ClpXP-Dependent RpoS Degradation Enables Full Activation of Type III Secretion System, Amylovoran Production, and Motility in *Erwinia amylovora*

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ABSTRACT

Erwinia amylovora, the causal agent of fire blight disease of apple and pear, employs intracellular proteases, including Lon and ClpXP, for posttranslational regulation of various cellular proteins. It has been shown that Lon plays a critical role in *E. amylovora* virulence by directly targeting type III secretion system (T3SS) proteins and the Rcs phosphorelay system. In this study, we genetically examined the role of ClpXP and its potential interaction with Lon in *E. amylovora*. Mutation in *clpXP* diminished the expression of the T3SS, reduced exopolysaccharide amylovoran production and motility, and resulted in delayed disease progress. Western blot analyses showed highly accumulated RpoS proteins in the *clpXP*

mutant. Moreover, mutation of *rpoS* in the *clpXP* mutant background rescued the expression of the T3SS and amylovoran production, suggesting that ClpXP-dependent RpoS degradation positively affects virulence traits. Interestingly, lack of both ClpXP and Lon resulted in significantly reduced virulence but increased expression of the T3SS and amylovoran production. However, this phenomenon was independent of RpoS accumulation, suggesting that ClpXP and Lon are indispensable for full virulence in *E. amylovora*.

Additional keywords: σ factor.

Fire blight disease, caused by an enterobacterium *Erwinia amylovora*, is one of the most economically important diseases in the plant family Rosaceae. It has been demonstrated that *E. amylovora* utilizes two major virulence factors: the hypersensitive response and pathogenicity (*hrp*)-type III secretion system (T3SS) and the exopolysaccharide (EPS) amylovoran (Khan et al. 2012; Oh and Beer 2005). The *hrp*-T3SS directly injects virulence-associated proteins into host cells to overcome host defense response and promote disease development (Nissinen et al. 2007). The expression of *hrp*-T3SS genes is activated by the master regulator HrpL, a member of the extracytoplasmic functions subfamily of σ factors (McNally et al. 2012; Wei and Beer 1995). Recent studies showed that the alternative σ factor 54 (RpoN), its modulation protein YbhH, enhancer binding protein HrpS, and the nucleoid-associated protein IHF are all essential for the expression of *hrpL* and other *hrp*-T3SS genes (Ancona et al. 2014; Lee and Zhao 2016; Lee et al. 2016). Moreover, the RpoN-HrpL alternative σ factor cascade is further activated by the bacterial alarmone (p)ppGpp-mediated stringent response under nutrient-limited conditions (Ancona et al. 2015). On the other hand, the EPS amylovoran is a heteropolymer composed of pentasaccharide-repeating units and contributes to pathogenesis through biofilm formation and vessel blockage in plants (Koczan et al. 2009; Nimtz et al. 1996). Amylovoran biosynthesis genes are encoded by the 12-gene *ams* operon, which is positively regulated by the Rcs phosphorelay system (Bernhard et al. 1993; Wang et al. 2009, 2012).

ClpXP and Lon are two major proteases in bacteria, belonging to the AAA⁺ (ATPase-associated with diverse cellular activities) family proteins, and control the abundance and quality of intracellular proteins in response to a wide variety of environments (Sauer and

Baker 2011). Previous studies have indicated that proteolytic activities from ClpXP and Lon are required during bacterial pathogenesis (Bretz et al. 2002; Iyoda and Watanabe 2005; Losada and Hutcheson 2005; Yamamoto et al. 2001). It was recently reported that Lon plays a major role in regulation of virulence in *E. amylovora* by directly targeting HrpS and HrpAT3SS proteins, as well as RcsA, an auxiliary response regulator of the Rcs phosphorelay system (Lee et al. in press). Furthermore, accumulation of RcsA/RcsB in the *lon* mutant led to upregulation of *hrpS* and *amsG* and downregulation of *flhD* and small RNA *csrB*. Suppression of *csrB* expression indirectly enhanced the activity of the RNA-binding carbon storage regulator A (CsrA) protein, which plays a central role in *E. amylovora* virulence (Ancona et al. 2016). However, the role of ClpXP in *E. amylovora* virulence remains unknown.

