ΔlrhA strains (Kernell Burke et al., 2015) suggesting a lack of transcriptional regulation. Here, EMSA analysis showed that His6-LrhA does not bind to the promoter of flhD/flhC (Fig. 2B), explaining the observed lack of transcriptional regulation.

Additional analysis of the LrhA-regulated transcriptome in P. stewartii revealed that LrhA repressed the expression level of several more downstream targets, including rcsA, CKS_0458, CKS_5208 and CKS_5211 (Kernell Burke et al., 2015). RcsA activates capsule production, a known virulence factor in P. stewartii (Kernell Burke et al., 2015; Minogue et al., 2005; Poetter & Coplin, 1991; Wehland et al., 1999). The putative roles of genes for fimbria encoded by CKS_0458, annotated as a putative fimbrial subunit, (and CKS_0459 located downstream in an operon) and for surfactant expression encoded by CKS_5208 and CKS_5211, annotated as a rhamnosyltransferase I subunit B and a putative alpha/beta superfamily hydrolase/acyltransferase, respectively, in plant colonization and/or virulence had not been established. However, it seemed plausible that they might also play roles in host association as they were some of the most highly LrhA-repressed genes, four-fold or greater (Kernell Burke et al., 2015). Therefore, the binding of LrhA to the promoters of these genes was also examined via EMSA. The direct binding of His6-LrhA to PrcsA, PCKS_0458 and PCKS_5211 (Figs. 2C–2E), was demonstrated via EMSAs while PCKS_5208 did not interact with His6-LrhA in vitro (Fig. 2F). Collectively, these findings identified four directly controlled gene targets in the LrhA regulon. The lack of LrhA regulation of FlhD$_2$C$_2$, the master regulator of flagellar-based motility and chemotaxis in E. coli, indicates a different role for LrhA in controlling P. stewartii motility. The direct binding of LrhA to the promoter of rcsA further links LrhA to P. stewartii pathogenesis. The role of the two other LrhA direct targets CKS_0458 and CKS_5211 remained to be established.

Examining the role of putative fimbrial and surfactant production genes in the surface motility and virulence of P. stewartii

To further investigate the role of the downstream targets of LrhA putatively involved in production of fimbriae and surfactant, a reverse genetic approach was utilized. Markerless deletions of CKS_0458/CKS_0459, CKS_5208, CKS_5211 and CKS_5211/CKS_5208 were successfully generated. Corresponding chromosomal complementation strains were also generated with the exception of a double deletion mutant of CKS_5211/CKS_5208 complementation strain, due to the length constraint of the DNA fragment containing the adjacent genes. In surface motility assays, the P. stewartii wild-type strain showed either uni-directional (Fig. 3A and Fig. S1A) or omni-directional (Fig. 3B and Fig. S1B) expansion from the inoculum sites as had been previously observed (Herrera et al., 2008; Kernell Burke et al., 2015). In comparison to the wild type, there is no obvious difference between the various deletion and complementation strains; they all possessed similar level of expansion on the agar surface (Figs. S1C–S1J). Therefore, these genes do not appear to play any detectable role in surface motility via this assay.

The same deletion and complementation strains of the genes putatively involved in fimbriae and surfactant production were also tested for virulence via in planta xylem infection assays. A lrhA deletion strain caused an intermediate level of disease severity in
Figure 3  Impact of RcsA and LrhA on surface motility of P. stewartii. The pictures show the analysis of surface motility in P. stewartii DC283 strains. Examples of wild type unidirectional (A) or omnidirectional surface motility (B) are shown as controls. The ΔlrhA/lrhA+ complementation strain (D) is similar to the control in (B), while the ΔlrhA strain has reduced surface motility expanding over a smaller surface area (C), as has been previously observed (Kernell Burke et al., 2015). Both ΔrcsA strains had dramatically reduced surface motility (E and G) as well as the ΔrcsA/rcsA+−2015 strain (F). The ΔrcsA/rcsA+−2017 strain was complemented for the defect in surface motility (H). All pictures were taken at the same magnification after 2 days of incubation at 30 °C in a closed plastic box.

corn seedlings during xylem-infection assays (Kernell Burke et al., 2015). However, similar to the surface motility assays, no significant impacts on the virulence of P. stewartii were observed in the strains with deletions in either the fimbriae or surfactant synthesis genes (Fig. S2). Hence, the contribution of these genes individually to the virulence of the phytopathogen could not be measured.

