Lon protease modulates virulence traits in *Erwinia amylovora* by direct monitoring of major regulators and indirectly through the Rcs and Gac-Csr regulatory systems

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SUMMARY

Lon, an ATP-dependent protease in bacteria, influences diverse cellular processes by degrading damaged, misfolded and short-lived regulatory proteins. In this study, we characterized the effects of *lon* mutation and determined the molecular mechanisms underlying Lon-mediated virulence regulation in *Erwinia amylovora*, an enterobacterial pathogen of apple. *Erwinia amylovora* depends on the type III secretion system (T3SS) and the exopolysaccharide (EPS) amylovoran to cause disease. Our results showed that mutation of the *lon* gene led to the overproduction of amylovoran, increased T3SS gene expression and the non-motile phenotype. Western blot analyses showed that mutation in *lon* directly affected the accumulation and stability of HrpS/HrpA and RcsA. Mutation in *lon* also indirectly influenced the expression of *flhD*, *hrpS* and *csrB* through the accumulation of the RcsA/RcsB proteins, which bind to the promoter of these genes. In addition, *lon* expression is under the control of CsrA, possibly at both the transcriptional and post-transcriptional levels. Although mutation in *csrA* abolished both T3SS and amylovoran production, deletion of the *lon* gene in the *csrA* mutant only rescued amylovoran production, but not T3SS. These results suggest that CsrA might positively control both T3SS and amylovoran production partly by suppressing Lon, whereas CsrA may also play a critical role in T3SS by affecting unknown targets.

Keywords: CsrA/RsmA, *csrB/rsmB*, GacS/GacA, HrpS/HrpR, post-translational regulation, RcsA/RcsB, T3SS.

INTRODUCTION

Erwinia amylovora, an enterobacterial plant pathogen, causes fire blight disease of apples and pears in more than 50 countries around the world. The type III secretion system (T3SS) and the exopolysaccharide (EPS) amylovoran are two major pathogenicity factors of the pathogen (Khan *et al.*, 2012; Zhao, 2014). The T3SS in *E. amylovora* is encoded by the hypersensitive response and pathogenicity (*hrp*) island. It has been demonstrated that expression of the *hrp*-T3SS genes is activated by the master regulator HrpL, a member of the exocytoplasmic functions (ECF) subfamily of sigma factors (McNally *et al.*, 2012; Wei and Beer, 1995). In turn, *hrpL* transcription is positively regulated by alternative sigma factor 54 (RpoN), its modulation protein YhbH, bacterial enhancer-binding protein (bEBP) HrpS and integration host factor IHF (Ancona *et al.*, 2014; Lee and Zhao, 2016). HrpS, a member of the NtrC family, activates RpoN-dependent transcription by mediating the isomerization of the RpoN–RNA polymerase (RNAP) complex, whereas the nucleoid-associated protein IHF enables the interaction between HrpS and RpoN (Bush and Dixon, 2012; Lee and Zhao, 2016; Lee *et al.*, 2016). Moreover, the RpoN–HrpL alternative sigma factor cascade is further activated by linear nucleotide second messengers (p)ppGpp, which are also essential for T3SS gene expression and virulence under nutrient stress conditions (Ancona *et al.*, 2015b).

In addition, amylovoran plays an important role in virulence, biofilm formation and survival of the bacterium (Koczan *et al.*, 2009; Sjulin and Beer, 1978). Genome-wide screening of two-component systems (TCSs) identified major regulators of amylovoran production in *E. amylovora* (Zhao *et al.*, 2009a). Among them, the enterobacterial-specific Rcs phosphorelay system is essential for pathogenicity and amylovoran production (Ancona *et al.*, 2015a; Bereswill and Geider, 1997; Bernhard *et al.*, 1990; Wang *et al.*, 2009, 2012). The Rcs system is an unusual complex TCS, comprising three core proteins RcsBCD and one auxiliary protein RcsA without the phosphorylation site (Bernhard *et al.*, 1990; Gottesman *et al.*, 1985; Majdalani and Gottesman, 2005; Wehland *et al.*, 1999). The RcsB homodimer or RcsA/RcsB heterodimer binds to the conserved RcsAB box to regulate gene expression, including *amsG*, the first gene of the amylovoran biosynthetic operon, and *flhD* in *E. amylovora* (Ancona *et al.*, 2015a; Bernhard *et al.*, 1993; Wehland *et al.*, 1999). Furthermore, the GacS/GacA (GrrS/GrrA and BarA/UvrY) system, a widely distributed TCS in Gammaproteobacteria, negatively regulates amylovoran biosynthesis and T3SS in *E. amylovora* (Li *et al.*, 2014). It has been shown recently that negative regulation of virulence by GacS/GacA in *E. amylovora* acts through the non-coding small regulatory RNA (sRNA) *csrB*, which binds to and neutralizes the positive effect of the RNA-binding protein CsrA on T3SS gene expression

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Table 1 Bacterial strains and plasmids used in this study.

Strains, plasmids	Description	Reference, source
Erwinia amylovora		
Ea1189	Wild-type, isolated from apple	Wang *et al.* (2009)
Δlon	*lon*::Cm; Cm^R-insertional mutant of *lon* of Ea1189	This study
$\Delta rcsA$	*rcsA*::Cm; Cm^R-insertional mutant of *rcsA* of Ea1189	Ancona *et al.* (2014)
$\Delta rcsB$	*rcsB*::Km; Km^R-insertional mutant of *rcsB* of Ea1189	Wang *et al.* (2009)
$\Delta lon/rcsA$	*rcsA*::Cm, *lon*::Km; Km^R-insertional mutant of *lon* of $\Delta rcsA$	This study
$\Delta lon/rcsB$	*rcsB*::Km, *lon*::Cm; Cm^R-insertional mutant of *lon* of $\Delta rcsB$	This study
$\Delta csrA$	*csrA*::Cm; Cm^R-insertional mutant of *csrA* of Ea1189	Ancona *et al.* (2016)
$\Delta csrB$	*csrB*::Cm; Cm^R-insertional mutant of *csrB* of Ea1189	Ancona *et al.* (2016)
$\Delta lon/csrA$	*csrA*::Km, *lon*::Cm; Km^R-insertional mutant of *csrA* of Δlon	This study
Escherichia coli		
DH10B	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L *nup*G λ–	Invitrogen (Carlsbad, CA, USA)
Plasmids		
pKD46	ApR, PBAD *gam bet exo* pSC101 oriTS	Datsenko and Wanner (2000)
pKD32	Cm^R, FRT *cat* FRT tL3 oriR6Kγ *bla rgnB*	Datsenko and Wanner (2000)
pKD13	Km^R, FRT *kan* FRT tL3 oriR6Kγ *bla rgnB*	Datsenko and Wanner (2000)
pWSK29	Ap^R, cloning vector, low copy number	Wang and Kushner (1991)
pLon	3077-bp DNA fragment containing promoter sequence of *lon* gene in pWSK29	This study
pHrpS-His6	1537-bp DNA fragment containing promoter sequence of *hrpS* gene and C-terminal His-tag in pWSK29	This study
pHrpA-His6	803-bp DNA fragment containing promoter sequence of *hrpA* gene and C-terminal His-tag in pWSK29	Ancona *et al.* (2015b)
pRcsA-His6	1058-bp DNA fragment containing promoter sequence of *rcsA* gene and C-terminal His-tag in pWSK29	This study
pRcsB-His6	1142-bp DNA fragment containing promoter sequence of *rcsB* gene and C-terminal His-tag in pWSK29	This study
pLon-His6	2877-bp DNA fragment containing promoter sequence of *lon* gene and C-terminal His-tag in pWSK29	This study

and amylovoran production, indicating a critical role of CsrA in *E. amylovora* virulence (Ancona *et al.*, 2016). However, the targets of CsrA remain unknown in *E. amylovora*.

Lon is a highly conserved cytosolic protease belonging to the AAA^+ superfamily of ATPase, and acts as a major player in general protein quality control by degrading damaged or misfolded proteins (Chung and Goldberg, 1981). The proteolytic activity of Lon also contributes to the post-translational regulation of functional proteins. To recognize potentially deleterious proteins, Lon tends to bind a cluster of aromatic and non-polar residues, which are embedded in most native proteins (Gur and Sauer, 2008). As one well-characterized Lon substrate, the RcsA protein level is generally maintained low by HN-S-mediated transcriptional repression and Lon-dependent degradation (Sledjeski and Gottesman, 1995; Torres-Cabassa and Gottesman, 1987). Increased stability of RcsA and its associated overproduction of EPS in the *lon* mutant of some enterobacterial species lead to mucoid colonies (Gottesman *et al.*, 1985; Lai *et al.*, 2003). Lon also controls the SulA protein level, which inhibits cell division as part of the SOS response under DNA damage-inducing conditions (Huisman and D'Ari, 1981). Failure to remove accumulated SulA in the *lon* mutant strain blocks cell division, leading to the irradiation sensitivity phenotype (Gottesman *et al.*, 1981; Mizusawa and Gottesman, 1983).

