Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2014) **15**(1), 58–66 DOI: 10.1111/mpp.12065

Alternative sigma factor RpoN and its modulation protein YhbH are indispensable for *Erwinia amylovora* virulence

VERONICA ANCONA†, WENTING LI† AND YOUFU ZHAO*

Department of Crop Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

SUMMARY

In *Erwinia amylovora*, ECF (extracytoplasmic functions) alternative sigma factor HrpL regulates the transcription of *hrp* (*hypersensitive response and pathogenicity*)-type III secretion system (T3SS) genes by binding to a consensus sequence known as the *hrp* box in *hrp* gene promoters. In turn, the expression of *hrpL* has been proposed to be positively controlled by alternative sigma factor 54 (σ^{54}) (RpoN) and HrpS, a member of the σ^{54} enhancer-binding proteins (EBPs). However, the function of RpoN has not been characterized genetically in *E. amylovora*. In this study, we investigated the role of RpoN, a nitrogen limitation sigma factor, and its modulation protein YhbH, a novel ribosome-associated protein, in *E. amylovora* virulence. Our results showed that mutations in *hrpS*, *hrpL*, *rpoN* and *yhbH*, but not *yfiA* and *rmf3*, resulted in a non-pathogenic phenotype on immature pear fruits and apple shoots. Consistently, the expression of T3SS genes, including *hrpL*, *dspE*, *hrpN* and *hrpA*, was barely detected in *hrpS*, *hrpL*, *rpoN* and *yhbH* mutants. These mutants were also not capable of eliciting a hypersensitive response (HR) on tobacco; however, the overexpression of *hrpL* using an inducible promoter rescued the HR-eliciting abilities of these mutants. These results suggest that a sigma factor cascade exists in the regulatory networks of *E. amylovora* and regulates important virulence factors. On the basis of this study and previously reported data, a model is proposed for the regulation of T3SS in *E. amylovora*.

INTRODUCTION

Fire blight disease of apples and pears has been considered the most destructive disease of pome fruit trees since its discovery in the 1880s (Vanneste, 2000). Its causal agent, *Erwinia amylovora*, is a highly virulent necrogenic vascular pathogen and is closely related to many important mammalian enterobacterial pathogens, such as *Escherichia coli*, *Yersinia pestis* and *Salmonella enterica*. In the past decade or so, extensive genetic and genomic studies have demonstrated that *E. amylovora* utilizes two essential virulence factors, the exopolysaccharide (EPS) amylovoran and a *hypersensitive response and pathogenicity* (*hrp*)-type III secretion system (T3SS), to cause disease (Khan *et al*., 2012; Koczan *et al*., 2009; Wang *et al*., 2009; Zhao and Qi, 2011).

Like many other Gram-negative plant pathogenic bacteria, *E. amylovora* contains an *hrp*-T3SS that delivers effector proteins into host plants. The *hrp*-T3SS gene cluster in *E. amylovora* can be divided into three subregions: the *hrp/hrc* region, the Hrc effector and elicitors (HEE) region and the Hrp-associated enzymes (HAE) region (Oh and Beer, 2005; Oh *et al*., 2005). The *hrp/hrc* region contains 25 genes, including four regulatory genes (*hrpL*, *hrpS* and *hrpXY*) and genes encoding structural components of T3SS. So far, the T3SS of *E. amylovora* has been found to secrete at least 15 virulence-associated proteins, including HrpA, HrpN, DspE, HrpW, HopC1, AvrRpt2 and Eop1 (Bogdanove *et al*., 1998; Nissinen *et al*., 2007; Zhao *et al*., 2005, 2006).