Re-examining the role of RcsA in the capsule production, surface motility and virulence of P. stewartii

The important finding that LrhA directly binds to the promoter of rcsA, led to a reexamination of the previous findings about the physiological role of RcsA in P. stewartii. In prior work, rcsA deletion and complementation strains of DC283 had been constructed (ΔrcsA−2015 and ΔrcsA/rcsA+−2015) (Kernell Burke et al., 2015). However, complete assembly of the genome of P. stewartii DC283 (Duong, Stevens & Jensen, 2017) revealed that there is a large deletion, ~66 kb containing 68 genes (Table S2), in the ΔrcsA−2015 and ΔrcsA/rcsA+−2015 strains. This deletion was not obvious using the incomplete genome sequence (NCBI GenBank accession no. AHIE0000000.1), but was found during a re-analysis of previously generated RNA-Seq data (Kernell Burke et al., 2015) using the new genome sequence (NCBI GenBank accession no. CP017581).

Therefore, a new set of rcsA deletion and complementation strains was re-constructed (ΔrcsA−2017 and ΔrcsA/rcsA+−2017) and shown to include the 66-kb region using PCR.
Figure 4 Impact of RcsA and LrhA on capsule production of *P. stewartii*. All pictures were taken at the same magnification after two days of incubation at 30 °C after cross-streaking on casamino acid, peptone, glucose (CPG) agar plates. Differences in capsule production are apparent in the regions between the arms of the X-cross streak.

(data not shown). These new strains then were subjected to three assays to establish the true phenotypes of the *rcsA* deletion strain. First, capsule production assays have re-confirmed that RcsA regulates EPS production, as was shown for the 2015 strains (*Kernell Burke et al., 2015*). Both the Δ*rcsA*-2015 (Fig. 4D) and Δ*rcsA*-2017 strains (Fig. 4F) are not as mucoid as the parental wild-type strain (Fig. 4A) or the pair of *lrhA* deletion and complementation strains (Figs. 4B and 4C) as assessed by visual observation. The chromosomal complementation strains Δ*rcsA/rcsA^+*-2015 (Fig. 4E) and Δ*rcsA/rcsA^+*-2017 (Fig. 4G) had mucoid levels as high or higher than those seen in the wild type (Fig. 4A).

Second, surface motility assays were performed. These original strains Δ*rcsA*-2015 and Δ*rcsA/rcsA^+*-2015 strains had not previously been examined for surface motility (*Kernell Burke et al., 2015*), but both were surprisingly defective for this phenotype (Figs. 3E and 3F). The fact that surface motility was not complemented by addition of *rcsA* back into the chromosome provided further evidence for the importance of the 66-kb region deletion that had been discovered initially through bioinformatics analysis. The new Δ*rcsA*-2017 also has severely reduced surface movement (Fig. 3G) while its complementation strain (Fig. 3H) restored motility levels similar to the wild-type strain (Figs. 3A and 3B). Thus, it has been demonstrated that RcsA plays a previously unappreciated role in the surface motility of *P. stewartii*. The defect in surface motility associated with the deletion of *rcsA* (Fig. 3G) appears to be greater than the defect in Δ*lrhA* (Fig. 3C). The Δ*lrhA/lrhA^+* strain has restored levels of surface motility (Fig. 3D), similar to the wild type (Figs. 3A and 3B), as previously reported (*Kernell Burke et al., 2015*).
Finally, the xylem infection assays for the newly constructed ΔrcsA-2017 strain and its complement, with the inclusion of the wild-type strain and PBS as controls, indicated that the absence of rcsA significantly \( (p < 0.05) \) reduces the severity of the disease compared to the wild-type and complementation strains (Fig. 5) \( (p < 0.05) \). These results have similar trends with those reported for the 2015 strains \( (Kernell Burke et al., 2015) \) which confirms the role of rcsA in virulence of this phytopathogen. However, strains from the 2015 study that were missing the 66-kb region were reduced in their average disease severity (score \( \sim 0 \) and \( \sim 1.5 \) for the deletion and complementation strains, respectively) in comparison to the new 2017 strains (score \( \sim 1.5 \) and \( \sim 3.5 \) for the deletion and complementation strains, respectively) while the wild-type control had similar levels in both studies, implicating a role for the 66-kb region in virulence as well as surface motility.