Furthermore, Lon has been implicated in the regulation of the T3SS in several important Gram-negative bacteria (Bretz *et al.*, 2002; Jackson *et al.*, 2004; Takaya *et al.*, 2005). In *Yersinia pestis*, Lon positively regulates the T3SS by degrading YmoA, a small histone-like protein that suppresses T3SS gene expression (Jackson *et al.*, 2004). In *Pseudomonas syringae*, Lon acts as a negative regulator of the T3SS by degrading HrpR and effector proteins. HrpR, a homologue of HrpS, forms a heterohexamer with HrpS and is maintained low by Lon under non-inductive conditions (Bretz *et al.*, 2002). Once HrpL-dependent T3SS gene expression is activated, Lon affects the stability of effector proteins, thereby modulating the secretion rate (Losada and Hutcheson, 2005). In *E. amylovora*, Lon is involved in EPS regulation and UV tolerance, but is not required for the infection of apple seedlings (Eastgate *et al.*, 1995). However, the effect of *lon* mutation on *E. amylovora* virulence and its underlying molecular mechanisms has not been fully characterized.

The purpose of this study was to characterize the effect of *lon* mutation in *E. amylovora*, to identify potential targets of Lon and to determine the molecular mechanisms underlying Lon-mediated virulence regulation. Our results showed that mutation of the *lon* gene led to amylovoran overproduction, increased T3SS gene expression and the non-motile phenotype by direct targeting of RcsA and HrpS/HrpA, and indirectly by affecting the expression of the *flhD*, *hrpS* and *csrB* sRNA genes through the accumulation of the RcsA/RcsB proteins. Moreover, mutation of the *csrA* gene led to the up-regulation of *lon* expression, suggesting that positive regulation of T3SS and amylovoran production by CsrA could occur partly through suppression of *lon* expression.

RESULTS

Characterization of the Ea1189 *lon* mutant in *E. amylovora*

It has been reported previously that *E. amylovora* Lon is involved in EPS production, but is not required for the infection of apple seedlings (Eastgate *et al.*, 1995). In this study, we generated an insertional mutant strain with a defect in the *lon* gene in the background of the wild-type (WT) strain Ea1189 (Table 1). Consistent with previous reports, the Ea1189 *lon* mutant exhibited a mucoid colony on growth medium (Fig. S1A, see Supporting Information) and produced about 10 times more amylovoran than that of the WT strain, which could be partially complemented (Fig. 1A). Transcript levels of *rcsA* (a rate-limiting regulatory gene) and *amsG* (the first gene in the *ams* operon for amylovoran biosynthesis) were about five- and 40-fold higher, respectively, in the *lon* mutant (Fig. 1B). The Ea1189 *lon* mutant induced a typical

hypersensitive response (HR) lesion on tobacco (data not shown) and was as pathogenic as WT on immature pears, although the disease progress was similar or slightly faster in the mutant at 4 days post-inoculation (Fig. 1C). We also found that expression of the T3SS regulatory (*hrpL*) and effector (*hrpA*) genes was about six-fold higher in the *lon* mutant relative to the WT (Fig. 1D). These results indicate that Lon suppresses the expression of the genes required for T3SS and amylovoran in *E. amylovora*, but is not indispensable for its virulence.

Lon negatively regulates the T3SS by targeting HrpS and HrpA

Previous studies in *P. syringae* have shown that Lon negatively regulates the T3SS by targeting HrpR and effector proteins, but not HrpS (Bretz *et al.*, 2002; Losada and Hutcheson, 2005). *Erwinia amylovora* only contains HrpS, which shares about 40%–44% amino acid identity with HrpR and HrpS of *P. syringae*,
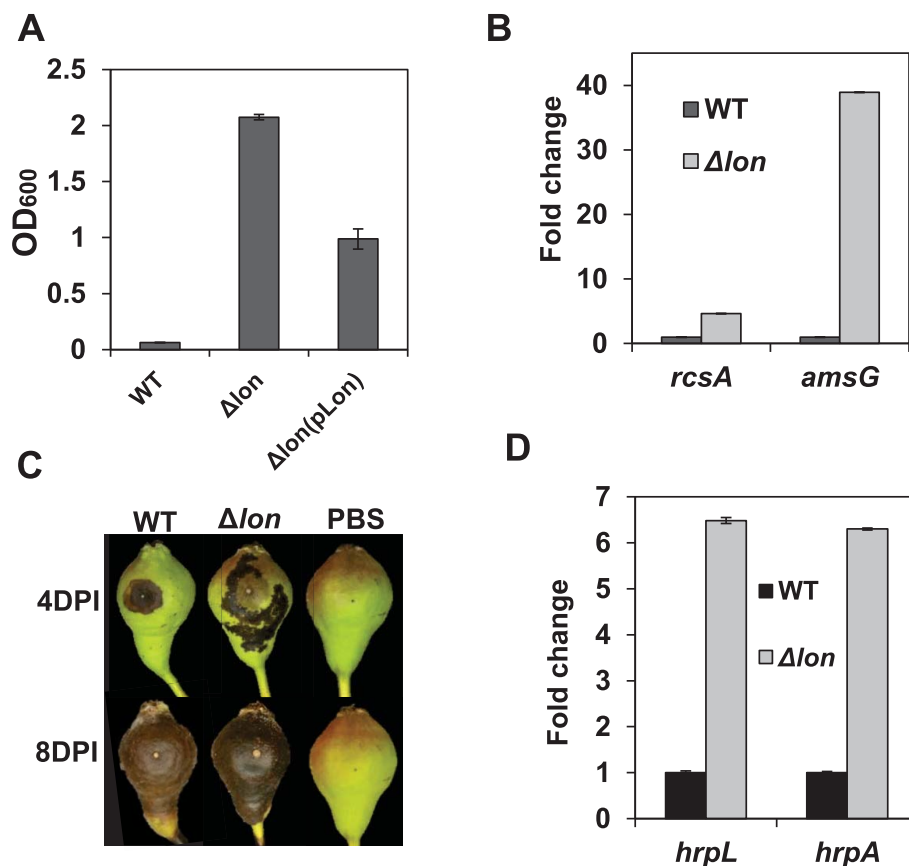

Fig. 1 Characterization of the *lon* mutant. (A) Amylovoran production of the wild-type (WT), the *lon* mutant and its complementation strain grown in MBMA medium for 24 h at 28 °C. (B) Relative gene expression of the *rcsA* and *amsG* genes in the *lon* mutant compared with the WT grown in MBMA medium for 18 h at 28 °C. (C) Disease symptoms caused by the WT and the *lon* mutant on immature pear fruits at 4 and 8 days post-inoculation (DPI). (D) Relative gene expression of the *hrpL* and *hrpA* genes in the *lon* mutant compared with the WT grown in *hrp*-inducing medium (HMM) for 6 h at 18 °C. The optical densities at 600 nm (OD_{600}) (A) and the relative fold change (B, D) are the means of three replicates, and similar results were obtained from repeated experiments. A representative of three independent experiments is presented for the virulence assay (C). Error bars indicate standard deviation.
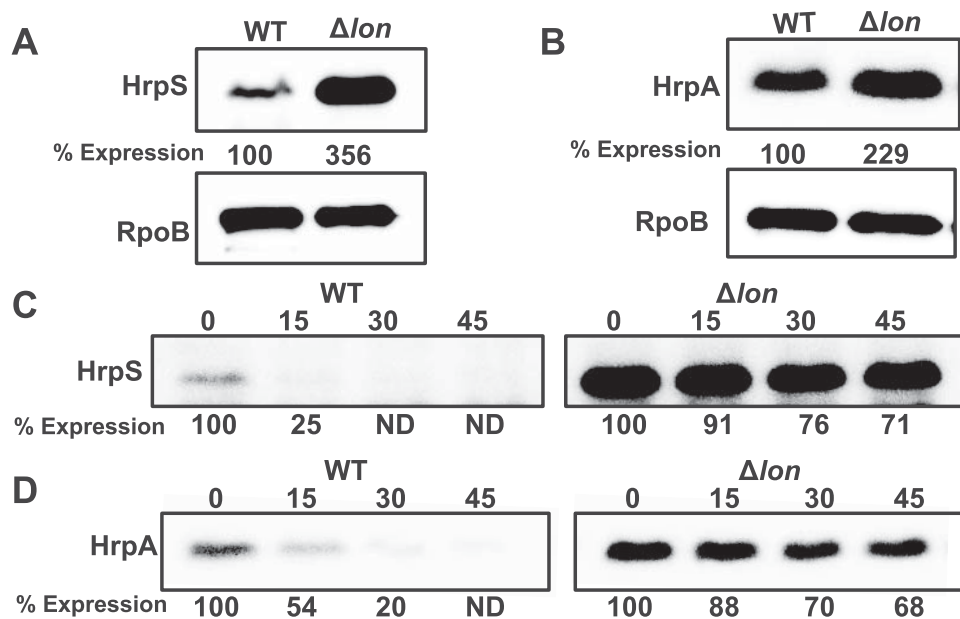
Fig. 2 Lon-dependent degradation of HrpS/HrpA regulates type III secretion system (T3SS) gene expression in *Erwinia amylovora*. Abundance of HrpS-His6 (A) and HrpA-His6 (B) proteins in the wild-type (WT) and *lon* mutant strains grown in *hrp*-inducing medium (HMM) for 6 h at 18 °C; the abundance of the RpoB protein was used as a loading control. Half-life of HrpS-His6 (C) and HrpA-His6 (D) proteins in the WT and *lon* mutant strains. For protein stability test, cells were grown in HMM for 6 h at 18 °C. Translation was stopped by the addition of tetracycline, and samples were taken at the indicated time points (min) at the top. The relative protein abundance at the bottom of each lane was calculated using ImageJ software. Cropped gel blots and percentage expression values are representatives of three independent experiments. ND, not detected.

