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e-Xtra*

ClpXP Protease Regulates the Type III Secretion System of *Dickeya dadantii* 3937 and Is Essential for the Bacterial Virulence

Yan Li,[1,2] Akihiro Yamazaki,[2] Lifang Zou,[2] Eulandria Biddle,[2] Quan Zeng,[2] Yongjun Wang,[3] Haiping Lin,[3] Qi Wang,[1] and Ching-Hong Yang[2]

[1]Department of Plant Pathology, College of Agronomy & Biotechnology, China Agricultural University, Beijing 100193, China; [2]Department of Biological Sciences, University of Wisconsin–Milwaukee 53211, U.S.A.; [3]College of Forestry and Biotechnology, Zhejiang Forestry University, Lin'an 311300, China

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The type III secretion system (T3SS) is considered one of the major virulence factors in many bacterial pathogens. This report demonstrates that RssB, ClpXP, and RpoS play a role in T3SS regulation of *Dickeya dadantii* 3937. ClpP is a serine-type protease which associates with the ClpX chaperone to form a functional Clp proteolytic complex for degradation of proteins. With the assistance of recognition factor RssB, ClpXP degrades the RpoS sigma factor. RpoS positively regulates the expression of the *rsmA* gene encoding an RNA-binding regulatory protein. By interacting with the *hrpL* mRNA, RsmA reduces HrpL production and downregulates the T3SS genes in the HrpL regulon. In addition, ClpXP, RssB, and RpoS affect pectinolytic enzyme production in *D. dadantii* 3937, probably through RsmA. The ClpXP and RssB proteins are essential for bacterial virulence.

Dickeya dadantii 3937 (formerly named *Erwinia chrysanthemi*), a member of the *Enterobacteriaceae* family, is an opportunistic necrotrophic pathogen that causes soft rot, wilts, and blight diseases on a wide range of plants, including ornamental plants and economically important vegetable crops (Ma et al. 2007). Several virulence determinants have been discovered in *D. dadantii*. These include the well-studied extracellular enzymes such as pectate lyases (Pel), polygalacturonases (Peh), cellulases, and proteases (Prt), with Pel as the major virulence determinant among these isozymes (Collmer and Keen 1986; Roy et al. 1999; Herron et al. 2000; Kazemi-Pour et al. 2004). Siderophore-dependent iron uptake systems and motility of the bacterial strains are also required for pathogenicity (Franza et al. 1999; Antunez-Lamas et al. 2009). In addition to the above-named virulence factors, the type III protein secretion system (T3SS) is reported to play an important role in the initial infection of the bacterium in plant hosts (Bauer et al. 1994; Yang et al. 2002). In gram-negative pathogens, the T3SS translocates effector proteins directly into the host cell cytosol to cause disease symptoms (Hueck 1998; He et al. 2004; Mota et al. 2005).

In *D. dadantii* 3937, at least two main regulatory pathways leading to the activation of the T3SS in the bacterium have been proposed (Fig. 1) (Yap et al. 2005; Tang et al. 2006; Yang et al. 2008a,b; Li et al. 2009). In the HrpX/Y-HrpS-HrpL regulatory pathway, the two-component system HrpX/HrpY activates *hrpS*, which encodes a σ^{54}-enhancer binding protein. HrpS interacts with the σ^{54} (RpoN)-containing RNA polymerase holoenzyme and initiates transcription of *hrpL* (Chatterjee et al. 2002; Yap et al. 2005; Tang et al. 2006). HrpL is a member of the extracytoplasmic function (ECF) family of alternative sigma factors and is the central regulator required for activation of many genes downstream of the T3SS regulatory cascade, such as *hrpA*, *dspE*, and *hrpN*, which encode a structural protein of the T3SS pilus, a T3SS effector, and a T3SS harpin, respectively (Wei and Beer 1995; Chatterjee et al. 2002; Tang et al. 2006). In the Rsm-mediated regulatory pathway, RsmA (a small RNA-binding protein) can bind to the *hrpL* mRNA to facilitate its degradation in *Pectobacterium carotovorum* (Chatterjee et al. 1995, 2002; Cui et al. 1995) (Fig. 1). Alternatively, an untranslated regulatory RNA molecule, *rsmB*, binds to RsmA and neutralizes its degradation effect on *hrpL* mRNA (Liu et al. 1998; Chatterjee et al. 2002). Another global two-component system, GacS/A, upregulates expression of *rsmB*, which in turn, inactivates RsmA, resulting in elevated levels of *hrpL* mRNA (Tang et al. 2006; Yang et al. 2008b).

Proteolysis plays a critical role in maintaining cellular homeostasis by controlling the levels of various proteins, including global regulators involved in numerous biological functions, as well as misfolded and damaged proteins (Jenal and Hengge-Aronis 2003; Ehrmann and Clausen 2004). In bacteria, most intracellular proteolysis is performed by the energy-dependent proteases Clp and Lon (Gottesman 1996, 2003; Porankiewicz et al. 1999; Jenal and Hengge-Aronis 2003). Proteases are grouped into different categories according to an active site residue or metal involved in the catalytic activity, such as serine or threonine proteases, and metalloproteases (Ehrmann and Clausen 2004). ClpP, a serine-type protease, is highly conserved throughout prokaryotes and eukaryotes. ClpP typically interacts with the ATP-dependent AAA+ chaperones, ClpX or ClpA. These chaperones determine the substrate specificity and also unfold and translocate the substrate to the proteolytic chamber where proteins are degraded into short peptides (Dougan et al. 2002; Yu and Houry 2007).