In *E. amylovora*, the transcription of *hrp*-T3SS genes is activated by the master regulator HrpL, a member of the ECF (extracytoplasmic functions) subfamily of sigma factors (Wei and Beer, 1995). HrpL binds to a consensus sequence, known as the *hrp* box (GGAACC–N_{16}–CCACNNA), in *hrp* gene promoters. Most T3SS structural (such as *hrpA*) and effector (such as *hrpN* and *dspE*) genes are subject to direct HrpL regulation (McNally *et al*., 2012; Nissinen *et al*., 2007). The expression of *hrpL* is believed to be activated by both HrpS and a two-component regulatory system: HrpX (sensor) and HrpY (response regulator) (Wei *et al*., 2000). However, recent bioinformatics and genetic studies have suggested that HrpXY may not be involved in the regulation of *hrpL* expression as *hrpXY* mutants remain virulent (Zhao *et al*., 2009b). Furthermore, as HrpS belongs to the NtrC family of sigma 54 (σ^{54}) enhancer-binding proteins (EBPs) and the *hrpL* gene contains a σ^{54} promoter, it has been proposed that σ^{54} (RpoN) might also be involved in the regulation of *hrpL* gene expression (Wei *et al*., 2000). We have also demonstrated that *rpoN* expression is induced during the infection of immature pear fruit tissue (Zhao *et al*., 2005). However, the exact role of RpoN (and also HrpS) has not been characterized genetically in *E. amylovora*.

In bacteria, core RNA polymerase (RNAP) requires sigma factors for promoter recognition to initiate transcription (Ghosh *et al*., 2010; Österberg *et al*., 2011). Sigma factors can be classified into two major families, the sigma 70 (σ^{70}) and σ^{54} families. Most sigma factors belong to the extensive σ^{70} family, including 'housekeeping' sigma factor σ^{70}(RpoD) and alternative sigma factors [σ^{38}(RpoS), σ^{32}(RpoH) and σ^{24}(RpoE)], and direct the binding of

**Correspondence:* Email: zhao888@illinois.edu
†These authors contributed equally to this work.

RNAP to the consensus −10 (TATAAT) and −35 (TTGACA) sequences for transcription initiation (Gruber and Gross, 2003; Österberg *et al*., 2011). In contrast, the σ^{54} family contains only one single member, RpoN(σ^{54}), which is structurally and functionally distinct from all other sigma factors and directs the binding of RNAP to the conserved −12 (TGC) and −24 (GG) promoter elements (Barrios *et al*., 1999). The σ^{54}–RNAP holoenzyme complex forms a closed loop, which is transcriptionally silent, and requires bacterial EBPs, such as HrpS, to open the closed DNA loop and start transcription (Bush and Dixon, 2012; Schumacher *et al*., 2006).

Although initially identified for its role in nitrogen assimilation, RpoN has been found to control many other physiological processes through different EBPs, especially the regulation of *hrp* genes and other virulence factors (Bush and Dixon, 2012; Kazmierczak *et al*., 2005; Shingler, 2011). In *Pseudomonas syringae*, the expression of *hrpL* was completely abolished in an *rpo*N mutant, which was also defective in causing disease (Hendrickson *et al*., 2000a, b). The expression of *Pantoea stewartii hrp* genes was reduced in an *E. coli rpoN* mutant strain (Frederick *et al*., 1993). In *Pectobacterium carotovorum*, studies have shown that the expression of *hrpL* and other T3SS genes is dependent on both RpoN and HrpS (Chatterjee *et al*., 2002). Furthermore, in *P. syringae* and *Pseudomonas aeruginosa*, the *rpoN* mutant has lost its ability to produce the phytotoxin coronatine and EPS alginate, respectively (Alarcón-Chaidez *et al*., 2003; Hendrickson *et al*., 2000a, b, c; Peñaloza-Vázquez *et al*., 2004). However, *Vibrio cholera* and *Vibrio anguillarum rpoN* mutants lack flagella and are nonmotile (Dong and Mekalanos, 2012; O'Toole *et al*., 1997).