respectively. We examined the abundance and stability of HrpS and HrpA proteins in the WT and *lon* mutant in *hrp*-inducing medium (HMM) using Western blot. The *lon* mutant accumulated about three- and two-fold more HrpS and HrpA protein, respectively, than that in the WT (Fig. 2A,B). The half-life of HrpS *in vivo* was increased to more than 45 min in the *lon* mutant from less than 15 min in the WT (Fig. 2C), and the half-life of HrpA was also increased from about 15 min to more than 45 min in the absence of Lon (Fig. 2D). These results indicate that HrpS and HrpA are directly targeted by Lon-dependent degradation.

Lon negatively regulates amylovoran by targeting RcsA, but not RcsB

In enterobacteria, previous reports have shown that Lon negatively regulates EPS biosynthesis by the degradation of RcsA, an auxiliary protein of the Rcs system (Gottesman *et al.*, 1985; Torres-Cabassa and Gottesman, 1987). Therefore, we examined the abundance and stability of RcsA, as well as RcsB, in the WT and *lon* mutant grown in MBMA medium using Western blot. As expected, the RcsA protein was 2.4-fold more abundant and exhibited a longer half-life (>45 min) in the *lon* mutant (Fig. 3A,C), indicating that RcsA is also negatively regulated by Lon-dependent degradation in *E. amylovora*. Interestingly, the RcsB protein was 1.6-fold more abundant in the *lon* mutant, but RcsB

protein stability was not significantly affected (Fig. 3B,D), suggesting that RcsB might not be directly targeted by Lon, but its expression might be subject to feedback regulation of the Rcs system.

RcsA/RcsB accumulation suppresses motility and *flhD* transcription in the *lon* mutant

In addition, we found that the *lon* mutant was non-motile (Fig. 4A). The diameter of the WT on the motility plate was 9 mm at 24 h and 29.6 mm at 48 h, whereas no circular movement was observed in the *lon* mutant, which remained at about 6 mm (Fig. 4B). Complementation of the *lon* mutant restored the motile phenotype (Fig. 4A), indicating that Lon is essential for motility in *E. amylovora*.

In order to identify suppressors of the *lon* mutant in controlling motility, we performed a transposon mutagenesis screening in the *lon* mutant background and obtained nine mutants with partially restored motility (Table 2). Among them, transposon insertion in the *rcsA* gene showed the strongest recovery in motility in the *lon* mutant (Table 2). In order to confirm this result, we constructed the *lon/rcsA* and *lon/rcsB* double deletion mutants. Interestingly, both the *lon/rcsA* and *lon/rcsB* double mutants partially recovered motility (Fig. 4B). Expression of the *flhD* gene, the master regulator of flagellar biosynthesis, was also recovered to the WT level in the *lon/rcsA* and *lon/rcsB* double mutants, but decreased five-fold

Fig. 3 Lon-dependent degradation of RcsA regulates amylovoran production in *Erwinia amylovora*. Abundance of RcsA-His6 (A) and RcsB-His6 (B) proteins in the wild-type (WT) and *lon* mutant strains grown in MBMA medium for 24 h at 28 °C; the abundance of the RpoB protein was used as a loading control. Half-life of RcsA-His6 (C) and RcsB-His6 (D) proteins in the WT and *lon* mutant strains. For protein stability test, cells were grown in MBMA medium for 24 h at 28 °C. Translation was stopped by the addition of tetracycline, and samples were taken at the indicated time points (min) at the top. Relative protein abundance at the bottom of each lane was calculated using ImageJ software. Cropped gel blots and percentage expression values are representatives of three independent experiments.
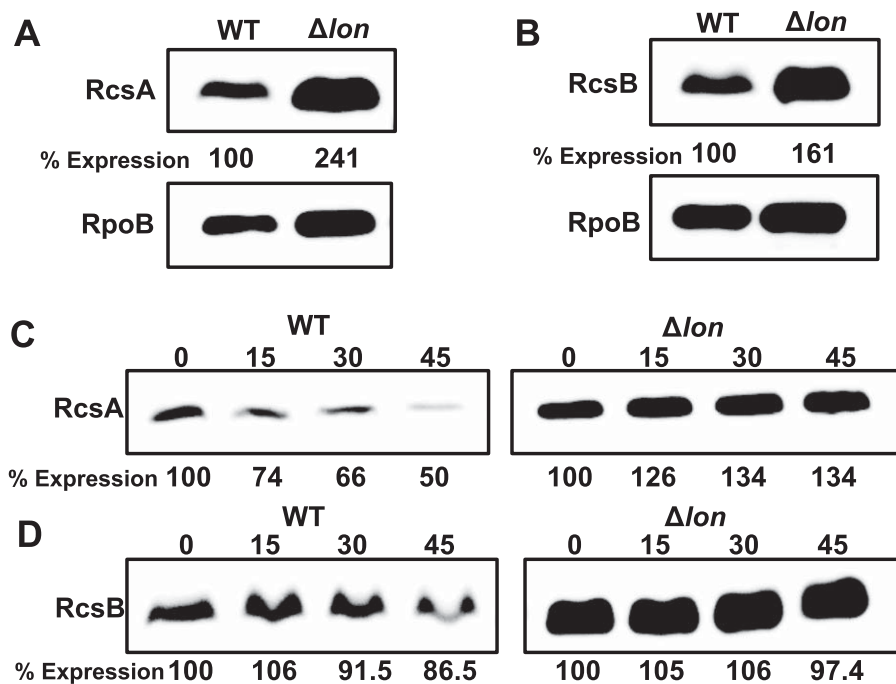
in the *lon* mutant (Fig. 4C). The transcript levels of the *flhD* gene increased slightly in the *rcsA* and *rcsB* single mutants, although they exhibited irregular and slightly decreased motility (Wang *et al.*, 2009). These results indicate that the accumulation of RcsA/ RcsB negatively regulates *flhD* transcription, and thus suppresses motility in the *lon* mutant. In addition, other regulators, such as the RNA chaperone Hfq and cholera toxin transcription activator YqeI, also contribute to the suppression of motility in the *lon* mutant, but the functional relevance in these situations was not determined in this study (Table 2).

Expression of *hrpS* is transcriptionally activated by RcsA/RcsB

A previous microarray study has shown that RcsB is required for full T3SS gene expression, but the mechanism remains uncertain (Wang *et al.*, 2012). Bioinformatic analyses of promoters found a potential RcsAB box (TAGGA-N$_4$-TCTTA) located 350 bp upstream of the *hrpS* start site. Indeed, *hrpS* gene expression was down-regulated in both *rcsA* and *rcsB* mutants in HMM, whereas it was up-regulated by about two-fold in the *lon* mutant (Fig. 5A). Deletion of either *rcsA* or *rcsB* in the *lon* mutant diminished this up-regulation of *hrpS* gene expression in the *lon* mutant, suggesting that *hrpS* gene expression is transcriptionally activated by RcsA/ RcsB. Binding of RcsA/RcsB to the *hrpS* upstream sequence was assessed by electrophoretic mobility shift assay (EMSA), as described previously (Ancona *et al.*, 2015a). A distinct band shift of the *hrpS* DNA probe was observed with RcsB and RcsA/RcsB proteins, but not with the RcsA protein alone (Fig. 5B). Western

blot analyses also showed that the abundance of RcsA and RcsB proteins was increased in the *lon* mutant grown in HMM when compared with the WT (Fig. 5C). These results indicate that the accumulation of RcsA/RcsB proteins leads to the up-regulation of *hrpS* transcription in the *lon* mutant.