In response to environmental signals, the ClpP protease performs important and diverse roles in bacteria by rapid regulation of cellular levels and activities of regulatory proteins or sigma factors. This system allows the cell to alter its pattern of gene expression to meet changing needs. For example, ClpXP

Corresponding authors: Ching-Hong Yang; Telephone: +1-414-229-6331; Fax: +1-414-229-3926; E-mail: chyang@uwm.edu; and Qi Wang; E-mail: wangqi@cau.edu.cn

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protease regulates the activity of RpoE by degrading the cytoplasmic domain of the single-pass inner membrane protein RseA, which frees RpoE to interact with RNA polymerase (Ades et al. 1999). RpoE is an ECF family sigma factor involved in sensing cell envelope stress and plays a role in the virulence of some bacteria (Missiakas et al. 1997; Hayden and Ades 2008). In addition, the virulence-associated T3SS was reported to be indirectly regulated by ClpXP through regulation of sigma factor RpoS (σ^S), GrlR, GadE, and GadX in enterohemorrhagic *Escherichia coli* (Iyoda and Watanabe 2005; Tomoyasu et al. 2005). The RpoS protein is a master regulator for the general stress response in bacteria (Loewen et al. 1998; Hengge-Aronis 2002; McMeechan et al. 2007; Sandercock and Page 2008), and is present at very low levels during exponential cell growth in media, largely due to its rapid degradation by ClpXP protease (Lange and Henggearonis 1994; Schweder et al. 1996). RpoS degradation by ClpXP takes place with the assistance of a recognition factor, RssB, which delivers RpoS to ClpXP for degradation (Zhou et al. 2001). In *P. carotovorum*, RpoS was reported to affect the extracellular enzyme levels, $hrpN_{Ecc}$ expression, and virulence of the bacterium (Mukherjee et al. 1998).

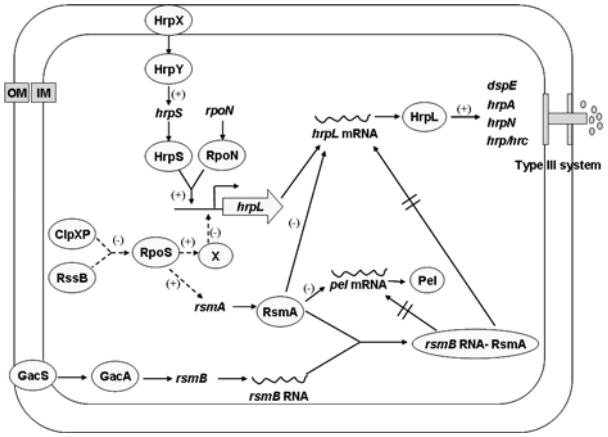

Fig. 1. Regulatory network controlling the *Dickeya dadantii* 3937 type III secretion system (T3SS) and the production of pectate lyases. The *D. dadantii* 3937 T3SS is regulated by the HrpX/HrpY-HrpS-HrpL and the GacS/GacA-*rsmB*-HrpL regulatory pathways. Dashed lines represent regulators identified in this article. ClpXP and RssB promote RpoS (an alternative sigma factor) degradation. RpoS negatively regulates *hrpL* by downregulating the promoter activity of *hrpL* and upregulating global repressor RsmA. RsmA can bind to and cause the degradation of the *hrpL* mRNA and, in turn, downregulate genes in the HrpL regulon, such as *hrpA*, *hrpN*, and *dspE*. RsmA also regulates the production of pectate lyases (Pel). Symbols: + designates positive regulation and – designates negative regulation.

Limited information is available about the regulatory effect of ClpXP on T3SS and the role of ClpXP in the virulence of phytobacteria. This study demonstrates that, with the help of RssB, ClpXP positively regulates T3SS through RpoS degradation. In addition to the regulation of T3SS, ClpXP protease, RssB, and RpoS play a role in pectinolytic enzyme production and virulence of *D. dadantii* 3937.

RESULTS AND DISCUSSION

Regulatory effect of ClpXP on T3SS.

A *clpP* mutant of *D. dadantii* 3937 was obtained by insertion of the transposon mini*Himar RB1*, on the basis of an altered *hrpA* expression in minimal medium (MM) (data not shown). In order to investigate the role of *clpP* in T3SS regulation, we constructed a *clpP* deletion mutant (Ech156) (Table 1) and evaluated the effects of this mutation on expression of the T3SS genes *hrpA*, *hrpN*, and *dspE*. These genes are located downstream in the HrpL regulon of *D. dadantii* 3937. Employing a green fluorescent protein (GFP) reporter system, promoter activities of *hrpA, hrpN*, and *dspE* of Ech156 were measured by flow cytometry. Deletion of *clpP* resulted in reduced expression of *hrpA*, *hrpN*, and *dspE* compared with the wild type (Table 2). The promoter activity of *hrpA* was restored to wild-type level when Ech156 was complemented with pMLclpP (Table 3). Because *hrpA*, *hrpN*, and *dspE* are all regulated by HrpL, *hrpA* was selected as the representative T3SS gene for the Northern blot and complementation assays. The effect of ClpP on *hrpA* expression was confirmed by Northern blot. Compared with the wild type, a smaller amount of *hrpA*

Table 1. Strains and plasmids used in this study

Strains and plasmids	Characteristics[a]	Reference or source
Strains		
Dickeya dadantii		
3937	Wild type, *Saintpaulia* (African violet) isolate	N. Hugouvieux-Cotte-Pattat
Ech156	Δ*clpP::kan*, Km^r	This work
Ech157	Δ*clpX::kan*, Km^r	This work
Ech158	Δ*clpXP::kan*; Km^r	This work
Ech159	Δ*rpoS::kan*, Km^r	This work
Ech160	Δ*rssB::kan*, Km^r	This work
Ech161	Δ*rpoE::kan*, Km^r	This work
Escherichia coli		
E. coli S17-1 λ-pir	λ-pir Lysogen of S17-1, Sp^r	Victor de Lorenzo, Spain
Plasmids		
pPROBE-AT	Promoter-probe vector, Ap^r	Miller et al. 2001
phrpA	pProbe-AT derivative with PCR fragment containing *hrpA* promoter region, Ap^r	Yang et al. 2008b
phrpN	pProbe-AT derivative with PCR fragment containing *hrpN* promoter region, Ap^r	Yang et al. 2007
pdspE	pProbe-AT derivative with PCR fragment containing *dspE* promoter region, Ap^r	Peng et al. 2006
phrpS	pProbe-AT derivative with PCR fragment containing *hrpS* promoter region, Ap^r	Yang et al. 2007
phrpL	pProbe-AT derivative with PCR fragment containing *hrpL* promoter region, Ap^r	Yang et al. 2007
pWM91	Sucrose-based counter-selectable plasmid, Ap^r	Metcalf et al. 1996
pML122/123	RSF1010-derived expression and *lac*-fusion broad host-range vector, Gm^r	Labes et al. 1990
pMLclpP	pML122 derivative with PCR fragment containing *clpP*, Gm^r	This work
pMLclpXP	pML123 derivative with PCR fragment containing *clpXP*, Gm^r	This work
pMLrpoS	pML123 derivative with PCR fragment containing *rpoS*, Gm^r	This work
pMLrssB	pML123 derivative with PCR fragment containing *rssB*, Gm^r	This work
pKD4	Template plasmid for kanamycin cassette, Km^r	Datsenko and Wanner 2000