On the basis of the genome sequence of *E. amylovora* strain Ea273, *rpoN* (*Eam_3104*) belongs to a large eight-gene operon (from *Eam_3098* to *Eam_3105*, Fig. 1A). The first six genes in the operon (from *yrbG* to *yhbG*) encode for a sodium exchanger protein, an arabinose 5-phosphate isomerase, a phosphatase, two exported proteins and an ABC transporter, respectively (Fig. 1A). The last gene in the operon is *yhbH* (*Eam_3105*), which encodes a small protein with 95 amino acids and has been annotated as a σ^{54} modulation protein (Smits *et al*., 2010). The deduced YhbH protein shares 84% identity with that of *E. coli*. In *E. coli*, YhbH has been characterized as a ribosomal binding protein and recently renamed as hibernation promoting factor (HPF) (Ueta *et al*., 2005). HPF is involved in ribosome stabilization and preservation in the stationary phase by binding specifically to 90S ribosome to form 100S ribosome, which has no translational activity (Ueta *et al*., 2005, 2008). Other ribosome-associated proteins involved in this process include the ribosome modulation factor (RMF) and YfiA. The binding of RMF causes the dimerization of 70S ribosomes into 90S particles, which are then stabilized as 100S dimers on HPF binding, whereas YfiA, which shares 40% homology to HPF and has the same binding sites within the ribosome as HPF, works antagonistically by preventing 90S ribosome formation (Polikanov

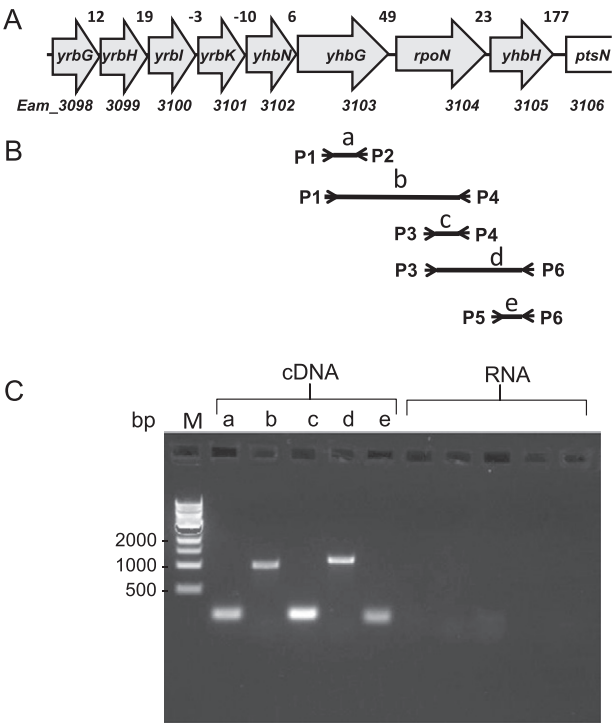

Fig. 1 Operon determination. (A) Schematic maps of *rpoN*, *yhbH* and upstream genes (from *yrbG* to *yhbG*) in the *Erwinia amylovora* genome. The numbers between the arrows represent the bases separating the corresponding genes. Not drawn to scale. (B) Schematic representation of the localization of the primers used to amplify polymerase chain reaction (PCR) products from cDNAs. (C) PCR amplification of cDNAs using primer pairs P1–P2, P1–P4, P3–P4, P3–P6 and P5–P6 for a, b, c, d and e PCR products, respectively. RNA template was used as a negative control with the same primer pairs. M, molecular marker.

et al., 2012; Ueta *et al*., 2005). In addition, YhbH has been shown to be up-regulated by quorum sensing in *E. coli* and during biofilm formation in *Bacillus cereus* (DeLisa *et al*., 2001; Oosthuizen *et al*., 2002). However, no evidence has shown whether YhbH interacts directly with σ^{54} or how YhbH is involved in σ^{54}-mediated gene regulation.

The purpose of this study was to determine the role of RpoN and its modulation protein YhbH in *E. amylovora* virulence. This is the first study involving YhbH in plant pathogenic bacteria, and the results show that YhbH, together with RpoN and HrpS, functions upstream of HrpL to regulate T3SS gene expression and plays a vital role in virulence.