Expression of the *csrB* sRNA is suppressed by RcsA/ RcsB accumulation in the *lon* mutant

The virulence gene expression of the *lon* mutant observed in this study was very similar to that reported for the *gacS/gacA* and *csrB* mutants in *E. amylovora* (Ancona *et al.*, 2016; Li *et al.*, 2014). We hypothesized that a connection between Lon and the GacS/GacA-Csr regulatory system exists. It has also been proposed that the Rcs system negatively regulates the expression of *rsmB*, a homologue of *csrB* sRNA, possibly by direct binding to its upstream sequence in *Pectobacterium* (Andresen *et al.*, 2010). Bioinformatic analysis of the *csrB* gene indeed found a potential RcsA/RcsB box (TACGA-N$_4$-TCTTA), which is located 172 bp upstream of the start site and is close to the GacA-binding site (Fig. 6A) (Lee and Zhao, 2016). A shifted band of the *csrB* DNA probe was observed with RcsB and RcsA/RcsB proteins, but not with RcsA alone (Fig. 6B). Transcript levels of *csrB* decreased about five-fold in the *lon* mutant, but increased 1.5-fold in the *rcsA* and *rcsB* mutants in MBMA medium (Fig. 6C). Deletion of the *rcsA/rcsB* gene in the *lon* mutant restored *csrB* expression to the WT level (Fig. 6C). However, *csrA* transcript levels were slightly increased in the five mutants tested relative to the WT. Similar expression patterns for both *csrA* and *csrB* were observed in these mutants
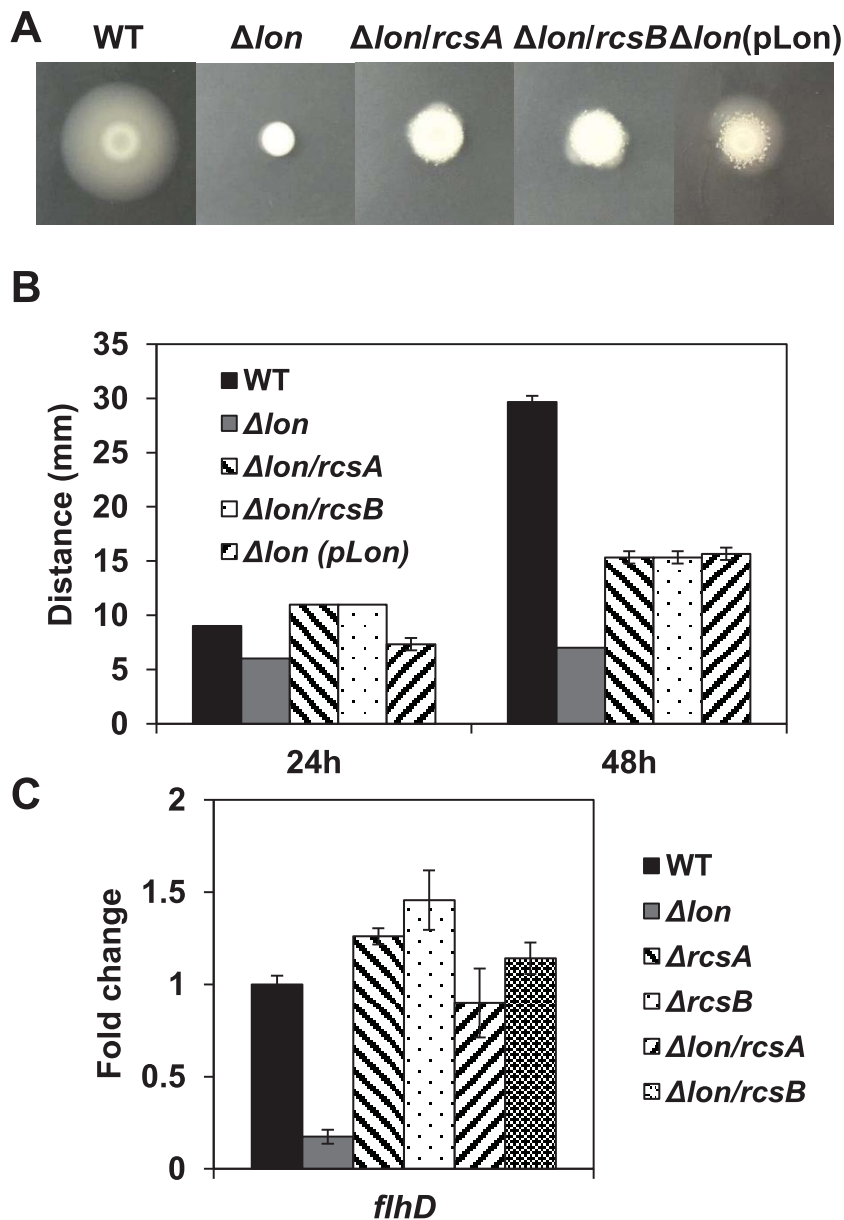
A

B

C

Fig. 4 RcsA/RcsB accumulation suppresses motility and *flhD* transcription in the *lon* mutant. (A) Movement of the wild-type (WT), *lon*, *rcsA*, *rcsB*, *lon*/*rcsA* and *lon*/*rcsB* mutants and the complementation strain of the *lon* mutant. Photographs were taken at 48 h post-incubation on a motility agar plate. (B) The moving distance of the WT, *lon*, *rcsA*, *rcsB*, *lon*/*rcsA* and *lon*/*rcsB* mutants and the complementation strain of the *lon* mutant. The diameters of the circle around the inoculation site (mm) were measured at 24 and 48 h post-inoculation. (C) Relative gene expression of *flhD* in the five mutant strains compared with the WT grown in MBMA medium for 18 h at 28 °C. The values of the relative fold change are the means of three replicates and the experiment was repeated three times with similar results. Error bars indicate standard deviation.

Table 2 Tn5 mutants with recovered motility in the *lon* mutant background.

| Accession	Gene	Description	Motility (24 h, mm)	Motility (48 h, mm)
EAM_0994	*lon*	Lon protease	6	7
EAM_1482	*rcsA*	Colanic acid capsular biosynthesis activation protein A	11.7 ± 0.58	16.3 ± 0.58
EAM_0427	*bspA*	Mechanosensitive ion channel protein	6.7 ± 0.58	11.7 ± 0.58
EAM_0436	*hfq*	RNA chaperone	8.7 ± 0.58	14
EAM_3422	*yqeI*	Cholera toxin transcription activator	7.3 ± 0.58	12.3 ± 0.58
EAM_3014		Hypothetical protein	6	10.7 ± 0.58
EAM_2896	*hrpI*	Type III secretion protein	5	9.3 ± 0.58
EAM_2178	*udk*	Uridine kinase	5	8
EAM_0009		Hypothetical protein	5	8
EAM_0609		Acetyltransferase	5.8 ± 0.58	9
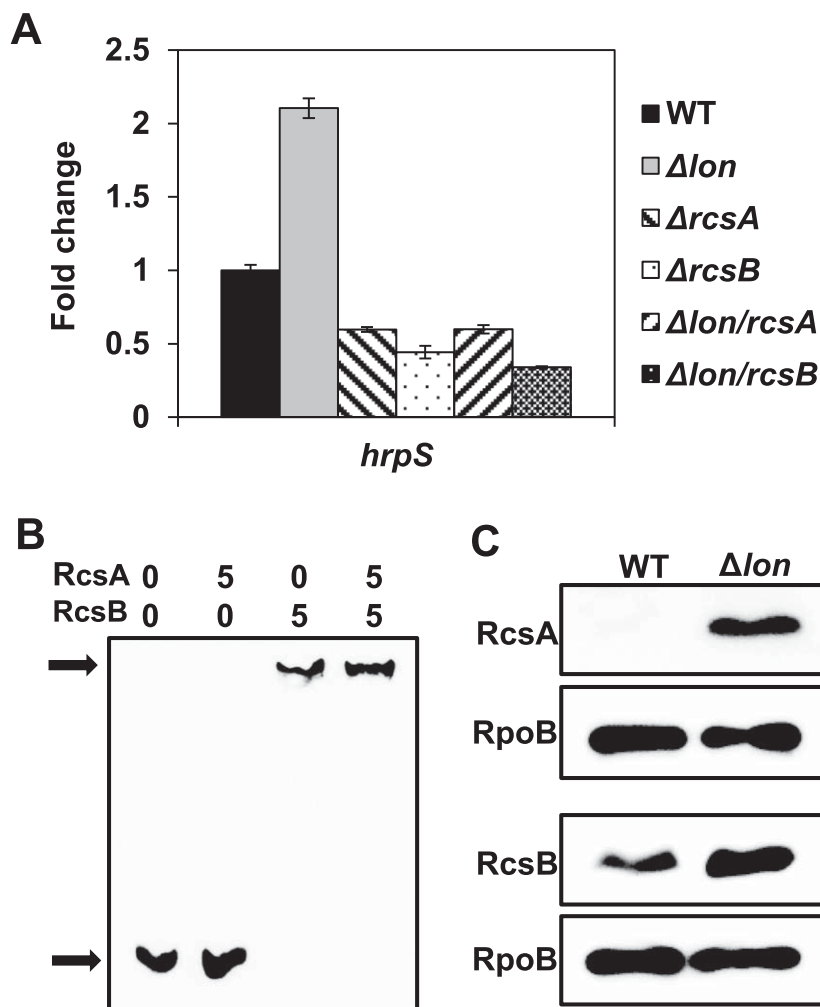
Fig. 5 Lon-dependent degradation of RcsA regulates the expression of *hrpS* in *Erwinia amylovora*. (A) Relative gene expression of the *hrpS* gene in the wild-type (WT), *lon*, *rcsA*, *rcsB*, *lon/rcsA* and *lon/rcsB* mutants compared with the WT grown in *hrp*-inducing medium (HMM) for 6 h at 18 °C. The values of the relative fold change are the means of three replicates and the experiment was repeated three times with similar results. Error bars indicate standard deviation. (B) Electrophoretic mobility shift assay for a 58-bp fragment of the *hrpS* upstream region and RcsA/RcsB proteins. Black arrows at the bottom and top indicate the free probe and the protein–DNA complex, respectively. The concentration of RcsA and RcsB (pmol) is indicated above each lane. (C) Abundance of RcsA-His6 and RcsB-His6 proteins in the *lon* mutant strains compared with the WT grown in HMM for 6 h at 18 °C. The abundance of the RpoB protein was used as a loading control. Cropped gel blots are representatives of three independent experiments.