[a] Ap^r, Km^r, Sp^r, and Gm^r indicate resistance to ampicillin, kanamycin, spectinomycin, and gentamicin, respectively. PCR = polymerase chain reaction.

mRNA was observed in Ech156 (Fig. 2A). In addition, the *hrpA* mRNA level in Ech156 was restored to wild-type level upon complementation of Ech156 with pMLclpP (Fig. 2A).

ClpX is a Clp ATPase, which associates with ClpP to form a functional proteolytic complex for the degradation of proteins (Ortega et al. 2004; Butler et al. 2006). *clpX* and *clpP* of *D. dadantii* 3937 are arranged in an operon. To investigate whether an interaction between ClpX and ClpP is necessary for regulation of the T3SS in *D. dadantii*, a *clpX* deletion mutant (Ech157) and a *clpX*, *clpP* double-deletion mutant (Ech158) were constructed. Compared with *D. dadantii* 3937, *hrpA* promoter activity was reduced by factors of 5 to 13 in Ech157 and Ech158 using the GFP reporter system (Table 2). When pMLclpXP containing the entire *clpXP* operon was provided in trans, the *hrpA* expression level was restored in both mutants (Table 3). These results indicate that the ATP-dependent proteolytic complex ClpXP acts as a positive regulator of T3SS genes.

ClpXP regulates *hrpL* at transcriptional and post-transcriptional levels.

Given that ClpXP positively regulates *hrpA* and *hrpN*, we also investigated the regulatory effect of ClpXP on key components of the T3SS regulatory pathways. Because HrpL is the main regulator required for activation of genes downstream of the regulatory pathways, such as *hrpA*, *hrpN*, and *dspE* (Wei and Beer 1995; Chatterjee et al. 2002; Tang et al. 2006), we examined the effect of the *clpP* mutation (Ech156) on *hrpL* by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). In Ech156, the amount of *hrpL* mRNA was only 0.23-fold that of the wild-type strain (Fig. 3A). In addition, a decrease in *hrpL* promoter activity was observed in Ech156, Ech157, and Ech158 compared with the wild type at 12 and 24 h using the GFP reporter system in combination with flow cytometry (Table 2). These results suggest that ClpXP positively regulates *hrpL* expression.

Table 2. Expression of type III secretion system genes in *Dickeya dadantii* 3937 (3937), *clpP* mutant (Ech156), *clpX* mutant (Ech157), *clpXP* mutant (Ech158), *rpoS* mutant (Ech159), and *rssB* mutant (Ech160)[a]

Strain	Average MFI ± standard deviation at 12 h	Average MFI ± standard deviation at 24 h
3937 (phrpA)	55.3 ± 6.1	77.3 ± 10.4
Ech156 (phrpA)	8.7 ± 0.5*	7.0 ± 0.3*
3937 (phrpN)	32.7 ± 10.7	56.4 ± 9.8
Ech156 (phrpN)	4.6 ± 0.4*	3.6 ± 0.1*
3937 (pdspE)	20.1 ± 0.7	30.1 ± 0.6
Ech156 (pdspE)	4.8 ± 0.2*	4.0 ± 0.3*
3937 (phrpL)	9.0 ± 0.3	11.0 ± 0.4
Ech156 (phrpL)	6.5 ± 0.5*	5.6 ± 0.2*
3937 (phrpA)	52.6 ± 9.3	88.9 ± 13.5
Ech157 (phrpA)	11.4 ± 0.2*	11.4 ± 0.4*
3937 (phrpN)	42.3 ± 1.8	73.1 ± 3.2
Ech157 (phrpN)	6.3 ± 0.3*	5.9 ± 0.2*
3937 (phrpL)	13.1 ± 0.1	19.1 ± 0.6
Ech157 (phrpL)	9.4 ± 0.8*	9.7 ± 0.8*
3937 (phrpA)	57.1 ± 4.6	72.1 ± 6.9
Ech158 (phrpA)	7.4 ± 0.4*	5.2 ± 0.3*
3937 (phrpN)	54.2 ± 4.0	71.3 ± 5.1
Ech158 (phrpN)	4.0 ± 0.4*	3.0 ± 0.3*
3937 (phrpL)	14.9 ± 0.6	20.8 ± 1.1
Ech158 (phrpL)	5.1 ± 0.6*	6.7 ± 0.5*
3937 (phrpA)	55.3 ± 6.1	77.3 ± 10.4
Ech159 (phrpA)	116.6 ± 15.0*	189.6 ± 6.9*
3937 (phrpN)	32.7 ± 10.7	56.4 ± 9.8
Ech159 (phrpN)	88.6 ± 4.3*	180.1 ± 7.5*
3937 (pdspE)	20.1 ± 0.7	30.1 ± 0.6
Ech159 (pdspE)	33.6 ± 2.5*	87.5 ± 2.2*
3937 (phrpL)	9.0 ± 0.3	11.0 ± 0.4
Ech159 (phrpL)	22.8 ± 3.6*	32.4 ± 0.4*
3937 (phrpA)	52.6 ± 9.3	88.9 ± 13.5
Ech160 (phrpA)	9.8 ± 0.3*	11.2 ± 0.1*
3937 (phrpN)	42.3 ± 1.8	73.1 ± 3.2
Ech160 (phrpN)	5.2 ± 0.5*	5.4 ± 0.3*
3937 (phrpL)	13.1 ± 0.1	19.1 ± 0.6
Ech160 (phrpL)	10.2 ± 0.2*	11.5 ± 0.2*
3937 (pPROBE-AT)	2.9 ± 0.0	4.9 ± 0.1

[a] Promoter activities at 12 and 24 h of bacterial growth were determined. Green fluorescent protein (GFP) mean fluorescence intensity (MFI) was determined for gated populations of bacterial cells by flow cytometry. Values are representative of two or three experiments, and three replicates were used for each experiment. Asterisks indicate statistically significant differences in GFP MFI between the wild type (3937) and mutants ($P < 0.01$, Student's *t* test).