RESULTS

Mutations in *rpoN*, *yhbH*, *hrpL* and *hrpS* render *E. amylovora* nonpathogenic

In order to determine the role of RpoN and YhbH in *E. amylovora* virulence, we generated *rpoN*, *yhbH*, *hrpS* and *hrpL*, as well as *yfiA*

(*Eam_2617*) and *rmf3* (between *Eam_1373* and *Eam_1374* of Ea273; *Eamy_1382* of CFBP1430), mutants by red cloning (Zhao *et al*., 2009a). These mutants were tested for virulence on immature pear fruits and 1-year-old apple shoots. In *E. amylovora*, the YfiA protein has 112 amino acids and was also annotated as σ^{54} modulation protein, whereas the small protein Rmf3 contains 55 amino acids and was not annotated in the genome of Ea273 (but is in CFBP1430). BLAST search using the *rmf3* (*Eamy_1382*) gene sequence identified the *rmf3* gene locus between *Eam_1373* and *Eam_1374* in strain Ea273. The deduced YfiA and Rmf3 proteins are identical in 12 sequenced *E. amylovora* strains (Mann *et al*., 2013), and share 82% and 80% identity, respectively, with those of *E. coli*.

For the wild-type (WT) strain, disease symptoms appeared at the inoculation site 2 days after inoculation, the necrotic lesion turned black with visible ooze formation at 4 days post-inoculation and blackened necrotic areas covered almost the whole pear fruits at 8 days (Fig. 2). For *rpoN*, *yhbH*, *hrpL* and *hrpS* mutant strains, no symptoms were observed (Fig. 2A). However, *yfiA* and *rmf3* mutants caused similar disease to the WT strain (Fig. 2C). Similarly, the WT strain caused typical 'shepherd's crook' symptoms and visible necrosis, and the length of necrosis reached 31.7 ± 3.05 cm at 7 days post-inoculation. No symptoms were observed on apple shoots inoculated with *rpoN*, *yhbH*, *hrpL* or *hrpS* mutants (data not shown).

In order to confirm that the absence of disease symptoms on the four mutants was caused by mutations in *rpoN*, *yhbH*, *hrpL* and *hrpS* genes, we transformed the four mutants with plasmids containing *rpoN*, *yhbH*, *hrpL* and *hrpS* genes, respectively. To complement the *yhbH* mutant, we used the plasmid containing both *rpoN* and *yhbH* genes (Fig. 1). The four complementation strains produced comparable disease progress and symptoms as the WT strain on immature pear fruits (Fig. 2B), and the lengths of necrotic tissue on apple shoots were measured as 23 ± 4.79, 30.69 ± 4.68, 35.75 ± 5.51 and 40.75 ± 4.92 cm for *yhbH*, *hrpL*, *hrpS* and *rpoN* complementation strains, respectively. These results indicate that RpoN, YhbH, HrpL and HrpS are all required for *E. amylovora* to cause disease.