grown in HMM (Fig. S1B). These results indicate that RcsA/RcsB accumulation in the *lon* mutant leads to the suppression of *csrB* transcription.

Transcription of *lon* is suppressed by CsrA

Given that mutations in *lon* and *csrA* caused opposite virulence gene expression patterns in *E. amylovora* (Ancona *et al.*, 2016), we further hypothesized that increased Lon activity might contribute to the decrease in T3SS and amylovoran production observed in the *csrA* mutant. Transcript levels of *lon* in the *csrA* mutant were about 2.5-fold up-regulated in both MBMA and HMM growth conditions, whereas no significant changes were observed in the *csrB* mutant (Figs 6D and S1C). However, Western blot analyses showed slightly increased Lon protein levels in the *csrA* mutant, but not in the *csrB* mutant (Figs 6E and S1D). These results suggest that CsrA might mainly regulate the expression of *lon* at the transcriptional level, but the possibility that CsrA post-transcriptionally affects *lon* translation could not be excluded.

To determine the effects of the increased Lon levels on the *csrA* mutant, we generated a *lon/csrA* double deletion mutant. The *lon/csrA* mutant still failed to cause disease on immature pears (Fig. 7A), but exhibited significantly increased amylovoran production (Fig. 7B). Western blot analyses revealed that the *lon/csrA* mutant showed slightly increased HrpS protein expression when compared with the *csrA* mutant, but HrpA proteins were barely detected in both mutants (Fig. 7C,D). These results suggest that CsrA might positively control both T3SS and amylovoran production partly by suppressing Lon, whereas CsrA may also play a critical role in T3SS by affecting unknown targets.

DISCUSSION

In bacteria, the abundance and quality of functional proteins are constantly monitored to meet the physiological needs of the cell by robust and highly selective post-translational degradation and modification. As one of the major ATP-dependent proteases in bacteria, Lon has been found to influence diverse cellular processes. In this study, we not only corroborated that Lon directly
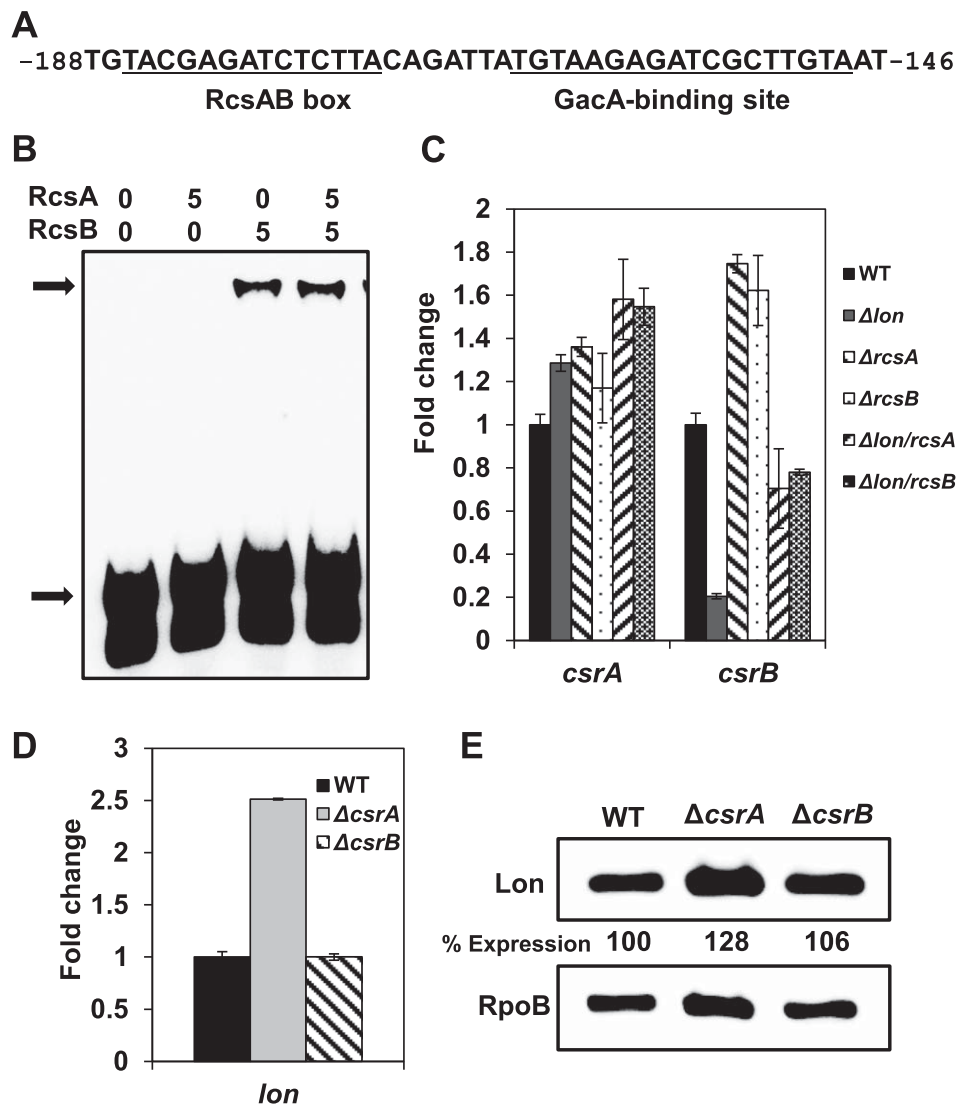
A

−188TG<u>TACGAGATCTCTTA</u>CAGATTA<u>TGTAAGAGATCGCTTGTA</u>AT−146

RcsAB box   **GacA-binding site**

B

C

D

E

Fig. 6 RcsA/RcsB accumulation suppresses *csrB* sRNA expression and the effect of *csrA* mutation on the *lon* gene. (A) The consensus RcsA/RcsB box and GacA-binding site on the *csrB* upstream region. Numbers refer to the nucleotide position relative to the start site of the *csrB* gene. (B) Electrophoretic mobility shift assay for a 58-bp fragment of the *csrB* upstream region and RcsA/RcsB proteins. Black arrows at the bottom and top indicate the free probe and the protein–DNA complex, respectively. The concentration of RcsA and RcsB (pmol) is indicated above each lane. (C) Relative gene expression of *csrA* and *csrB* in the *lon*, *rcsA*, *rcsB*, *lon/rcsA* and *lon/rcsB* mutants compared with the wild-type (WT) grown in MBMA medium for 18 h at 28 °C. (D) Relative expression of *lon* in the *csrA* and *csrB* mutants compared with the WT grown in MBMA medium for 18 h at 28 °C. The values of the relative fold change are the means of three replicates and the experiment was repeated twice with similar results. Error bars indicate standard deviation. (E) Abundance of Lon-His6 protein in the *csrA* and *csrB* mutant strains compared with the WT grown in MBMA for 18 h at 28 °C. The abundance of the RpoB protein was used as a loading control. Relative protein abundance at the bottom of each lane was calculated using ImageJ software. Cropped gel blots and percentage expression values are representatives of three independent experiments.

degrades RcsA, but also demonstrated that HrpA and HrpS, an activator of RpoN-dependent transcription of the T3SS, are direct targets of Lon in *E. amylovora*. We further provided evidence that the accumulation of RcsA/RcsB proteins in the *lon* mutant represses motility by inhibiting *flhD* expression, and promotes EPS production and T3SS gene expression by suppressing *csrB* sRNA expression and activating *hrpS* expression. Moreover, we documented that the expression of *lon* is under the control of CsrA,

possibly at both the transcriptional and post-transcriptional levels. These results are novel and further suggest that CsrA contributes to the activation of both T3SS and amylovoran production partly by suppressing Lon.