Table 3. Expression of type III secretion system gene *hrpA* in *Dickeya dadantii* 3937 (3937), *clpP* mutant (Ech156), *clpX* mutant (Ech157), *clpXP* mutant (Ech158), *rpoS* mutant (Ech159), and *rssB* mutant (Ech160) containing pML122/123, or complementation plasmid[a]

Stain[b]	Average MFI ± standard deviation at 12 h	Average MFI ± standard deviation at 24 h
3937 (pML122)	21.4 ± 1.4	39.0 ± 3.6
Ech156 (pML122)	9.0 ± 0.7*	7.2 ± 0.3*
Ech156 (pMLclpP)	23.8 ± 4.3	39.1 ± 7.2
3937 (pML123)	43.3 ± 1.5	47.8 ± 2.2
Ech157 (pML123)	18.2 ± 0.2*	14.3 ± 0.5*
Ech157 (pMLclpXP)	51.7 ± 5.4	84.8 ± 7.9*
3937 (pML123)	43.3 ± 1.5	47.8 ± 2.2
Ech158 (pML123)	12.0 ± 0.6*	8.3 ± 0.3*
Ech158 (pMLclpXP)	45.5 ± 4.2	113.6 ± 5.5*
3937 (pML123)	27.2 ± 4.7	62.2 ± 7.8
Ech159 (pML123)	56.1 ± 1.0*	121.4 ± 1.5*
Ech159 (pMLrpoS)	9.9 ± 0.4*	10.1 ± 0.3*
3937 (pMLrpoS)	10.7 ± 0.5*	9.7 ± 0.2*
3937 (pML123)	43.3 ± 1.5	47.8 ± 2.2
Ech160 (pML123)	15.9 ± 1.3*	14.5 ± 0.4*
Ech160 (pMLrssB)	92.0 ± 0.4*	106.2 ± 4.5*

[a] Promoter activity of *hrpA* was compared at 12 and 24 h of bacterial growth in strains containing empty vector pML122/123 or complementation plasmid. Green fluorescent protein (GFP) mean fluorescence intensity (MFI) was determined for gated populations of bacterial cells by flow cytometry. Values are representative of two experiments, and three replicates were used for each experiment. Asterisks indicate statistically significant differences in GFP MFI between the wild-type strain (3937) with empty vector and mutants with empty vector or complementation plasmid ($P < 0.01$, Student's *t* test).

[b] Stains carrying two plasmids (one was GFP reporter phrpA and the other was pML122/123 or their derivatives) were used in this study.

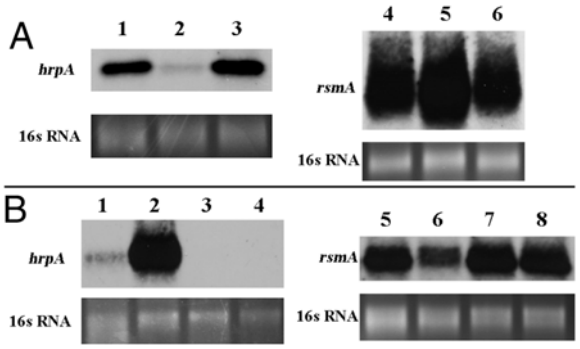

Fig. 2. Northern blot analysis of *hrpA* and *rsmA* mRNA levels. **A,** *hrpA* and *rsmA* mRNA levels in lanes 1 and 4, *Dickeya dadantii* 3937 with pML122; lanes 2 and 5, Ech156 (*clpP* mutant) with pML122; and lanes 3 and 6, Ech156 with complementation plasmid pMLclpP. **B,** *hrpA* and *rsmA* mRNA levels in lanes 1 and 5, *D. dadantii* 3937 with pML123; lanes 2 and 6, Ech159 (*rpoS* mutant) with pML123; lanes 3 and 7, Ech159 with complementation plasmid pMLrpoS; and lanes 4 and 8, *D. dadantii* 3937 with pMLrpoS. Cells were cultured in minimal medium for 12 h before the RNA isolation. 16S rRNA was used as an internal control.

The T3SS of *D. dadantii* 3937 is controlled by two major regulatory pathways, HrpX/Y-HrpS and GacS/A-*rsmB*-RsmA (Yap et al. 2005; Tang et al. 2006; Yang et al. 2008a,b; Li et al. 2009). To determine the T3SS regulatory pathway through which ClpXP controls *hrpL*, the expression of *hrpS* was examined in the wild type and the *clpP* mutant (Ech156) by qRT-PCR. The *hrpS* mRNA level in Ech156 was comparable with that in the wild-type strain (Fig. 3A), indicating that the regulatory effect on *hrpL* by ClpXP is probably not through the HrpX/Y-HrpS pathway.

We also evaluated the effect of ClpXP on T3SS gene expression through the RsmA-*rsmB*–dependent regulatory pathway. The RNA levels of *rsmA* and *rsmB* were examined in the wild type and Ech156. Northern blot analysis revealed a considerable increase in *rsmA* mRNA in Ech156 at 12 h of growth in MM (Fig. 2A) but the level of *rsmB* RNA in Ech156 was indistinguishable from that detected in the wild type (Supplementary Fig. 1). Complementation of Ech156 with pMLclpP restored *rsmA* mRNA roughly to the wild-type level (Fig. 2A). In *P. carotovorum*, RsmA negatively regulates *hrpL* at the post-transcriptional level to ultimately regulate genes in the HrpL regulon (Cui et al. 1995; Chatterjee et al. 2002). Our results indicate that ClpXP functions upstream in the post-transcriptional regulatory pathway and positively regulates *hrpL* and genes in the HrpL regulon through downregulation of *rsmA* expression (Fig. 1).