ClpXP is an ATP-dependent cytosolic protease, composed of AAA⁺ ATPase (ClpX) and proteolytic chamber (ClpP). ClpP alone can degrade damaged or misfolded peptides but its proteolytic activity becomes selective and tightly controlled only after association with ClpX (Frees et al. 2013). A number of proteins involved in diverse cellular processes, including metabolic enzymes and transcription factors, have been identified as ClpXP substrates (Flynn et al. 2003). Most importantly, ClpXP-mediated proteome changes are largely achieved by regulating RpoS activity (Schweder et al. 1996). RpoS is an alternative σ factor induced during stationary phase and under stress conditions, and plays major roles in stress response and virulence regulation by altering stress sensitivity and virulence-associated phenotypes (Badger and Miller 1995; Fang et al. 1992; Flavier et al. 1998; Solis et al. 2006). A recent study showed that the *rpoS* mutant of *E. amylovora* exhibited increased sensitivity to oxidative, osmotic, acid, and heat stresses (Santander et al. 2014). On the other hand, RpoS activity is tightly controlled by multiple layers of regulatory elements, including ClpXP-dependent degradation, during favorable growth conditions (Battesti et al. 2011). The rate-limiting factor for RpoS degradation is the adaptor protein RssB, which facilitates RpoS recognition by ClpXP (Becker et al. 1999; Pruteanu and Hengge-Aronis 2002). Under stress conditions or during stationary phase, RssB inhibitors and low ATP

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levels release RpoS from degradation by ClpXP (Bougdour et al. 2006, 2008; Peterson et al. 2012). This process leads to increased RpoS levels and allows cells to activate genes involved in cell survival. A defect in controlling RpoS level has been reported to cause reduced virulence in enterohemorrhagic *Escherichia coli* (EHEC) and *Dickeya dadantii* (Iyoda and Watanabe 2005; Li et al. 2010).

We aimed to examine the role of ClpXP in *Erwinia amylovora* virulence and its potential interaction with Lon. Our results showed that RpoS accumulation in the *clpXP* mutants contributed to the suppression of T3SS, amyloporan production, and motility. Deletion of both *clpXP* and *lon* genes led to significantly reduced virulence independent of RpoS level and other virulence factors.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) broth was used for routine growth of *E. amylovora* and *Escherichia coli* strains. An *hrp*-inducing medium (HMM)—1 g of (NH₄)₂SO₄, 0.246 g of MgCl₂ · 6H₂O, 0.1 g of NaCl, 8.708 g of K₂HPO₄, and 6.804 g of KH₂PO₄—supplemented with 10 mM galactose as carbon source was used for T3SS gene expression, whereas a modified basal medium A (MBMA)—3 g of KH₂PO₄, 7 g of K₂HPO₄, 1 g of (NH₄)₂SO₄, 2 ml of glycerol, 0.5 g of citric acid, and 0.03 g of MgSO₄—supplemented with 1% sorbitol was used for amyloporan production (Ancona et al. 2014; Wang et al. 2009). When required, antibiotics were used at the following concentrations: ampicillin at 100 µg ml⁻¹, kanamycin (Km) at 50 µg ml⁻¹, and chloramphenicol (Cm) at 10 µg ml⁻¹. Primer sequences used in this study for mutant construction, mutant confirmation, and quantitative reverse-transcription polymerase chain reaction (PCR) are listed in Supplementary Table S1.

Mutant generation by λ-red recombinase cloning. To generate *Erwinia amylovora* Ea1189 mutant strains, the λ-red recombinase cloning method was performed, as described previously (Zhao et al. 2009a). Briefly, competent cells of *E. amylovora* strain carrying pKD46 were prepared by subculturing in LB with 0.1% arabinose to exponential phase (optical density at 600 nm [OD₆₀₀] = 0.8, approximately 8 × 10⁸ CFU/ml) and washing with cold sterile water. These cells were transformed by electroporation with recombinant DNA fragments, which contain a selection marker (Cm^R or Km^R) flanked with a 50-nucleotide homology from the target gene or

region. Plasmids pDK32 or pKD13 were used as a template. Double mutant strains were generated using single mutants as a background. For complementation, the genomic region containing the native promoter and coding sequence of the target gene was PCR amplified and cloned into pWSK29. The resulting plasmids were verified by sequencing at the University of Illinois at Urbana-Champaign Core sequencing facility.