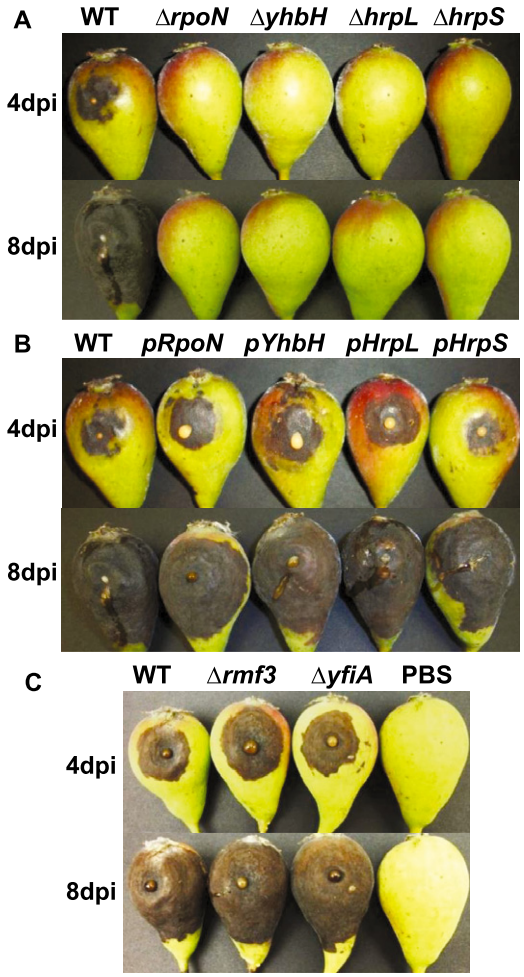

Fig. 2 Virulence assays of *Erwninia amylovora* wild-type (WT) strain, mutants and complementation strains on immature pears. (A) Symptoms caused by WT, *ΔrpoN*, *ΔyhbH*, *ΔhrpL* and *ΔhrpS* mutants on immature pears. (B) Symptoms caused by WT and complementation strains of *ΔrpoN* (pRpoN), *ΔyhbH* (pYhbH), *ΔhrpL* (pHrpL) and *ΔhrpS* (pHrpS) mutants. (C) Symptoms caused by *ΔyfiA* and *Δrmf3*. dpi, days post-inoculation.

Expression of *hrpL* is under the control of RpoN, YhbH and HrpS, but expression of *hrpS* and *rpoN* is independent from each other

Although we predicted that the expression of *rpoN* is under the control of the promoter of an eight-gene operon (from *Eam_3098 to yhbH*) using the operon prediction tool (Okuda *et al*., 2006), it has also been reported that *rpoN* contains its own promoter for independent expression in *E. coli* (Sperandeo *et al*., 2006). Based on our complementation studies described above, we confirmed that *rpoN* indeed contains its own promoter in *E. amylovora*. To further determine whether *rpoN* and *yhbH* were transcribed from both its own promoter and the operon promoter, polymerase chain reaction (PCR) amplifications were performed on cDNA derived from RNA extracted from *E. amylovora* WT. Products having the expected sizes for the combination of primer pairs were amplified from cDNA, but not from RNA, allowing the reconstitution of a continuous 1967-nucleotide-long transcript encompassing *yhbG* to *yhbH* (Fig. 1B,C).

Next, we examined how mutations of *rpoN* and *yhbH* affect *hrpL* and the expression of other T3SS genes using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). As expected, no expression of *rpoN*, *yhbH*, *hrpL* and *hrpS* genes was detected in the corresponding mutants both *in vitro* and *in vivo* (Fig. 3A,B). In *hrp*-inducing medium, T3SS genes, including *dspE*, *hrpL*, *hrpN* and *hrpA*, were barely detected in all four mutants (Fig. 3A). However, the *hrpS* gene was expressed at