In summary, a deletion in *clpP* (Ech156) led to an increase in the *rsmA* mRNA level and a decrease in the *hrpL* mRNA level as well as a decrease in *hrpL* promoter activity (Figs. 2 and 3; Table 2). These results suggest that ClpXP positively regulates *hrpL* at both transcriptional and post-transcriptional levels.

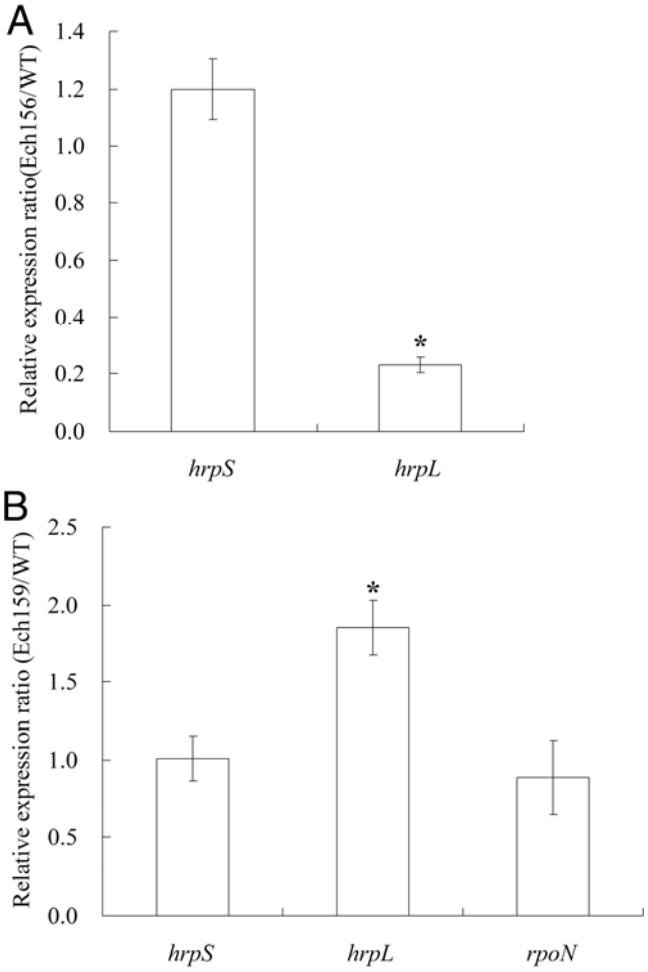

Fig. 3. Relative mRNA levels of type III secretion system (T3SS) genes in mutants and *Dickeya dadantii* 3937 (WT) as determined by quantitative reverse-transcription polymerase chain reaction. **A,** Relative mRNA level of *hrpS* and *hrpL* in WT and the *clpP* mutant (Ech156). **B,** Relative mRNA levels of *hrpS, hrpL*, and *rpoN* in WT and the *rpoS* mutant (Ech159). *rplU* was used as an endogenous control for data analysis. Asterisks indicate statistically significant differences in mRNA level of the mutants compared with WT ($P < 0.05$, Student's *t* test).

Effect of ClpXP on T3SS gene expression is not channeled via RpoE.

The activity of the stress responsive sigma factor RpoE is controlled by the level of anti-sigma factor RseA. The ClpXP protease degrades the cytoplasmic domain of the RseA protein in response to stress, which leads to increased levels of free RpoE that can interact with RNA polymerase (Ades et al. 1999). Our results show that ClpXP negatively regulates the level of *rsmA* mRNA but the regulatory mechanism is unclear. To investigate the possibility that ClpXP may regulate the expression of T3SS genes in an RpoE-dependent manner, an *rpoE* mutant (Ech161) was constructed. Using the GFP promoter probe, promoter activities of *hrpS*, *hrpL*, and *hrpN* were measured and found to be similar at 12 and 24 h in the wild-type strain and *rpoE* mutant grown in MM (Table 4). These results demonstrate that the effect of ClpXP on T3SS gene expression is not channeled via RpoE.

RpoS regulates T3SS by mediating *rsmA* expression.

In enterohemorrhagic *E. coli*, ClpXP was reported to control T3SS through degradation of RpoS (Iyoda and Watanabe 2005; Tomoyasu et al. 2005). In addition, RpoS was reported to regulate *rsmA* and harpin production in *P. carotovorum* (Mukherjee et al. 1998). Based on this information, we hypothesized that i) RpoS may regulate T3SS in *D. dadantii* 3937 and ii) the ClpXP proteolytic complex may also regulate T3SS through degradation of RpoS in this bacterium. To test the regulatory effect of RpoS on T3SS, an *rpoS* null mutant (Ech159) was constructed and the promoter activities of *hrp* genes were examined by flow cytometry using the GFP promoter probe. Compared with *D. dadantii* 3937, the expression levels of *hrpA*, *hrpN*, and *dspE* were increased two- to threefold in Ech159 (Table 2). The mRNA level of *hrpA* in *D. dadantii* 3937 and Ech159 was also examined by Northern blot. Compared with the wild-type strain, an increase in *hrpA* mRNA was observed in Ech159 (Fig. 2B). Moreover, *hrpA* expression was dramatically reduced by introduction of pMLrpoS into the *rpoS* mutant or the wild type compared with either strain containing only the vector (Table 3; Fig. 2B). These results demonstrate that RpoS negatively regu-

Table 4. Promoter activities of type III secretion system genes in *Dickeya dadantii* 3937 (3937) and *rpoE* mutant (Ech161)[a]

	Average MFI ± standard deviation at	
Stain	**12 h**	**24 h**
3937(phrpS)	50.9 ± 2.1	73.0 ± 1.6
Ech161(phrpS)	49.4 ± 2.4	76.7 ± 2.0
3937(phrpL)	7.7 ± 0.4	12.6 ± 0.4
Ech161(phrpL)	8.5 ± 0.2	10.9 ± 0.7
3937(phrpN)	45.0 ± 6.7	65.8 ± 6.8
Ech161(phrpN)	37.8 ± 1.2	68.4 ± 4.7
3937(pPROBE-AT)	3.3 ± 0.4	4.3 ± 0.6

[a] Promoter activities at 12 and 24 h of bacterial growth were determined. Green fluorescent protein (GFP) mean fluorescence intensity (MFI) was determined for gated populations of bacterial cells by flow cytometry. Values are representative of three experiments, and three replicates were used for each experiment.

lates expression of genes encoding T3SS effectors and structural components.