Virulence assay on immature pear. Overnight cultures of *E. amylovora* wild-type (WT), mutant, and complementation strains in LB were harvested and suspended in 0.5× phosphate-buffered saline (PBS) to OD₆₀₀ = 0.1 and then diluted 100 times (approximately 10⁶ CFU/ml) (Zhao et al. 2009b). Immature ‘Bartlett’ pear fruit (*Pyrus communis* L.) were surface sterilized with 10% bleach for 10 min, rinsed with sterile distilled water, and air dried. Pear fruit were pricked with a sterile needle, inoculated with 2 µl of bacterial suspension, and incubated at 28°C in a humidified chamber in darkness. Disease symptoms were recorded at 4 and 8 days post-inoculation (DPI). The experiments were repeated at least twice in triplicate.

Cetylpyrimidinium chloride assay. Amyloporan production was measured using cetylpyrimidinium chloride (CPC), as described previously (Bellemann et al. 1994; Zhao et al. 2009b). Overnight cultures of *E. amylovora* WT, mutant, and complementation strains in LB were harvested, washed, and inoculated into 5 ml of MBMA medium to OD₆₀₀ = 0.2 (approximately 2 × 10⁸ CFU/ml). After 24 h of incubation at 28°C with shaking, 1 ml of each culture was centrifuged at 4,500 × g for 10 min, and 50 µl of CPC at 50 mg/ml was added to the supernatant. After 10 min of incubation, amyloporan production was quantified by measuring OD₆₀₀ turbidity and normalized for a cell density of 1.0. Each experiment was performed in triplicate and repeated three times. Statistical analysis was performed using Student’s *t* test, with *P* < 0.05 considered to be statistically significant.

Motility assay. Overnight cultures of *E. amylovora* WT, mutant, and complementation strains in LB were harvested, washed, and suspended in 0.5× PBS to OD₆₀₀ = 1 (approximately 10⁹ CFU/ml). The bacterial suspensions were then plated onto the center of the motility plates (Zhao et al. 2009b)—10 g of Bacto tryptone (Becton Dickinson), 5 g of NaCl, and 2.5 g of agar (plant tissue culture agar; PhytoTechnology Laboratories) per liter—and incubated at 28°C. Diameters of movement were measured at 24 and 48 h postinoculation, and the experiments were repeated three times with three

TABLE 1. Bacterial strains and plasmids used in this study

Strains, plasmids	Description ^a	Reference, source
<i>Erwinia amylovora</i>		
Ea1189	Wild type, isolated from apple	Wang et al. 2009
Δlon	lon::Cm; Cm ^R -insertional mutant of lon of Ea1189	Lee et al. in press
ΔclpXP	clpP1,clpX1,clpP2,clpX2::Cm; Cm ^R -insertional mutant of clpXP of Ea1189	This study
ΔclpXP/lon	clpP1,clpX1,clpP2,clpX2,lon::Cm; Cm ^R -insertional mutant of clpXP/lon of Ea1189	This study
ΔrpoS	rpoS::Cm; Cm ^R -insertional mutant of rpoS of Ea1189	This study
ΔrssB	rssB::Cm; Cm ^R -insertional mutant of rssB of Ea1189	This study
ΔclpXP/rpoS	rpoS::Cm, clpP1,clpX1,clpP2,clpX2::Km; Km ^R -insertional mutant of clpXP of ΔrpoS	This study
ΔclpXP/lon/rpoS	rpoS::Cm, clpP1,clpX1,clpP2,clpX2,lon::Km; Km ^R -insertional mutant of clpXP/lon of ΔrpoS	This study
ΔrssB/rpoS	rpoS::Cm, rssB::Km; Km ^R -insertional mutant of rssB of ΔrpoS	This study
<i>Escherichia coli</i>		
DH10B	F ⁻ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara leu) 7697 galU galK rpsL nupG λ ⁻	Invitrogen
Plasmids		
pKD46	Ap ^R , 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
pXP1	2848-bp DNA fragment containing promoter sequence of clpP1 and clpX1 genes in pWSK29	This study
pXP2	2618-bp DNA fragment containing promoter sequence of clpP2 and clpX2 genes in pWSK29	This study
pRpoS-His6	1893-bp DNA fragment containing promoter and coding sequence of rpoS gene and c-terminal His-tag in pWSK29	This study
pHrpA-His6	803-bp DNA fragment containing promoter and coding sequence of hrpA gene and c-terminal His-tag in pWSK29	Ancona et al. 2015