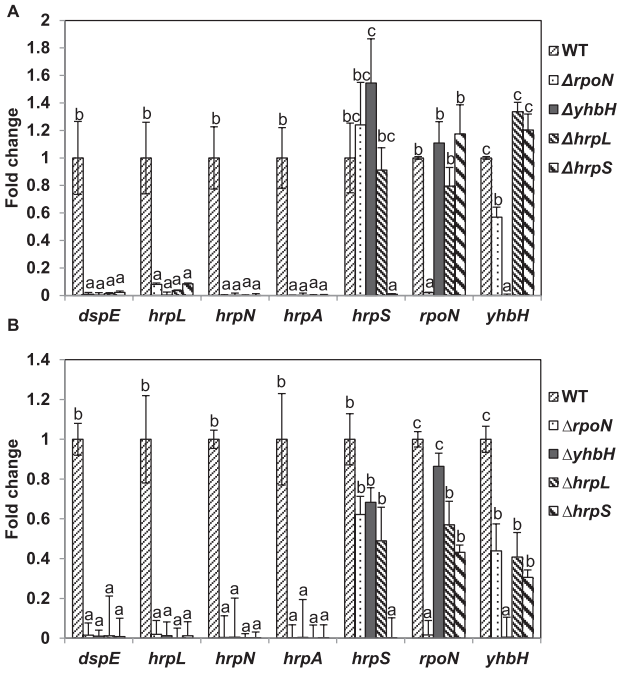

Fig. 3 Expression of selected genes *in vivo* and *in vitro* by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). (A) Relative gene expression of *rpoN*, *yhbH*, *hrpS* and *hrpL* genes and three type III secretion system (T3SS) genes in Δ*rpoN*, Δ*yhbH*, Δ*hrpL* and Δ*hrpS* mutant strains compared with the wild-type (WT) grown in *hrp* (*hypersensitive response and pathogenicity*)-inducing minimal medium at 18 °C for 6 h. (B) Relative expression of *rpoN*, *yhbH*, *hrpS* and *hrpL* genes and three T3SS genes in Δ*rpoN*, Δ*yhbH*, Δ*hrpL* and Δ*hrpS* mutant strains compared with WT inoculated onto immature pear fruits for 6 h. The relative fold change of each gene was derived from the comparison of mutant strains versus WT control. The 16S rDNA (*rrsA*) gene was used as a control. The values of the relative fold change were the means of three replicates. The experiments were repeated at least twice with similar results. Error bars indicate standard deviation. One-way analysis of variance (ANOVA) and Student–Newmans–Keuls test ($P = 0.05$) were used to analyse the qRT-PCR data. For each gene tested in WT and the four mutants, fold changes marked with the same letter do not differ significantly ($P < 0.05$).

a similar level in *rpoN*, *yhbH* and *hrpL* mutants when compared with WT. Similarly, the expression of the *rpoN* gene was not affected in *yhbH*, *hrpL* and *hrpS* mutants. Meanwhile, the expression of *yhbH* was about two-fold lower in the *rpoN* mutant than in WT, but no change was detected in *hrpL* and *hrpS* mutants (Fig. 3A). These results indicate that RpoN, HrpS and YhbH are required for the expression of *hrpL* and T3SS genes, but that the expression of *rpoN* and *hrpS* is independent from each other. Furthermore, it is possible that *yhbH* may also contain its own weak promoter.

Under *in vivo* conditions, T3SS genes, including *dspE*, *hrpL*, *hrpN* and *hrpA*, were also barely detected in all four mutants (Fig. 3B). The expression of *hrpS*, *rpoN* and *yhbH* in the four mutants showed similar trends as described above under *in vitro* conditions, except that the expression of *rpoN* and *yhbH* genes in the *hrpS* mutant was about two-fold lower than that in the WT strain (Fig. 3B), suggesting that HrpS may promote the expression of the *rpoN/yhbH* operon under *in vivo* conditions. Furthermore, the expression of *hrpS*, *rpoN* and *yhbH* was slightly lower in the *hrpL* mutant, suggesting a possible positive feedback loop. These data further demonstrate that RpoN, YhbH and HrpS activate *hrpL* expression, which then activates the expression of other T3SS genes.

Mutations in *rpoN*, *yhbH*, *hrpL* and *hrpS* abolish their abilities to elicit a hypersensitive response (HR)

Next, we tested whether RpoN and YhbH are also required for HR induction in tobacco. As expected, *rpoN*, *yhbH*, *hrpL* and *hrpS* mutants were unable to elicit HR on tobacco; meanwhile, normal tissue collapse was observed for all complementation strains, as well as WT, after 24 h (Fig. 4A). These results demonstrate that HrpL, HrpS, RpoN and YhbH are required to elicit HR on nonhost tobacco.