**DISCUSSION**

The role of the LrhA regulon in *P. stewartii* was further investigated in this study to understand how it is involved in the surface motility and virulence of the pathogen. Previous studies showed that surface motility in *P. stewartii* contributes to disease pathogenesis and this process involves both QS-controlled biofilm formation and flagella \( (Herrera et al., 2008) \). However, to date, there is no clear evidence to directly connect the synthesis of flagella to QS control in *P. stewartii*. Unlike *E. coli*, the QS-controlled transcription factor LrhA in *P. stewartii* does not regulate FlhD\(_2\)C\(_2\), the master activator of flagellar synthesis. This was suggested by earlier RNA-Seq data \( (Kernell Burke et al., 2015) \), but directly tested here through EMSA that confirmed the inability of LrhA to bind to the
flhD/flhC promoter. Additionally, LrhA activates its own expression in E. coli whereas autorepression was observed in P. stewartii. Even though P. stewartii LrhA has 77% amino acid identity to E. coli LrhA, the two have clearly evolved distinctive physiological roles in their host organisms.

In an attempt to define the function of the genes controlled by LrhA in P. stewartii, a reverse genetics approach was used to examine the role of select LrhA-regulated genes in surface motility and virulence of the phytopathogen. Multiple deletion and complementation strains of genes annotated as being involved in surfactant production (CKS_5208 and CKS_5211, initially annotated as a rhamnosyltransferase I subunit B and putative alpha/beta superfamily hydrolase/acyltransferase, respectively) and fimbriae assembly (CKS_0458 and CKS_0459, annotated as putative fimbrial subunits) were constructed and tested. Interestingly, none of these genes appear to play a fundamental role in surface motility and virulence individually. A LrhA deletion mutant impacting expression of multiple genes in the regulon produced noticeably decreased surface motility, but only intermediate virulence levels in comparison to the wild-type strain (Kernell Burke et al., 2015).

With regard to biosurfactant and fimbriae genes potentially associated with surface motility and adhesion, respectively, P. stewartii appears to utilize multiple levels of repression to ensure that the level of those genes’ expression is minimal. This low level of
expression was again confirmed by an in planta RNA-Seq analysis (Packard et al., 2017). In the LrhA deletion strain expression of these genes was elevated. Thus, deletion mutants might actually mimic wild-type levels of the expression of these genes, producing a wild-type phenotype. Alternatively, these genes are not functional in the wild-type strain (indeed the new genome sequence (Duong, Stevens & Jensen, 2017) suggests that CKS_5211 is a pseudogene) or they may serve another function for the bacterium that was not examined in this study. Biofilm/adhesion assays were inconclusive (data not shown). Interestingly, some Pantoea species have been demonstrated to produce biosurfactants when grown on hydrocarbons (Vasileva-Tonkova & Gesheva, 2007). How this might impact bacterial surface motility or survival in planta is unclear.

It has been demonstrated that both RcsA and LrhA play an essential role to the surface motility of the wild-type strain of P. stewartii. The observed intermediate impact of a LrhA deletion on virulence may be due primarily to its direct control of RcsA and thereby its indirect control on the levels of stewartan extracellular polysaccharide produced during growth within the plant. However, it could be that some of the other genes regulated by LrhA that were not examined in this work were actually contributing to the observed phenotypes in the LrhA deletion strain. RNA-Seq analysis of the transcriptome controlled by LrhA revealed 23 additional genes, in addition to the ones examined in this study, that were differentially expressed four-fold or more in comparison to the wild-type strain (Kernell Burke et al., 2015). Overall, the majority of the genes in the LrhA regulon code for hypothetical proteins and phage-related proteins, 57.7% (15/26) and 15.4% (4/26) respectively. The possible role of these genes with regard to surface motility and virulence remains to be established, but LrhA clearly regulates these processes.