The complex enterobacterial-specific Rcs system was originally identified as a primary activator of EPS biosynthesis in *Escherichia coli* (Gottesman *et al.*, 1985; Majdalani and Gottesman, 2005). It is also well established that Lon negatively regulates the Rcs
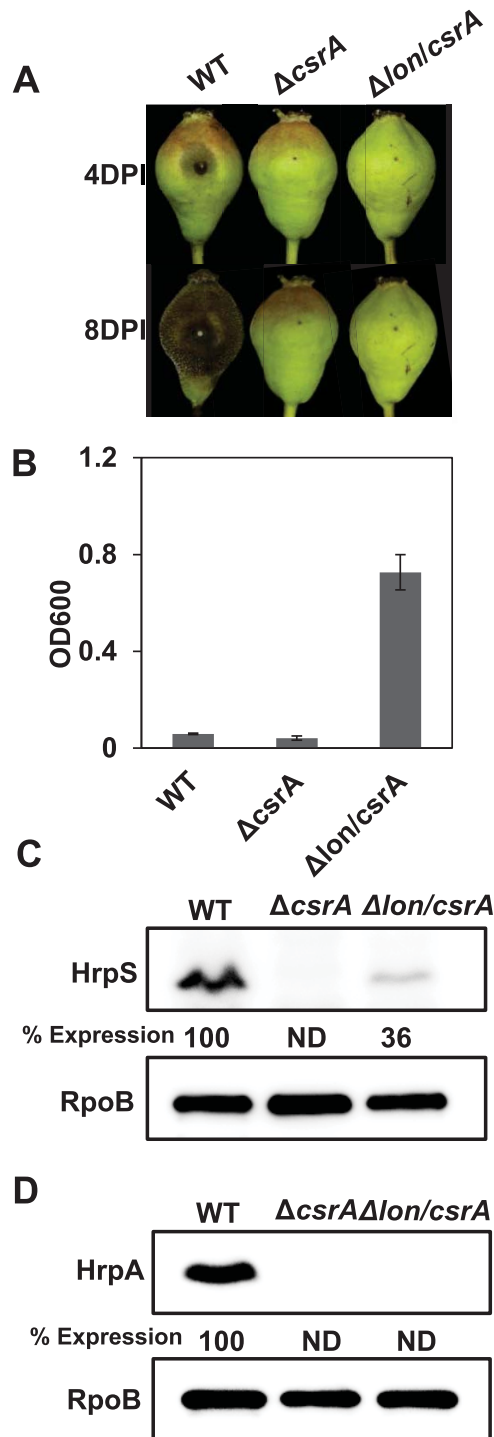
A

WT   ΔcsrA   Δlon/csrA

4DPI

8DPI

B

C

WT   ΔcsrA   Δlon/csrA

HrpS

% Expression   100   ND   36

RpoB

D

WT   ΔcsrA Δlon/csrA

HrpA

% Expression   100   ND   ND

RpoB

Fig. 7.

(Ancona *et al.*, 2015a; Wang *et al.*, 2009, 2012; Zhao *et al.*, 2009a). Here, we also provided novel insights into the role of the Rcs system in the regulation of *E. amylovora* virulence through the characterization of the *lon* mutant, which accumulates higher levels of RcsA/RcsB proteins.

Previous studies have reported that the Rcs system also negatively regulates motility through transcriptional repression of the flagellum master regulator *flhDC* (Francez-Charlot *et al.*, 2003; Wang *et al.*, 2007). In *E. coli*, the *rcsB* mutant is hypermotile, whereas the *rcsA* mutant exhibits a WT level of motility (Francez-Charlot *et al.*, 2003; Fredericks *et al.*, 2006). RcsA has been shown to affect *E. coli* motility when expressed at high levels (Fredericks *et al.*, 2006). The *rcsB* mutant of *Proteus mirabilis* also shows increased *flhDC* expression and motility (Clemmer and Rather, 2007). However, in *Salmonella*, RcsA is not involved in the regulation of *flhDC* expression, and thus mutation in *lon* has no effect on flagellum formation and motility (Takaya *et al.*, 2002; Wang *et al.*, 2007). In contrast, the *rcsB* mutant of *E. amylovora* is less motile than the WT despite increased *flhDC* promoter activity (Wang *et al.*, 2009; Zhao *et al.*, 2009a). Over-expression of RcsB$_{D56E}$, a phosphorylation mimic variant, significantly reduces motility in *E. amylovora* (Ancona *et al.*, 2015a). This is consistent with our current observations that the accumulation of RcsA/RcsB in the *lon* mutant leads to decreased *flhD* expression and motility, suggesting that the Rcs system in *E. amylovora* indeed acts as a negative regulator of motility, and this is dependent on its expression level.

HrpS in *E. amylovora* and HrpR/HrpS in *P. syringae* are bEBPs, critical for the activation of RpoN-dependent *hrpL* gene expression (Hutcheson *et al.*, 2001; Lee *et al.*, 2016; Wei *et al.*, 2000). In general, bEBP is regulated through its N-terminal regulatory domain that interacts with various signal transduction intermediates, including phosphoryl groups, ligands and anti-activator proteins (Bush and Dixon, 2012). However, HrpR and HrpS in *P. syringae* and HrpS in *E. amylovora* lack this regulatory domain. In *P. syringae*, HrpR is subject to Lon-dependent degradation,

system by targeting RcsA, and thus inhibits EPS overproduction (Torres-Cabassa and Gottesman, 1987). We confirmed that Lon also directly degrades RcsA in *E. amylovora*, the only plant pathogen known to require a functional Rcs system for its pathogenesis

whereas HrpS activity is suppressed by direct interaction with HrpV, which further interacts with a chaperone-like protein HrpG to relieve the suppression (Bretz *et al.*, 2002; Jovanovic *et al.*, 2011; Ortiz-Martín *et al.*, 2010; Preston *et al.*, 1998; Wei *et al.*, 2005). In *E. amylovora*, HrpG and HrpV form a stable heterodimeric complex *in vitro*, suggesting that a similar regulation mechanism may exist (Gazi *et al.*, 2015). In this study, we demonstrated that HrpS is regulated at least at two levels. Despite the low amino acid identity with *P. syringae* HrpR and HrpS, *E. amylovora* HrpS is a direct Lon substrate, and *hrpS* gene expression is under positive regulation of the Rcs system. This is consistent with previous microarray analysis of the *rcsB* mutant, which showed that the Rcs system, especially RcsB, is required for full T3SS gene expression (Wang *et al.*, 2012).

It has been proposed that type III effector proteins in the cytoplasm are maintained in an unfolded state to pass through the narrow secretion machinery, and are generally associated with specific chaperone(s) to prevent premature folding and aggregation (Page and Parsot, 2002; Stebbins and Galán, 2001). Meanwhile, effector proteins may also be exposed to Lon degradation, as features of unfolded proteins, such as hydrophobic peptides, can be easily recognized by Lon (Gur and Sauer, 2008). In *P. syringae*, Lon affects the stability of at least eight type III effector proteins, which becomes rate limiting for effector secretion (Losada and Hutcheson, 2005). The Hrp pilus of *P. syringae* and *E. amylovora* consists of HrpA subunits and extends to the plant cell by the addition of HrpA at the distal end (Jin and He, 2001; Li *et al.*, 2002). We showed here that HrpA stability is greatly enhanced in the *lon* mutant, suggesting that the HrpA protein is subject to Lon degradation, but we could not rule out the possibility that other T3SS proteins might also be targeted by Lon. Therefore, Lon, as a negative regulator of the T3SS in *E. amylovora*, could also function at multiple stages.

The widely distributed GacS/GacA system is a conserved global dual regulatory system, which specifically activates the expression of the *csrB/rsmB* sRNAs and antagonizes the activity of the CsrA/RsmA proteins (Lapouge *et al.*, 2008; Li *et al.*, 2014; Romeo *et al.*, 2013; Vakulskas *et al.*, 2015). At the transcriptional level, the expression of *csrB* also requires IHF, ppGpp and DksA in *E. coli* and *Salmonella enterica* (Edwards *et al.*, 2011; Martínez *et al.*, 2014; Zere *et al.*, 2015). In *E. amylovora*, the expression of *csrB* is positively mediated by GacS/GacA and IHF (Lee and Zhao, 2016). Here, we have provided evidence that the accumulation of RcsA/RcsB in the *lon* mutant inhibits *csrB* expression, suggesting that Lon indirectly activates *csrB* expression.