As suggested by the expression data (Fig. 3), it is obvious that *rpoN*, *hrpS* and *yhbH* regulate *hrpL* and the expression of other T3SS genes. We therefore cloned the *hrpL* gene under the control of an arabinose-inducible promoter, and the resulting plasmid pHrpL-30 was transformed into *rpoN*, *yhbH*, *hrpL* and *hrpS* mutants and tested for HR on tobacco under inducing conditions. As shown in Fig. 4B, all four mutants containing pHrpL-30 showed restored ability to elicit HR in tobacco under inducing conditions, but not without induction. These results demonstrate that RpoN, YhbH and HrpS function upstream of HrpL to regulate *hrp* gene expression.

DISCUSSION

As a breakthrough discovery in modern molecular plant bacteriology, T3SS and effector biology represent the centre stage in the study of the pathogenesis of plant pathogenic bacteria (He *et al.*, 2004). A full understanding of T3SS, such as the structure, gene expression, regulation, effector function, secretion and translocation, will greatly increase our knowledge in elucidating the genetic, molecular and physiological basis for pathogenesis during interactions with eukaryotic hosts (Zhao and Qi, 2011). In this study, we investigated the role of an alternative sigma factor RpoN and, for the first time, a novel ribosome-associated protein YhbH in *E. amylovora* virulence. We demonstrated that RpoN and YhbH, together with HrpS, are all essential for virulence on host plants, HR induction on tobacco and the expression of *hrpL* and other T3SS genes. Therefore, it is apparent that a sigma factor cascade controls T3SS in *E. amylovora* regulatory networks, suggesting that the activation of one sigma factor by another might allow the pathogen to integrate diverse environmental signals or to

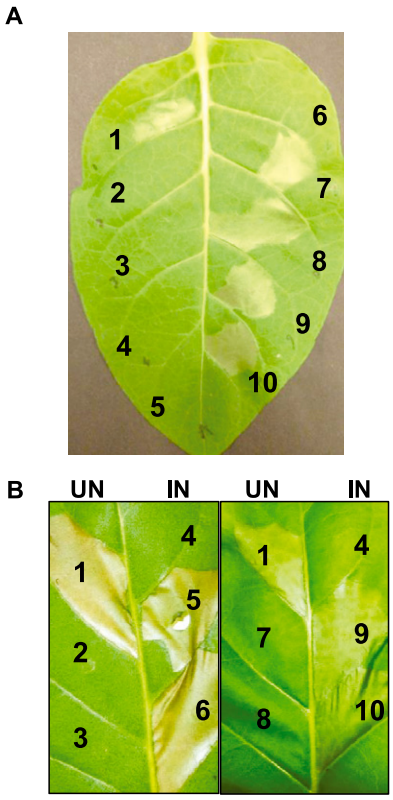

Fig. 4 Hypersensitive response (HR) assay on tobacco leaves. (A) Eight-week-old tobacco leaves were infiltrated with wild-type, mutant strains and complementation strains with cell suspensions at an optical density at 600 nm (OD_{600}) of 0.1:1, Ea1189 WT; 2, *ΔrpoN*; 3, *ΔyhbH*; 4, *ΔhrpL*; 5, *ΔhrpS*; 6, phosphate-buffered saline (PBS); 7, Δ*rpoN*(pRpoN); 8, *ΔyhbH* (pYhbH); 9, Δ*hrpL* (pHrpL); 10, Δ*hrpS* (pHrpS). (B) Eight-week-old tobacco leaves were infiltrated with strains carrying pHrpL-30 driven by an arabinose-inducible promoter: 1, Ea1189 WT; 2 and 5, Δ*rpoN*(pHrpL-30); 3 and 6, Δ*yhbH*(pHrpL-30); 7 and 9, Δ*hrpL*(pHrpL-30); 8 and 10, Δ*hrpS*(pHrpL-30); 4, PBS. UN, uninduced with arabinose; IN, induced with arabinose. Photographs were taken at 24 h post-infiltration. PBS was used as a negative control.

withstand specific stress conditions encountered in the host environment.