The newly discovered connection between RcsA and surface motility suggests coordination of the RcsA and LrhA regulons with regard to bacterial virulence in the corn host beyond promotion of capsule production. Capsule production is thought to be a factor impacting the ability of surface motility to occur in this phytopathogen, which may explain the need for integrated downstream regulation. The fact that the strain with the 66-kb deletion region could not be complemented by rcsA suggests that there are additional genes in this region that are essential to surface motility and virulence. Further work will be needed to identify these genes and to overall correlate to the ability of the phytopathogen to move inside the plant via surface motility in relation to virulence.

**CONCLUSIONS**

The findings of this study have further defined the tightly coordinated gene regulation that occurs in the QS regulon of the corn pathogen P. stewartii. The EsaR-activated transcription factor LrhA was found to directly auto-repress expression of its own gene as demonstrated through GFP-transcription fusions and EMSA experiments. In addition, the direct binding of LrhA to downstream targets, such as the promoters of genes coding for RcsA, and for putative biosurfactant synthesis (CKS_5211) and fimbrial production (CKS_0458), was also shown. This established a hierarchy of gene regulation in the QS network from the master regulator, EsaR, to the downstream transcription factors, RcsA and LrhA, which in turn
control the expression of their own targets. Intriguingly, EsaR also directly controls some of these same targets (Ramachandran et al., 2014; Ramachandran & Stevens, 2013) integrating with coherent type two (RcsA) and type three (LrhA) feed forward loops (Mangan & Alon, 2003) to regulate genes in the QS regulon in a manner that ensures precisely synchronized gene expression (Fig. 6).

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• Duy An Duong conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
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REFERENCES

Beck von Bodman S, Farrand SK. 1995. Capsular polysaccharide biosynthesis and pathogenicity in Erwinia stewartii require induction by an N-acylhomoserine lactone autoinducer. *Journal of Bacteriology* 177:5000–5008 DOI 10.1128/jb.177.17.5000-5008.1995.

Blumer C, Kleefeld A, Lehnen D, Heintz M, Dobrindt U, Nagy G, Michaelis K, Emody L, Polen T, Rachel R, Wendisch VF, Unden G. 2005. Regulation of type 1 fimbriae synthesis and biofilm formation by the transcriptional regulator LrhA of Escherichia coli. *Microbiology* 151:3287–3298 DOI 10.1099/mic.0.28098-0.

Braun EJ. 1982. Ultrastructural investigation of resistant and susceptible maize inbreds infected with Erwinia stewartii. *Phytopathology* 72:159–166 DOI 10.1094/Phyto-72-159.

Carlier A, Burbank L, Von Bodman SB. 2009. Identification and characterization of three novel Esal/EsaR quorum-sensing controlled stewartan exopolysaccharide biosynthetic genes in Pantoea stewartii ssp. stewartii. *Molecular Microbiology* 74:903–913 DOI 10.1111/j.1365-2958.2009.06906.x.

Carlier AL, Von Bodman SB. 2006. The rcsA promoter of Pantoea stewartii subsp. stewartii features a low-level constitutive promoter and an EsaR quorum-sensing-regulated promoter. *Journal of Bacteriology* 188:4581–4584 DOI 10.1128/JB.00211-06.

Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, Schweizer HP. 2005. A Tn7-based broad-range bacterial cloning and expression system. *Nature Methods* 2:443–448 DOI 10.1038/nmeth765.

Dolph PJ, Majerczak DR, Coplin DL. 1988. Characterization of a gene cluster for exopolysaccharide biosynthesis and virulence in Erwinia stewartii. *Journal of Bacteriology* 170:865–871 DOI 10.1128/jb.170.2.865-871.1988.