^a Abbreviations: Cm^R, Km^R, and Ap^R indicate resistant to chloramphenicol, kanamycin, and ampicillin, respectively.

replicates. Statistical analysis was performed using Student's *t* test, with *P* < 0.05 considered as statistically significant.

Western blot. Western blotting analyses of RpoS and HrpA proteins were performed as previously described (Ancona et al. 2016; Lee et al. in press). Briefly, equal amount of cell lysates from *E. amylovora* cultures were separated by sodium dodecyl sulfate polyacrylamide gels followed by transfer to polyvinylidene fluoride membrane (Millipore). After blocking with 5% milk in PBS, membranes were incubated with rabbit anti-His antibodies at 1.0 µg/ml (GeneScript) or rabbit anti-RNA polymerase β antibodies (1:2000 dilution; Abcam) and then horseradish peroxidase-linked antirabbit immunoglobulin G antibodies (1:10,000 dilution; Amersham Bioscience). Protein bands were detected using enhanced chemiluminescence reagents (Pierce) and an ImageQuant LAS 4010 CCD camera (GE Healthcare). This experiment was repeated three times with similar results.

RESULTS

ClpXP positively contributes to virulence in *E. amylovora*. Based on published genome sequences (Bell et al. 2004; Blattner et al. 1997; Glasner et al. 2011; Smits et al. 2010a,b), *E. amylovora* and its closely related species *E. pyrifoliae* contain two sets of *clpXP* genes, whereas *Escherichia coli* and two related enterobacterial plant pathogens, *Pectobacterium atrosepticum* and *D. dadantii*, contain only one set of *clpXP* genes (Fig. 1A). However, the location of the *clpXP* genes (i.e., between *tig*, encoding a chaperone protein, and *lon*) is conserved among these bacteria. The deduced amino acids of *Erwinia amylovora* ClpP1, ClpX1, and ClpX2 share high identities and similarities with their homologs of *Escherichia coli*, *P. atrosepticum* and *D. dadantii* (Table 2), whereas *Erwinia amylovora*

Fig. 1. Comparison of ClpXP in *Erwinia amylovora* and other related species. **A**, Schematic maps of the *clpP* and *clpX* genes. For complementation of the mutant strains, plasmids pXP1, containing *clpP1* and *clpX1*, and pXP2, containing *clpP2* and *clpX2*, were constructed. **B**, Phylogenetic tree of ClpP proteins. Based on deduced amino acid sequences, a phylogenetic neighbor-joining tree was generated using MEGA 5.0, and bootstrap values were indicated at each node. Ea = *Erwinia amylovora*, Ep = *Erwinia pyrifoliae*, Ec = *Escherichia coli*, Pa = *Pectobacterium atrosepticum*, and Dd = *Dickeya dadantii*.

ClpP2 is less conserved in both length and deduced amino acid sequence, and the gene also lies in the opposite direction relative to other *clpXP* genes (Fig. 1A). Phylogenetic analysis also separated ClpP2 of *E. amylovora* and *E. pyrifoliae* from other ClpP homologs and clustered into another clade (Fig. 1B).

To determine the role of ClpXP in *E. amylovora* virulence, a *clpXP* mutant was generated by deleting all four genes (Fig. 1A). For complementation, each set of *clpXP* genes (i.e., *clpXP1* and *clpXP2*) was cloned and transformed into the mutant (Fig. 1A). A virulence assay on immature pear fruit showed that the *clpXP* mutant caused slightly delayed disease progress as compared with the WT (Fig. 2A), indicating that ClpXP contributes to full virulence in *E. amylovora*. The complementation strain carrying pXP1 (containing the *clpXP1* genes) but not pXP2 (containing the *clpXP2* genes) completely restored disease symptoms, comparable with the WT (Fig. 2A), indicating that the first set of *clpXP* genes in *E. amylovora* is functional under tested conditions.