However, CsrA positively activates T3SS and amylovoran production in *E. amylovora*, but the molecular mechanisms of CsrA regulation remain enigmatic (Ancona *et al.*, 2016). CsrA generally binds to GGA motifs in the 5′ untranslated region of target transcripts and affects the translation rate or stability of target mRNAs

either positively or negatively (Vakulskas *et al.*, 2015). CsrA also promotes premature transcription termination by altering the Rho-dependent transcript structure in *E. coli*, such as *pgaA* mRNA, which encodes a polysaccharide adhesin export protein (Figueroa-Bossi *et al.*, 2014). Recent studies have shown that *lon* mRNA could be co-purified with CsrA protein in *E. coli*, and (sequencing by cross-linking immunoprecipitation) CLIP-seq data from *Salmonella* have shown that CsrA binds to the coding region of *lon* mRNA, which is conserved in *E. amylovora* (Edwards *et al.*, 2011; Holmqvist *et al.*, 2016). Our results suggest that Lon is possibly under the control of CsrA at both the transcriptional and post-transcriptional levels. It is reasonable to speculate that Lon might be a direct target of CsrA, as deletion of *lon* in the *csrA* mutant background fully restores amylovoran production. However, although CsrA may indirectly promote HrpS stability and *hrpS* gene expression by suppressing Lon, deletion of *lon* in the *csrA* mutant background fails to restore the T3SS and virulence, suggesting that CsrA may also target other unknown regulators in addition to Lon. Therefore, further studies are needed to determine the molecular targets of CsrA in the regulation of the T3SS in *E. amylovora*.

Based on our results, it is tempting to speculate that Lon-mediated suppression of RcsA and HrpS/HrpA activities could effectively block *E. amylovora* pathogenesis, allowing the bacteria to utilize cellular resources in other processes under non-pathogenic conditions. Increased motility might also enable the bacteria to reach infection sites. Several studies have shown that environmental stimuli, such as phosphate molecules, can regulate Lon activity. Inorganic phosphate (polyP) forms a complex with Lon and promotes the degradation of ribosomal proteins under nutrient starvation (Kuroda *et al.*, 2001). Cardiolipin and lipopolysaccharide (LPS), found in inner and outer membranes, respectively, in Gram-negative bacteria, directly bind to Lon through their phosphate groups and inhibit Lon activity (Minami *et al.*, 2011; Sugiyama *et al.*, 2013). The Lon activity *in vitro* can also be inhibited by polyP, cyclic adenosine monophosphate (cAMP), (p)ppGpp and c-di-GMP (Osbourne *et al.*, 2014). In *E. amylovora*, (p)ppGpp and c-di-GMP have been shown to positively regulate T3SS and amylovoran production, respectively, suggesting potential involvement of the nucleotide second messengers in the post-translational regulation of virulence (Ancona *et al.*, 2015b; Edmunds *et al.*, 2013), which warrants further investigation.

In summary, we propose the following working model for Lon-mediated virulence regulation, and its interaction with Rcs and Gac-Csr regulatory systems, in *E. amylovora* (Fig. 8). Lon broadly impacts *E. amylovora* virulence by the negative regulation of amylovoran production and T3SS, and its positive effect on motility. These could mainly be achieved through the direct targeting of major regulators of amylovoran (RcsA) and the T3SS (HrpS/HrpA)
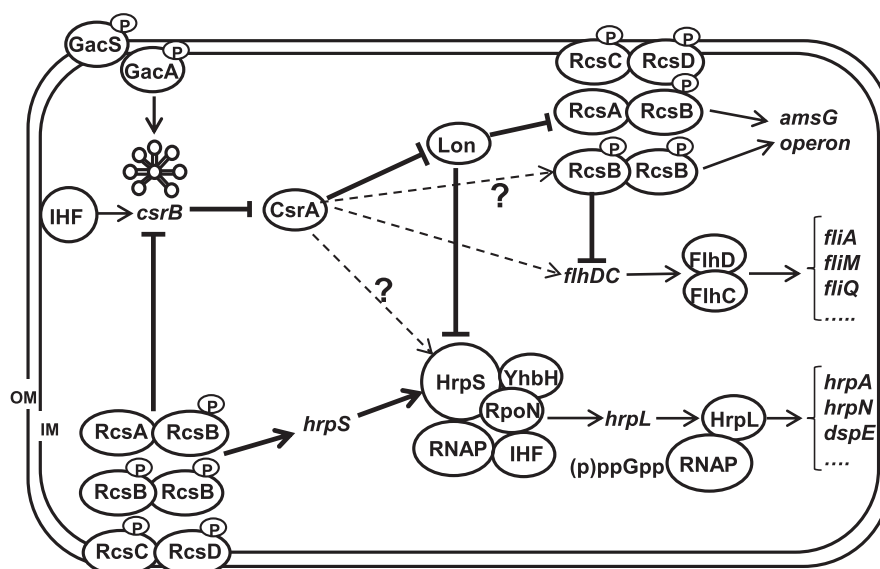
Fig. 8 A working model illustrating Lon-mediated virulence regulation and its interaction with Rcs and Gac-Csr regulatory systems in *Erwinia amylovora*. This model is based on findings obtained in this study as well as those reported in previous studies (Ancona *et al.*, 2014; 2015a, b, 2016; Lee and Zhao, 2016; Lee *et al.*, 2016; Li *et al.*, 2014; Wang *et al.*, 2009, 2012; Zhao *et al.*, 2009a). FlhD/C, master regulator of flagellar formation; HrpL, an exocytoplasmic functions (ECF) sigma factor and master regulator of the type III secretion system (T3SS); HrpS, σ^{54}-dependent enhancer-binding protein; IHF, integration host factor; RpoN, σ^{54} alternative sigma factor; YhbH, σ^{54} modulation protein (ribosome-associated protein); RNAP, RNA polymerase; (p)ppGpp, guanosine tetraphosphate and guanosine pentaphosphate; GacS/GacA and RcsA/B/C/D, two-component regulatory systems; *csrB*, small non-coding regulatory RNA; CsrA, RNA-binding protein; OM, outer membrane; IM, inner membrane; P, phosphorylation. Symbols: ↓, positive effect; ⊥, negative effect; dashed line with/without ?, unknown mechanism.

and, indirectly, through suppression of the Rcs system. Over-activation of the Rcs system inhibits flagellar formation and *csrB* sRNA expression, and activates T3SS (*hrpS*) expression. However, CsrA protein positively regulates both amylovoran and T3SS, partly by the suppression of Lon activity and of other unknown regulators. The balance of CsrA and Lon activities is further monitored by the regulatory circuit between the Rcs and Gac-Csr systems. Future research should focus on understanding the molecular mechanisms underlying the regulation of Lon by CsrA and the identification of other CsrA and Lon targets.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Erwinia amylovora* and *Escherichia coli* strains were routinely grown in Luria-Bertani (LB) broth. For T3SS gene expression, HMM [1 g (NH$_4$)$_2$SO$_4$, 0.246 g MgCl$_2$.6H$_2$O, 0.1 g NaCl, 8.708 g K$_2$HPO$_4$, 6.804 g KH$_2$PO$_4$], supplemented with 10 mM galactose as carbon source, was used (Ancona *et al.*, 2014). For amylovoran production, MBMA minimal medium [3 g KH$_2$PO$_4$, 7 g K$_2$HPO$_4$, 1 g (NH$_4$)$_2$SO$_4$, 2 mL glycerol, 0.5 g citric acid, 0.03 g MgSO$_4$], supplemented with 1% sorbitol, was used (Wang *et al.*, 2009). Antibiotics were used at the following concentrations when appropriate: 100 μg/mL ampicillin (Ap), 50 μg/mL kanamycin (Km) and 10 μg/mL chloramphenicol (Cm). Primer sequences used for mutant construction, mutant confirmation, quantitative reverse transcription real-time

polymerase chain reaction (qRT-PCR), inverse PCR, cloning and EMSA in this study are listed in Table S1 (see Supporting Information).

Mutant generation by λ-Red recombinase cloning

As described previously, *E. amylovora* Ea1189 mutant strains were generated using the λ phage recombinase method (Zhao *et al.*, 2009b). Briefly, competent cells were prepared from overnight cultures of *E. amylovora* strains carrying pKD46, which were subcultured to exponential phase [optical density at 600 nm (OD$_{600}$) = 0.8] in LB containing 0.1% arabinose. Recombination DNA fragments, which contained a Cm^R or Km^R gene with their own promoter flanked with a 50-nucleotide homology from the target genes, were generated by PCR using plasmids pDK32 or pKD13 as template and transformed into competent cells by electroporation. The resulting mutants were selected on LB plates with appropriate antibiotics, and confirmed by PCR. In the corresponding mutant strains, the coding regions of the target genes were deleted, except for the first and last 50 nucleotides. Double mutant strains were generated using single mutants as a background strain as indicated.