Duong DA, Stevens AM, Jensen RV. 2017. Complete genome assembly of Pantoea stewartii subsp. stewartii DC283, a corn pathogen. *Genome Announcements* 5:e00435-17 DOI 10.1128/genomeA.00435-17.

Esker PD, Nutter FW. 2002. Assessing the risk of Stewart’s disease of corn through improved knowledge of the role of the corn flea beetle vector. *Phytopathology* 92:668–670 DOI 10.1094/PHYTO.2002.92.6.668.

Gibson KE, Silhavy TJ. 1999. The LysR homolog LrhA promotes RpoS degradation by modulating activity of the response regulator sprE. *Journal of Bacteriology* 181:563–571.

Grant SG, Jessee J, Bloom FR, Hanahan D. 1990. Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. *Proceedings of the National Academy of Sciences of the United States of America* 87:4645–4649 DOI 10.1073/pnas.87.12.4645.

Ham JH, Majerczak DR, Arroyo-Rodriguez AS, Mackey DM, Coplin DL. 2006. WtsE, an AvrE-family effector protein from Pantoea stewartii subsp. stewartii, causes disease-associated cell death in corn and requires a chaperone protein for stability. *Molecular Plant-Microbe Interactions* 19:1092–1102 DOI 10.1094/MPMI-19-1092.
Herrera CM, Koutsoudis MD, Wang X, Von Bodman SB. 2008. *Pantoea stewartii* subsp. *stewartii* exhibits surface motility, which is a critical aspect of Stewart’s wilt disease development on maize. *Molecular Plant-Microbe Interactions* 21:1359–1370 DOI 10.1094/MPMI-21-10-1359.

Kernell Burke A, Duong DA, Jensen RV, Stevens AM. 2015. Analyzing the transcriptomes of two quorum-sensing controlled transcription factors, RcsA and LrhA, important for *Pantoea stewartii* virulence. *PLOS ONE* 10:e0145358 DOI 10.1371/journal.pone.0145358.

Kvitko BH, Bruckbauer S, Prucha J, McMillan I, Breland EJ, Lehman S, Mladinich K, Choi KH, Karkhoff-Schweizer R, Schweizer HP. 2012. A simple method for construction of *pir*+ Enterobacterial hosts for maintenance of R6K replicon plasmids. *BMC Research Notes* 5:157 DOI 10.1186/1756-0500-5-157.

Labes M, Puhler A, Simon R. 1990. A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for Gram-negative bacteria. *Gene* 89:37–46 DOI 10.1016/0378-1119(90)90203-4.

Lehnen D, Blumer C, Polen T, Wackwitz B, Wendisch VF, Unden G. 2002. LrhA as a new transcriptional key regulator of flagella, motility and chemotaxis genes in *Escherichia coli*. *Molecular Microbiology* 45:521–532 DOI 10.1046/j.1365-2958.2002.03032.x.

Mangan S, Alon U. 2003. Structure and function of the feed-forward loop network motif. *Proceedings of the National Academy of Sciences of the United States of America* 100:11980–11985 DOI 10.1073/pnas.213381100.

Miller WG, Leveau JH, Lindow SE. 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. *Molecular Plant-Microbe Interactions* 13:1243–1250 DOI 10.1094/MPMI.2000.13.11.1243.

Minogue TD, Carlier AL, Koutsoudis MD, Von Bodman SB. 2005. The cell density-dependent expression of stewartan exopolysaccharide in *Pantoea stewartii* ssp. *stewartii* is a function of EsaR-mediated repression of the *rcsA* gene. *Molecular Microbiology* 56:189–203 DOI 10.1111/j.1365-2958.2004.04529.x.

Nimtz M, Mort A, Wray V, Domke T, Zhang Y, Coplin DL, Geider K. 1996. Structure of stewartan, the capsular exopolysaccharide from the corn pathogen *Erwinia stewartii*. *Carbohydrate Research* 288:189–201 DOI 10.1016/S0008-6215(96)90797-1.