In *P. carotovorum*, RpoN (σ^{54}) is required for the transcription of *hrpL* (Chatterjee et al. 2002). We speculated that RpoS may regulate the expression of *rpoN* and, in turn, the RpoN protein regulates *hrpL* expression. To test this hypothesis, the *rpoN* mRNA level was measured in the wild type and the *rpoS* mutant by qRT-PCR. We found that the *rpoN* mRNA level in Ech159 was similar to that in the wild type (Fig. 3B), suggesting that RpoS does not regulate the expression of *rpoN*.

To elucidate the regulatory mechanism of RpoS on T3SS, the expression of key T3SS regulatory genes *hrpS*, *rsmA*, *rsmB*, and *hrpL* were examined in the wild type and Ech159. Northern blot analysis revealed a considerable decrease in *rsmA* mRNA in cell extracts of Ech159 grown in MM for 12 h (Fig. 2B) but no difference was observed in the level of *rsmB* RNA. Complementation of Ech159 with pMLrpoS restored *rsmA* mRNA almost to the wild-type level (Fig. 2B). As observed by qRT-PCR, the *hrpS* mRNA level in Ech159 was comparable with that in the wild-type strain but the *hrpL* mRNA level was significantly higher in Ech159 (Fig. 3B). Finally, the promoter activity of *hrpL* was examined by GFP promoter probe. After growth in MM, the promoter activity of *hrpL* was increased two- to threefold in Ech159 compared with the wild type at 12 and 24 h (Table 2).

In summary, the *rpoS* mutant (Ech159) exhibited a decrease in *rsmA* mRNA and an increase in the levels of *hrpL* mRNA and *hrpL* promoter activity (Figs. 2 and 3; Table 2). These results indicate that RpoS regulates the expression of genes encoding T3SS effectors and structural components by mediating *rsmA* expression, which results in a reduction of *hrpL* mRNA stability. RpoS also negatively regulates *hrpL* at the transcriptional level. At this stage, how RpoS affects the promoter activity of *hrpL* is unclear. It is possible that RpoS upregulates an unidentified transcriptional repressor of *hrpL* which further controls the expression of *hrpL* (Fig. 1).

ClpXP regulates *rsmA* through RssB-mediated RpoS degradation.

In several gram-negative bacteria, the RpoS (σ^S) protein is a master regulator of the general stress response (Loewen et al. 1998; McMeechan et al. 2007; Sandercock and Page 2008). With the assistance of the recognition factor RssB, RpoS is rapidly degraded by ClpXP protease during exponential cell growth in *E. coli* (Lange and Henggearonis 1994; Schweder et al. 1996; Zhou et al. 2001). To verify the regulatory effect of RssB on T3SS genes of *D. dadantii* 3937, a null mutant of *rssB* (Ech160) was generated. The promoter activities of *hrpA* and *hrpN* were measured by flow cytometry. A decrease in *hrpA* and *hrpN* expression was observed in Ech160 compared with *D. dadantii* 3937 (Table 2). The expression of *hrpA* was restored to the wild-type level when Ech160 was complemented with pMLrssB (Table 3). In addition, a decrease in *hrpL* expression was observed in Ech160 when promoter activity was monitored by GFP promoter probe (Table 2). These results demonstrate that RssB acts as a positive regulator of T3SS. This also suggests that RssB may play a role as a direct recognition factor in ClpXP-dependent RpoS proteolysis in *D. dadantii* 3937.

We hypothesized that ClpXP may regulate T3SS through degradation of RpoS in *D. dadantii* 3937. To determine whether attenuated *hrpA* expression in *clpP*, *clpX*, and *rssB* mutants was due to accumulation of RpoS, protein expression was analyzed by Western blot using anti-$RpoS_{Dd}$ antibodies and total cell extracts from Ech156 (*clpP* mutant), Ech157 (*clpX* mutant), and Ech160 (*rssB* mutant) grown in MM. Western blot analysis revealed that, compared with the wild type, RpoS accumulated to a higher level in Ech156, Ech157, and Ech160 (Fig. 4), suggesting that ClpXP and RssB are responsible for the degradation of RpoS. The diminished *hrpA* expression level in strains Ech156, Ech157, and Ech160 is likely due to increased amounts of RpoS. Together, the results above demonstrate that ClpXP regulates T3SS through degradation of RpoS in *D. dadantii* 3937.

Effect of ClpXP, RssB, and RpoS on pectinolytic enzyme production and virulence.

In addition to their effect on the T3SS, ClpXP, RssB, and RpoS were evaluated for their role in Pel production in *D. dadantii*. Cultures of Ech156 (*clpP* mutant), Ech157 (*clpX* mutant), Ech158 (*clpXP* mutant), Ech159 (*rpoS* mutant), Ech160 (*rssB* mutant), and the wild-type strain were tested for total Pel activity by a spectrophotometric assay. Compared with the wild-type strain, Ech156, Ech157, Ech158, and Ech160 showed a considerable reduction in Pel activity (Fig. 5). However, an increase in Pel activity was observed in Ech159 (Fig. 5). Because RsmA was reported to suppress the production of pectinolytic enzymes in *P. carotovorum* (Cui et al. 1995), the effect of ClpXP, RssB, and RpoS on Pel production in *D. dadantii* 3937 may be due to their effect on *rsmA* expression (Fig. 1).