Our previous studies showed that Lon plays a major role in *E. amylovora* virulence by negatively regulating T3SS and amylovoran production (Lee et al. in press). In order to determine the effect of lacking both Lon and ClpXP, a *clpXP/lon* mutant was generated. Interestingly, the mutant exhibited a noticeable reduction of necrotic lesions on immature pear fruit at 8 DPI, which could also be rescued by pXP1, but not pXP2 (Fig. 2B), further suggesting that ClpXP1 are functional under tested conditions and that lack of both ClpXP and Lon leads to noticeable delayed disease progress.

Accumulation of RpoS contributes to delayed disease progress in the *clpXP* mutant. RpoS has been regarded as one of the physiologically important ClpXP substrates (Battesti et al. 2011; Flynn et al. 2003). Under favorable growth conditions and at early growth stages, degradation of RpoS by ClpXP with the aid of RssB restricts its activity posttranslationally (Schweder et al. 1996). To determine whether the lack of ClpXP led to RpoS accumulation and thus, reduced virulence, deletion mutants of *rpoS* in the WT and *rssB*, *clpXP*, and *clpXP/lon* mutant backgrounds were generated. Virulence assay showed that the *rpoS*, *rssB*, and *rssB/rpoS* double mutants were as pathogenic as the WT (Fig. 2C). Interestingly, the *clpXP/rpoS* mutant exhibited disease progress comparable with the WT, whereas deletion of *rpoS* in the *clpXP/lon* mutant did not change the *clpXP/lon* mutant in causing reduction of necrotic lesions at 8 DPI (Fig. 2C).

To verify whether RpoS is accumulated in the *clpXP* mutant and contributes to disease progress, the RpoS protein level at different time points in HMM was determined by Western blot. *E. amylovora* grew slowly at the first 12 h of incubation (hoi) and reached the stationary phase at 24 hoi in HMM (Supplementary Fig. S1A). The intracellular level of RpoS in *E. amylovora* was maintained at a low level until 12 hoi and increased starting at 18 hoi, approximately the late exponential or early stationary phase (Fig. 3A). Compared with the WT, RpoS accumulation was approximately 16- and 7-fold higher in the *clpXP* and *clpXP/lon* mutants, respectively, and *rssB* mutant at 6 hoi (Fig. 3B). These results indicate that RpoS accumulation is growth-dependent and under the control of RssB and ClpXP. These results also suggest that higher accumulation of RpoS in the *clpXP* mutant might influence its virulence. However, reduced virulence in the *clpXP/lon* and *clpXP/lon/rpoS* mutants might be caused by unknown factors independent of RpoS.

TABLE 2. Percentage of identity and similarity of deduced amino acid sequences for ClpXP in *Erwinia amylovora* and related enterobacterial species

Protein ^a	Length	Identity	Similarity	Protein ^a	Length	Identity	Similarity
Ea ClpP1	207	...	...	Ea ClpX1	424	...	...
Ea ClpP2	198	36.4	68.2	Ea ClpX2	424	98.3	99.1
Ep ClpP1	207	99	99	Ep ClpX1	424	98.8	99.8
Ep ClpP2	198	36.4	68.9	Ep ClpX2	424	97.2	98.6
Ec ClpP	207	87.4	96.6	Ec ClpX	424	91.5	97.6
Pa ClpP	207	88.4	96.1	Pa ClpX	424	90.3	97.2
Dd ClpP	207	87.9	96.6	Dd ClpX	424	89.2	96.5

^a Ea = *E. amylovora*, Ep = *E. pyrifoliae*, Ec = *Escherichia coli*, Pa = *Pectobacterium atrosepticum*, and Dd = *Dickeya dadantii*.