Virulence, amylovoran production and motility assays

Virulence assay on immature Bartlett pear fruits (*Pyrus communis* L. cv. Bartlett) was performed as described previously (Ancona *et al.*, 2014, 2016; Wang *et al.*, 2009). Briefly, bacterial inoculum was prepared from overnight cultures and resuspended in phosphate-buffered saline (PBS) to OD$_{600}$ = 0.1 and then diluted 100 times [approximately 10^6 colony-forming units (CFU)/mL]. Surface-sterilized immature pears were air dried,

pricked with a sterile needle, inoculated with 2 μL of cell suspensions and incubated in a humidified chamber at 28 °C. Symptoms were recorded at 4 and 8 days post-inoculation. Pears were assayed in triplicate for each strain, and the experiments were repeated three times.

Amylovoran production was determined using the cetylpyrimidinium chloride (CPC) method, as described previously (Bellemann *et al.*, 1994; Zhao *et al.*, 2009a). Briefly, overnight-grown cultures were re-inoculated into 5 mL of MBMA medium to OD_{600} = 0.2. After 24 h of incubation at 28 °C with shaking, 1 mL of each culture was centrifuged at 4500 *g* for 10 min, and 50 μL of 50 mg/mL CPC were added to the supernatant. After 10 min of incubation at room temperature, the amylovoran concentration was quantified by measuring OD_{600} turbidity and normalized to a cell density of 1.0. Each experiment was performed in triplicate and repeated three times.

Motility was performed on motility agar plates (10 g tryptone, 5 g NaCl and 2.5 g agar per litre), as described previously (Zhao *et al.*, 2009a). Diameters were measured at 24 and 48 h post-inoculation, and each experiment was performed in triplicate and repeated three times.

qRT-PCR

For *in vitro* gene expression of T3SS and amylovoran production, RNA was isolated from cultures in HMM for 6 h at 18 °C, and in MBMA medium for 18 h at 28 °C, respectively (Ancona *et al.*, 2016; Wang *et al.*, 2012). To avoid RNA degradation, 4 mL of RNA protect reagent (Qiagen, Hilden, Germany) was added to 2 mL of bacterial cell cultures and the cells were harvested by centrifugation. RNA was extracted using an RNeasy® mini kit (Qiagen) following the manufacturer's instructions, and DNase I treatment was performed with a TURBO DNA-free kit (Ambion, Austin, TX, USA). RNA was quantified using a Nano-Drop ND100 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA).

Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Power SYBR® Green PCR master mix (Applied Biosystems, Foster City, CA, USA) with appropriate primers (Table S1) was mixed with cDNAs of selected genes, and qRT-PCR was performed using the StepOne-Plus Real-Time PCR system (Applied Biosystems) under the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The dissociation curve was measured after the program was completed, and the relative gene expression was calculated with the relative quantification ($\Delta\Delta Ct$) method using the *rpoD* gene as an endogenous control. The experiment was repeated least twice.

Western blot

Western blot was performed as described previously (Ancona *et al.*, 2015b, 2016). The genomic DNA regions containing the promoter and coding sequences of the *hrpS*, *hrpA*, *rcsA*, *rcsB* and *lon* genes with a 6-His tag at the C-terminus were cloned into pWSK29. Genes were oriented opposite to vector promoters, and the expression of genes was driven only by their native promoters. The resulting plasmids were sequenced at the University of Illinois at Urbana-Champaign core sequencing facility and transformed by electroporation into the WT and mutants. For Western blot, equal amounts of *E. amylovora* cells grown in HMM at 18 °C for 6 h or MBMA medium at 28 °C for 24 h were collected. To test protein

stability, tetracycline was added to cell cultures with a final concentration of 50 μg/mL, and equal amounts of cells were collected at different time points. Cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking with 5% milk in PBS, membranes were probed with 1.0 μg/mL rabbit anti-His antibodies (GenScript, Piscataway, NJ, USA) or rabbit anti-RNA polymerase beta (*E. coli* RpoB) antibodies (Abcam, Cambridge, MA, USA) diluted 1 : 2000, followed by horseradish peroxidase-linked anti-rabbit IgG antibodies (Amersham Bioscience, Uppsala, Sweden) diluted 1 : 10 000. Immunoblots were developed using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA) and visualized using an ImageQuant LAS 4010 CCD camera (GE Healthcare, South Plainfield, NJ, USA). The experiment was performed at least three times.

Transposon mutagenesis and screening for the motile mutants

The EZ::T5™ <KAN-2> Tnp Transposome™ kit (Epicentre, Madison, WI, USA) was used for random mutagenesis following the manufacturer's instructions. Briefly, to prepare the competent cells, an overnight culture of the *lon* mutant was subcultured to exponential phase (OD_{600} = 0.8) in LB. Cells were then harvested, washed with cold sterile water and transformed with 1 μL of the EZ-Tn5 <Kan> Tnp transposome by electroporation. After 3 h of incubation at 28 °C, transformants were plated on LB with Km. A total of 1045 colonies was picked and stored at −20 °C. For screening, the WT, *lon* mutant and Tn5 mutant strains were grown overnight in LB in 96-well microwell plates, and 2 μL of each culture were directly inoculated on the motility agar plate. After 24 and 48 h of incubation at 28 °C, the mutants with restored motility were selected and the motility of the mutants was re-examined as described above.

Inverse PCR was performed to determine the transposon insertion site as described previously with a few modifications (Martin and Mohn, 1999). Genomic DNA of overnight cell cultures was isolated using a MasterPure™ complete DNA & RNA purification kit (Epicentre), digested with the restriction enzyme (*Pst*I or *Pvu*I) and re-ligated using T4 DNA ligase. For PCRs, DNA samples treated with *Pst*I were amplified with the primer pair KAN-2 FP-1/KAN-2 RP-1, whereas DNA samples treated with *Pvu*I were amplified with the primer pair KAN-2 FP1/PvuI-right or KAN-2 RP1/PvuI-left (Table S1). The PCR products were gel purified and sequenced at the University of Illinois at Urbana-Champaign core sequencing facility. The flanking sequences of the transposon insertion site were analysed using BLAST search at the National Center for Biotechnology Information (NCBI).

EMSA

EMSAs for RcsA/RcsB binding to the upstream regions of the *rsmB* sRNA and the *hrpS* gene were performed as described previously (Ancona *et al.*, 2015a; Lee and Zhao, 2016). Briefly, complementary oligonucleotides (Table S1) were 3′ biotinylated using the biotin 3′ end DNA labelling kit (Pierce) and annealed before use. Reaction volumes of 10 μL with 20 fmol of labelled oligonucleotides were incubated with 5 pmol of either or both RcsA/RcsB proteins in 1 × binding buffer, 50 ng/μL Poly(dI·dC), 0.5 mM MgCl$_2$, 0.1% Nonidet P-40, 0.05 mg/mL bovine serum albumin and 5%

glycerol. Reactions were incubated for 20 min at room temperature, mixed with 2.5 μL of 5 × loading buffer and resolved into a 6% native polyacrylamide gel in 0.5 × Tris/Borate/EDTA; 0.089M Tris, 0.089M borate, 2 mM EDTA (TBE) buffer. The resolved reactions were transferred to a positively charged nylon membrane and UV cross-linked. The chemiluminescent signals were developed using the lightshift chemiluminescent EMSA kit (Pierce) and visualized using an ImageQuant LAS 4010 charge-coupled device (CCD) camera (GE Healthcare). The experiment was repeated at least twice.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 (A) Mucoid colony morphology of the *lon* mutant. The wild-type (WT) and *lon* mutant strains were grown in Luria-Bertani (LB) at 28 °C. Photographs were taken at 48 h after incubation. RcsA/RcsB accumulation suppresses *csrB* sRNA expression and the effect of *csrA* mutation on the *lon* gene. (B) Relative gene expression of *csrA* and *csrB* in the *lon* mutant compared with WT grown in *hrp*-inducing medium (HMM) for 6 h at 18 °C. (C) Relative expression of *lon* in the *csrA* and *csrB* mutants compared with WT grown in HMM for 6 h at 18 °C. The values of the relative fold change were the means of three replicates and the experiment was repeated twice with similar results. Error bars indicate standard deviation. (D) Abundance of the Lon-His6 protein in the *csrA* and *csrB* mutant strains compared with WT grown in HMM for 6 h at 18 °C. The abundance of the RpoB protein was used as a loading control. Relative protein abundance at the bottom of each lane was calculated using ImageJ software. Cropped gel blots and percentage expression values are representatives of three independent experiments.

Table S1 Primers used in this study.