Both pectinolytic enzymes and the T3SS play major roles in the pathogenicity of *D. dadantii* (Collmer and Keen 1986; Bauer et al. 1994; Roy et al. 1999; Herron et al. 2000; Yang et al. 2002; Kazemi-Pour et al. 2004). Ech156, Ech157, Ech158, Ech159, and Ech160 were evaluated for the ability to cause disease on Chinese cabbage. Compared with the wild-type strain, reduced maceration symptoms were observed in Ech156, Ech157, Ech158, and Ech160 (Fig. 6), which is likely due to the significant reduction in Pel production and T3SS expression

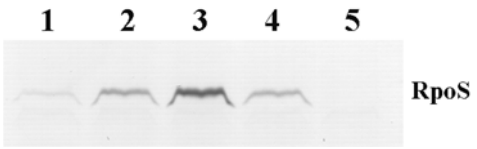

Fig. 4. Western blot with anti-$RpoS_{Dd}$ antibody. Cell extracts were prepared from cultures grown in minimal medium for 12 h at 28°C. Lane 1, *Dickeya dadantii* 3937 (WT); lane 2, Ech157 (*clpX* mutant); lane 3, Ech156 (*clpP* mutant); lane 4, Ech160 (*rssB* mutant), lane 5, Ech159 (*rpoS* mutant).

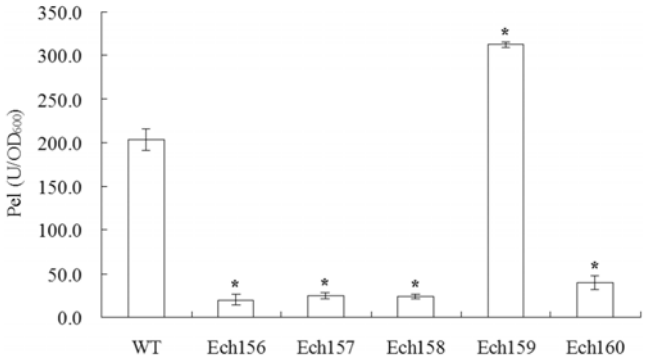

Fig. 5. Production of total pectate lyases (Pel) in *Dickeya dadantii* 3937 (WT), *clpP* mutant (Ech156), *clpX* mutant (Ech157), *clpXP* mutant (Ech158), *rpoS* mutant (Ech159), and *rssB* mutant (Ech160). Spectrophotometric quantification of Pel activity (unit per optical density at 600 nm) was examined as described (Matsumoto et al. 2003). Values are a representative of two individual experiments. Three replicates were used in each experiment. Value is presented as the average of three replicates. Asterisks indicate statistically significant differences in Pel activity of the mutants compared with the wild type ($P < 0.05$, Student's *t* test).

in these mutants. The *rpoS* mutant (Ech159) showed increased maceration ability (Fig. 6), probably because of the increase in Pel production and T3SS expression. During starvation and environmental stresses, the RssB activity is sequestered, which results in stabilization of the RpoS protein (Bougdour et al. 2006). An increase in the cellular levels of RpoS through RssB regulation suggests that RpoS may play a role in bacterial virulence in response to environmental conditions.

In summary, the regulatory roles of ClpXP, RssB, and RpoS in *D. dadantii* 3937 are depicted in Figure 1. With the assistance of the recognition factor RssB, ClpXP degrades the sigma factor, RpoS. RpoS negatively regulates promoter activity of *hrpL*. This regulatory action could possibly be indirect through an unidentified transcriptional repressor of *hrpL* which is upregulated by RpoS. In addition, RpoS positively regulates *rsmA* expression. RsmA promotes the degradation of *hrpL* and *pel* mRNA and, ultimately, leads to a reduction in T3SS gene expression and Pel production. Furthermore, ClpXP, RssB, and RpoS play an essential role in the virulence of *D. dadantii* 3937 by regulating the components necessary for T3SS gene expression and Pel production.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and media.

The bacterial strains and plasmids used in this study are listed in Table 1. *D. dadantii* 3937 and mutant strains were stored at –80°C in 15% glycerol. *D. dadantii* strains were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl), MG medium (1% mannitol, 0.2% glutamic acid, 0.05% potassium phosphate monobasic, 0.02% NaCl, and 0.02% $MgSO_4$) or low-nutrient T3SS-inducing MM at 28°C (Yang et al. 2007). *E. coli* strains were grown in LB at 37°C. Antibiotics were added at the following concentrations when required: ampicillin (100 μg/ml), kanamycin (50 μg/ml), gentamicin (10 μg/ml), and spectinomycin (50 μg/ml). Primers used for PCR in this report are listed in Supplementary Table 1.

Construction of plasmids and mutants.

The *clpP* (Ech156), *clpX* (Ech157), *clpXP* (Ech158), *rpoS* (Ech159), *rssB* (Ech160), and *rpoE* (Ech161) deletion mutants were constructed by marker exchange mutagenesis (Yang et al. 2002). Briefly, two fragments flanking each target gene were obtained. A kanamycin cassette, amplified from pKD4 (Table 1), was ligated with these two fragments and then cloned into *Bam*HI and *Xho*I sites in pWM91. This construct was transferred into *D. dadantii* 3937 by conjugation using *E. coli* S17-1. To select strains with chromosomal deletions, transconjugants with kanamycin and ampicillin resistance were plated on MG containing 5% sucrose and kanamycin. Colonies having sucrose and kanamycin resistance and ampicillin sensitivity were isolated and confirmed by PCR using outside primers. To construct plasmids for complementation, the coding regions of target genes were amplified and were cloned into low–copy number plasmids pML122 or pML123 (Table 1). All of the constructs and mutants described above were verified by PCR and DNA sequencing.

Flow cytometry analysis.

To screen for regulators that alter the expression of *hrpA*, the mini*Himar RB1* transposon (Bouhenni et al. 2005) was used to construct a mutant library of *D. dadantii* 3937. A reporter plasmid carrying a transcriptional fusion of the *hrpA* promoter controlling expression of *gfp* (phrpA) was mobilized into each transposon mutant and the *hrpA* promoter activity was monitored by flow cytometry as previously described (BD Biosciences, San Jose, CA, U.S.A.) (Peng et al. 2006). One of these mutants showed reduced expression of *hrpA*, and the site of the transposon insertion was identified as *clpP*.