RpoS accumulation leads to reduced expression of the T3SS. In *E. amylovora*, T3SS is activated at early growth stages in nutrient-limited conditions (Ancona et al. 2015; Wei et al. 2000). Expression of the T3SS genes *hrpL* and *hrpA* peaked at 6 hoi in vitro, and then the *hrpL* gene expression was reduced to basal level, whereas transcription of *hrpA* was maintained at a relatively high level until 24 hoi. To determine the effect of RpoS accumulation on T3SS in the *rssB*, *clpXP*, and *clpXP/lon* mutants, Western blot analysis of HrpA protein was performed (Fig. 3C). The abundance of HrpA was reduced approximately 3-, 12-, and 50-fold in the *rssB*, *clpXP*, and *clpXP/lon* mutants, respectively, as compared with the WT. Deletion of *rpoS* in the *rssB* and *clpXP* mutant backgrounds rescued HrpA expression to levels comparable with those in the *rpoS* mutant. Deletion of *rpoS* in the *clpXP/lon* mutant also increased the HrpA protein level similar to that in the *lon* mutant, which is approximately twofold higher than that of the WT (Lee et al. in press). These results indicate that RpoS accumulation led to reduced expression of the T3SS.

RpoS suppresses amylovoran production. Previous characterization of the *rpoS* mutant in *E. amylovora* strain CFBP 1430 proposed the regulatory role of RpoS in EPS production and motility (Santander et al. 2014); therefore, we determined EPS production in the *clpXP* mutant and its related mutant strains. Amylovoran

production was barely detected in the *clpXP* mutant but increased in its complementation strain with pXP1, indicating that ClpXP positively affects amylovoran production (Fig. 4A). Consistently, amylovoran production in the *clpXP/lon* mutant was lower than that in the *lon* mutant (Lee et al. in press) and in the *clpXP/lon* complementation strain with pXP1. In contrast, amylovoran production was approximately eightfold higher in the *rpoS* mutant as compared with that of the WT (Fig. 4B), indicating that RpoS negatively affects amylovoran production. Similar to the *clpXP* mutant, the *rssB* mutant also exhibited reduced amylovoran production, which could be rescued by deletion of *rpoS* in both mutant backgrounds. This further confirms that RpoS negatively affects amylovoran production. On the other hand, the *clpXP/lon/rpoS* mutant did not show any significant difference in amylovoran production as compared with the *clpXP/rpoS* mutant.

ClpXP is required for motility partially by inhibiting RpoS accumulation. Our recent study reported that the *lon* mutant is nonmotile mainly due to the accumulation of RcsA/RcsB proteins (Lee et al. in press). Interestingly, the *clpXP* mutant also exhibited a nonmotile phenotype, whereas the *clpXP/lon* mutant exhibited reduced motility as compared with the WT (Fig. 5A; Supplementary Fig. S2). Complementation of the *clpXP/lon* mutant with pXP1 returned to the nonmotile phenotype, similar to the *clpXP* or *lon* mutant, whereas the nonmotile phenotype of the *clpXP* mutant could not be rescued by pXP1. On the other hand, the *rpoS*, *rssB*, and *rpoS/rssB* mutants all exhibited reduced motility as compared with the WT (Fig. 5B). Deletion of *rpoS* rendered the cell motile in the *clpXP* mutant, indicating that ClpXP positively affects motility in *E. amylovora*, possibly by inhibiting RpoS accumulation. However, unexpectedly, the *clpXP/lon/rpoS* mutant also exhibited the nonmotile phenotype (Fig. 5B). Some of these results seemingly contradicted with each other, suggesting that explaining what controls motility might be difficult at this stage.

Fig. 2. ClpXP contributes to virulence in *Erwinia amylovora*, possibly by affecting RpoS accumulation. Disease symptoms caused by the wild-type (WT), mutants, and complementation strains on immature pear fruits at 4 and 8 days postinoculation (DPI). **A**, Column 1 = Ea1189 WT, 2 = *clpXP*, 3 = *clpXP* (pXP1), and 4 = *clpXP* (pXP2); **B**, column 5 = *lon*, 6 = *clpXP/lon*, 7 = *clpXP/lon* (pXP1), and 8 = *clpXP/lon* (pXP2); and **C**, column 9 = *rpoS*, 10 = *rssB*, 11 = *rssB/rpoS*, 12 = *clpXP/rpoS*, and 13 = *clpXP/lon/rpoS*.