Packard H, Kernell Burke A, Jensen RV, Stevens AM. 2017. Analysis of the *in planta* transcriptome expressed by the corn pathogen *Pantoea stewartii* subsp. *stewartii* via RNA-Seq. *PeerJ* 5:e3237 DOI 10.7717/peerj.3237.

Poetter K, Coplin DL. 1991. Structural and functional analysis of the *rcsA* gene from *Erwinia stewartii*. *Molecular & General Genetics* 229:155–160 DOI 10.1007/BF00264225.

Ramachandran R, Burke AK, Cormier G, Jensen RV, Stevens AM. 2014. Transcriptome-based analysis of the *Pantoea stewartii* quorum-sensing regulon and identification
of EsaR direct targets. Applied and Environmental Microbiology 80:5790–5800 DOI 10.1128/AEM.01489-14.

Ramachandran R, Stevens AM. 2013. Proteomic analysis of the quorum-sensing regulon in Pantoea stewartii and identification of direct targets of EsaR. Applied and Environmental Microbiology 79:6244–6252 DOI 10.1128/AEM.01744-13.

Roper MC. 2011. Pantoea stewartii subsp. stewartii: lessons learned from a xylem-dwelling pathogen of sweet corn. Molecular Plant Pathology 12:628–637 DOI 10.1111/j.1364-3703.2010.00698.x.

Schu DJ, Carlier AL, Jamison KP, Von Bodman S, Stevens AM. 2009. Structure/function analysis of the Pantoea stewartii quorum-sensing regulator EsaR as an activator of transcription. Journal of Bacteriology 191:7402–7409 DOI 10.1128/JB.00994-09.

Shong J, Huang YM, Bystroff C, Collins CH. 2013. Directed evolution of the quorum-sensing regulator EsaR for increased signal sensitivity. ACS Chemical Biology 8:789–795 DOI 10.1021/cb3006402.

Simon R, Prief U, Pühler A. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Nature Biotechnology 1:784–791 DOI 10.1038/nbt1183-784.

Stubb EV, Ruby EG. 2002. RP4-based plasmids for conjugation between Escherichia coli and members of the Vibrionaceae. Methods in Enzymology 358:413–426 DOI 10.1016/S0076-6879(02)58106-4.

Studier FW, Moffatt BA. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. Journal of Molecular Biology 189:113–130 DOI 10.1016/0022-2836(86)90385-2.

Vasileva-Tonkova E, Gesheva V. 2007. Biosurfactant production by antarctic facultative anaerobe Pantoea sp. during growth on hydrocarbons. Current Microbiology 54:136–141 DOI 10.1007/s00284-006-0345-6.

Von Bodman SB, Ball JK, Faini MA, Herrera CM, Minogue TD, Urbanowski ML, Stevens AM. 2003. The quorum sensing negative regulators EsaR and ExpR Ecc homologues within the LuxR family, retain the ability to function as activators of transcription. Journal of Bacteriology 185:7001–7007 DOI 10.1128/JB.185.23.7001-7007.2003.

Von Bodman SB, Bauer WD, Coplin DL. 2003. Quorum sensing in plant-pathogenic bacteria. Annual Review of Phytopathology 41:455–482 DOI 10.1146/annurev.phyto.41.052002.095652.

Von Bodman SB, Majerczak DR, Coplin DL. 1998. A negative regulator mediates quorum-sensing control of exopolysaccharide production in Pantoea stewartii subsp. stewartii. Proceedings of the National Academy of Sciences of the United States of America 95:7687–7692 DOI 10.1073/pnas.95.13.7687.

Wehland M, Kiecker C, Coplin DL, Kelm O, Saenger W, Bernhard F. 1999. Identification of an RcsA/RcsB recognition motif in the promoters of exopolysaccharide biosynthetic operons from Erwinia amylovora and Pantoea stewartii subspecies stewartii. Journal of Biological Chemistry 274:3300–3307 DOI 10.1074/jbc.274.6.3300.