Promoter-*gfp* constructs phrpA, phrpN, pdspE, phrpS, and phrpL were used to monitor promoter activities in *D. dadantii* strains by flow cytometry. Briefly, the bacterial cells carrying the promoter-GFP transcriptional fusions were grown in LB at 28°C overnight and transferred to MM to induce T3SS gene expression. Samples were collected and diluted to the appropriate concentration with 1× phosphate-buffered saline. The promoter activity was analyzed by measuring GFP intensity using flow cytometry.

qRT-PCR analysis.

qRT-PCR was used to measure transcript levels of target genes. Bacteria were cultured in MM for 12 h. Cells were harvested and total RNA was isolated by using TRI reagent (Sigma-Aldrich, St. Louis) in combination with the RNeasy Mini kit (Qiagen Sciences, Germantown, MD, U.S.A.) and the TURBO DNA-free kit (Ambion, Austin, TX, U.S.A.) as described elsewhere (Peng et al. 2006; Li et al. 2009). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's instructions. cDNA levels of target genes were quantified by qRT-PCR using RealMaster SYBR ROX Mix (5 PRIME, Gaithersburg, MD, U.S.A.), with *rplU* as an endogenous control for data analysis (Mah et al. 2003; Kuchma et al. 2005).

Northern blot analysis.

Bacterial strains were grown in MM for 12 h, and total RNA was isolated by using TRI reagent and treated with the TURBO DNA-free kit. RNA samples were analyzed by using biotin-labeled *hrpA, rsmB*, or *rsmA* probes and a biotin detection system (BrightStar Psoralen-Biotin and Bright Star BioDetect; Ambion). 16S rRNA was used as an internal control.

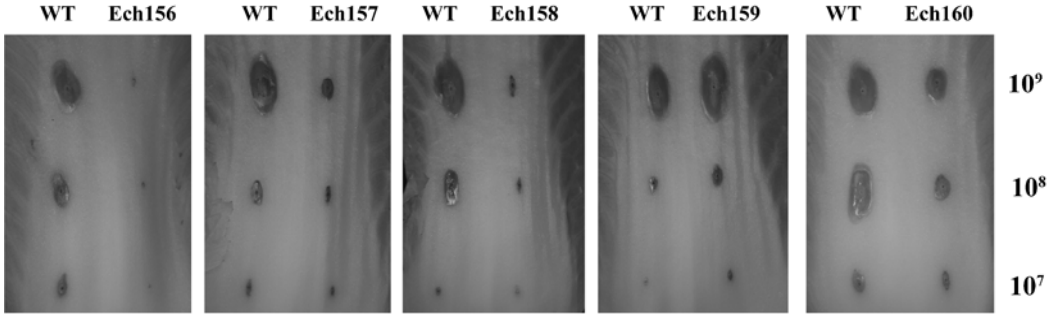

Fig. 6. Disease lesions caused by *Dickeya dadantii* 3937 (WT), *clpP* mutant (Ech156), *clpX* mutant (Ech157), *clpXP* mutant (Ech158), *rpoS* mutant (Ech159), and *rssB* mutant (Ech160) in leaves of Chinese cabbage. The leaves were inoculated at small wounds with a 10-μl bacterial suspension at concentrations of 10^7, 10^8, and 10^9 CFU/ml. Pictures were taken at 36 h after inoculation.

Western blot analysis.

Bacteria were cultured in MM at 28°C for 12 h. Cells were harvested and resuspended in bacterial protein extraction reagent (B-PER; Thermo Scientific, Rockford, IL, U.S.A.) supplemented with lysozyme (100 ng/ml). Samples were incubated for 30 min at room temperature and centrifuged to remove cell debris. The protein concentration was determined using a Bio-Rad protein assay (Bio-Rad) and normalized to an equivalent concentration by adding B-PER. Proteins from total cell lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to an Immobilion polyvinylidene diflouride transfer membrane (Millipore, Bedford, MA, U.S.A.) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The blot was sequentially probed with a 1:10,000 dilution of anti-$RpoS_{Dd}$ peptide antibody and a 1:500 dilution of an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (Millipore). Chromogenic detection was carried out with nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluine salt. To develop anti-$RpoS_{Dd}$ antibodies, a short-peptide fragment in the $RpoS_{Dd}$ protein sequence (ILQVQGLDIEELFRE) was designed, synthesized, conjugated to keyhole limpet hemocyanin, and purified by Proteintech Group, Inc. (Chicago). Rabbits were immunized against the short-peptide fragments and boosted four times after the primary immunization. The antibody value was tested further at different intervals after the primary immunization. Anti-$RpoS_{Dd}$ peptide antibodies were affinity purified using the synthesized peptides that were used for immunization (Proteintech Group, Inc.).

Enzyme activity assay.

Spectrophotometric quantification of Pel activity was performed essentially as previously described (Matsumoto et al. 2003). Briefly, cells from overnight cultures grown in LB were subcultured in MM containing 1% polygalacturonic acid (PGA) at 28°C for 20 h. The optical density of cell suspensions was adjusted to 1.0 at 600 nm (OD_{600}) by adding MM with 1% PGA. For total Pel activity, 1 ml of the culture was sonicated two times for 20 s (Virsonic 600; VirTis, Gardiner, NY, U.S.A.) on ice and then centrifuged at 15,000 rpm for 2 min to remove cell debris, and the resultant supernatant was used for the assay. In all, 10 µl of sample solution was added to 990 µl of the reaction buffer (0.05% PGA, 0.1 M Tris-HCl [pH 8.5], and 0.1 mM $CaCl_2$, prewarmed to 30°C) and thoroughly mixed. The OD_{230} was monitored for 3 min. One unit of Pel activity was equivalent to an increase of 1×10^{-3} OD_{230} in 1 min.

Leaf maceration assay.

The *D. dadantii* 3937 and mutant strains were grown in LB at 28°C overnight. Cells were harvested and resuspended in 50 mM phosphate buffer (pH 7.4) and adjusted to 10^7, 10^8, and 10^9 CFU/ml. Chinese cabbage leaves were used for the local maceration assay. For each Chinese cabbage leaf, the wild type and mutant strain were inoculated in parallel by addition of 10 µl of the appropriate culture. Leaves were observed for maceration symptoms 36 h after inoculation. Maceration assays were performed in triplicate.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

ASAP database and web interface: asap.ahabs.wisc.edu/asap/ASAP1.htm