Fig. 3. ClpXP/RssB-dependent RpoS degradation contributes to type III secretion system gene expression. **A**, Abundance of RpoS-His6 protein in the wild-type (WT) grown in *hrp*-inducing minimal medium (HMM) at different hours of inoculation at 18°C. **B**, Abundance of RpoS-His6 protein in the WT and mutant strains grown in HMM at 18°C for 6 h. **C**, Abundance of HrpA-His6 protein in the WT and mutant strains grown in HMM at 18°C for 6 h. Abundance of the RpoB protein was used as a loading control, and relative protein abundance (percent expression) was calculated using ImageJ software. These experiments were repeated three times with similar results.

DISCUSSION

Posttranslational regulation effectively controls various metabolic enzymes and transcription factors under changing environmental conditions (Sauer and Baker 2011). ClpXP, an intracellular protease belonging to the AAA⁺ family protein, is known to be responsible for regulating more than 50 functional proteins (Flynn et al. 2003). One of its major substrates is RpoS σ factor, which affects the expression of genes necessary for growth at stationary phase, stress responses, and virulence (Battesti et al. 2011; Patten et al. 2004). In this study, we showed that ClpXP contributes to *E. amylovora* virulence by delaying RpoS accumulation. If accumulated at a high level, RpoS suppresses T3SS gene expression, amylovoran production, and motility. In addition, *E. amylovora* lacking both ClpXP and Lon exhibited reduced virulence independent of RpoS, suggesting that ClpXP or Lon proteases are indispensable to maintain certain functions for *E. amylovora* virulence.

ClpXP has been shown to activate full T3SS gene expression in several enterobacterial pathogens. In EHEC, mutation in *clpXP* caused significantly reduced expression of the enterocyte effacement (LEE)-encoded T3SS genes due to accumulation of GrlR and RpoS (Iyoda and Watanabe 2005). Increased levels of GrlR in the *Escherichia coli* *clpXP* mutant directly inhibit the activity of GrlA, which positively regulates the LEE-encoded regulator (Ler), a master

regulator of T3SS genes (Deng et al. 2004). *Yersinia pestis* utilizes both ClpXP and Lon to activate T3SS by degrading a small histone-like protein (YmoA), which represses LcrF, another master regulator of T3SS genes (Hoe and Goguen 1993; Jackson et al. 2004; Lambert de Rouvroit et al. 1992). In the soft rot plant pathogen *D. dadantii*, ClpXP-dependent RpoS degradation has been proposed to positively regulate T3SS by lowering the expression of RNA-binding protein RsmA (repressor of secondary metabolites), a homolog of CsrA (Ancona et al. 2016; Li et al. 2010). Analysis of promoter sequence in *E. coli* showed that the *csrA* gene has two RpoS-dependent promoters (Yakhnin et al. 2011). In this study, we also found that excess accumulation of RpoS at an early growth stage suppresses T3SS gene expression. However, in *Erwinia amylovora*, CsrA positively regulates various virulence traits, including T3SS and amylovoran (Ancona et al. 2016).

Lon, AmyR (amylovoran repressor), and several two-component systems were previously identified as negative regulators of amylovoran production in *E. amylovora* (Lee et al. in press; Li et al. 2014; Wang et al. 2012; Zhao et al. 2009b). In this study, we provided evidence that RpoS also acts as a negative regulator of amylovoran production, as previously reported (Santander et al. 2014). This is also consistent with previous findings in *Escherichia coli* that the *rpoS* mutant overproduces EPS (Ferrieres et al. 2009; Ionescu and Belkin 2009). It has been suggested that EPS overproduction in the *rpoS* mutant is an adaptive response, because both RpoS and EPS

Fig. 4. ClpXP affects amylovoran production by inhibiting RpoS accumulation. **A** and **B**, Amylovoran production of the wild-type (WT), mutants, and complementation strains grown in modified basal medium A at 28°C for 24 h. Presented values are representative of three independent experiments with similar results. Error bars indicate standard deviation of three replicates. Values marked with the same letter do not differ significantly ($P < 0.05$).

Fig. 5. ClpXP affects motility partially by inhibiting RpoS accumulation. **A** and **B**, The moving distance of the wild-type (WT) and mutant strains on the motility plate. Diameters of the circle around the inoculation site (in millimeters) were measured at 24 and 48 h of inoculation. Presented values are representative of three independent experiments with similar results. Error bars indicate standard deviation of three replicates. Values marked with the same letter do not differ significantly ($P < 0.05$).

contribute to cell survival in stress conditions (Battesti et al. 2011; Flemming et al. 2007). Transcriptome analyses in *E. coli* revealed that the *rpoS* mutant exhibits increased transcript level of *rcaA*, which encodes RcsA, a rate-limiting factor in EPS production (Dong et al. 2011; Ionescu and Belkin 2009). On the other hand, the Rcs phosphorelay system also activates small RNA RprA to promote translation initiation of *rpoS* (Majdalani et al. 2001, 2002), suggesting that RpoS might negatively regulate EPS production through the Rcs phosphorelay system.

In EHEC and *Salmonella* spp. ClpXP negatively regulates FlhDC, the master regulator of flagellar gene expression, at the posttranslational levels, and the *clpXP* mutants are hyperflagellated (Kitagawa et al. 2011; Tomoyasu et al. 2002, 2003). In contrast, the *clpXP* mutant of *Erwinia amylovora* exhibited the nonmotile phenotype, which could be partially rescued by deletion of *rpoS*. Previously, we have demonstrated that accumulation of RcsA/RcsB in the *lon* mutant causes the nonmotile phenotype (Lee et al. in press). In *E. amylovora*, RcsA/RcsB binds to the promoter of *flhDC*, and its accumulation negatively regulates motility by inhibiting transcription initiation of *flhDC*. Interestingly, the *clpXP/lon* mutant recovered motility, whereas the *clpXP/lon/rpoS* mutant exhibited the nonmotile phenotype. Because mutation in the *rpoS* gene in the *clpXP* mutant rescued the amylovan production, the *clpXP/lon/rpoS* might have increased levels of RcsA/B, which would block motility. However, the effect of *lon* deletion on motility of the *clpXP* mutant remains unexplained.

Taken together, we provided evidence that ClpXP-dependent degradation of RpoS contributes to *E. amylovora* virulence. As a sixth dissociable subunit of RNAP, σ factor plays a critical role in the regulation of transcription initiation in bacteria. RpoD (σ factor 70) acts as a primary σ factor responsible for the expression of essential genes, whereas alternative σ factors such as RpoN and RpoS activate expression of the subset of genes implicated in diverse cellular functions, including virulence and stress responses (Gruber and Gross 2003). The RpoN-HrpL alternative σ factor cascade directs the expression of all T3SS genes in *E. amylovora* (Ancona et al. 2014; McNally et al. 2012; Wei and Beer 1995). Transcription of structural components of the flagella apparatus is also activated by FlhA (σ factor 28) (Liu and Matsumura 1995). Because the total level of σ factors in the cell exceeds that of core-RNAP, competition between σ factors occurs and leads to antagonistic effects on gene expression (Grigorova et al. 2006; Österberg et al. 2011; Piper et al. 2009). RpoS accumulation during the stationary phase downregulates the expression of several genes under the control of RpoD and FlhA (Patten et al. 2004; Weber et al. 2005). Mutation in *rpoS* also upregulates approximately 60% of RpoN-dependent genes (Dong et al. 2011; Patten et al. 2004). Therefore, it is reasonable to assume that higher levels of RpoS in the *rssB*, *clpXP*, and *clpXP/lon* mutants might outcompete other σ factors, resulting in overexpression of RpoS regulon and suppression of virulence genes.

In summary, our results showed that ClpXP positively contributes to *E. amylovora* virulence by maintaining certain levels of RpoS in the cell. However, reduced virulence of the *clpXP/lon* double mutant might be independent of RpoS. Future research should focus on understanding genes under the control of RpoS, ClpXP, and Lon that affect *E. amylovora* virulence.

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