Bacterial RNAP holoenzymes are composed of a core enzyme associated with one of a range of sigma factors (Ghosh *et al*., 2010; Österberg *et al*., 2011). Therefore, the central mechanism for the programming of transcription initiation in bacteria is that a pool of sigma factors competes for binding to RNAP to form holoenzymes, leading to the expression of a subset of genes controlled by the specific sigma factor (Österberg *et al*., 2011). It has been found that alternative sigma factor RpoN presents in a diverse phylogeny, including *Pseudomonas*, *Listeria*, *Campylobacter* and many enterobacteria, and controls a wide diversity of cellular processes, including nitrogen metabolism, flagellar motility and pilus-mediated attachment (Kazmierczak *et al*., 2005). Most importantly, RpoN plays an important role in controlling T3SS gene expression and virulence in *Pseudomonas*, *Pantoea* and *Pectobacterium* (Alarcón-Chaidez *et al*., 2003; Chatterjee *et al*., 2002; Hendrickson *et al*., 2000a, b, c; Peñaloza-Vázquez *et al*., 2004). In this study, we have demonstrated that the alternative sigma factor RpoN is essential for the regulation of *hrpL* and other T3SS genes, and thus controls *E. amylovora* virulence.

Unlike the σ^{70} family, binding of σ^{54} to the RNAP holoenzyme confers unique features on the holoenzyme and forms a transcriptionally silent closed loop complex, which could then be activated by EBPs, such as HrpS, which provide energy through ATP hydrolysis under appropriate environmental conditions (Bush and Dixon, 2012; Shingler, 2011). EBPs, also called σ^{54} activators, are members of the functionally versatile AAA+ (ATPases associated with various cellular activities) family of proteins, which are highly regulated in response to environmental cues (Bush and Dixon, 2012; Schumacher *et al*., 2006). Therefore, the evolutionary advantages of σ^{54}-dependent gene regulation through the activation of promoters by EBPs are that transcription is tightly regulated and occurs rapidly and specifically (Bush and Dixon, 2012; Shingler, 2011). As a consequence, σ^{54}-dependent gene expression is often responsible for the creation of swift and precise responses to environmental change and regulates stress response genes, such as T3SS and nitrogen assimilation genes (Jovanovic *et al*., 2011; Schumacher *et al*., 2006).

Some EBPs contain an N-terminal regulatory domain, such as NtrC, which is responsible for the sensing of environmental cues. However, some EBPs lack an N-terminal regulatory domain, such as HrpRS of *Pseudomonas* and HrpS of *Erwinia* (Hutcheson *et al*., 2001; Schumacher *et al*., 2006). In *P. syringae*, HrpR and HrpS form heteromeric complexes that bind to the *hrpL* promoter and stimulate transcription by σ^{54}–RNAP (Grimm *et al*., 1995; Jovanovic *et al*., 2011). HrpS, but not HrpR, is specifically bound by a negative regulator HrpV to prevent HrpRS from activating *hrpL* (Preston *et al*., 1998). HrpG, a chaperone-like protein that acts as an anti-anti-activator, binds to HrpV, preventing its association with HrpS (Stauber *et al*., 2012; Wei *et al*., 2005). In *E. amylovora*, HrpS forms homohexamers to control *hrpL* expression. However, as both HrpR and HrpS lack the regulatory domain, it is assumed that environmental or host signals are not sensed by HrpR or HrpS. Furthermore, HrpXY, which is not present in *P. syringae*, does not control *hrpS* expression and virulence in *E. amylovora* (Wei *et al*., 2000; Zhao *et al*., 2009b). Therefore, how *E. amylovora* and *P. syringae* sense unknown plant or environmental signals to activate the *hrp*-T3SS system is still a mystery.

In this study, we have also discovered that, for the first time, a novel ribosome-associated protein YhbH (HPF), but not YfiA and Rmf3, is critical in mediating the σ^{54}-dependent regulation of *hrp*-T3SS in *E. amylovora*. BLAST search using the National Center for Biotechnology Information (NCBI) database has shown that YhbH presents in a very diverse phylogeny, including proteobacteria, firmicutes and many other bacteria. Initial

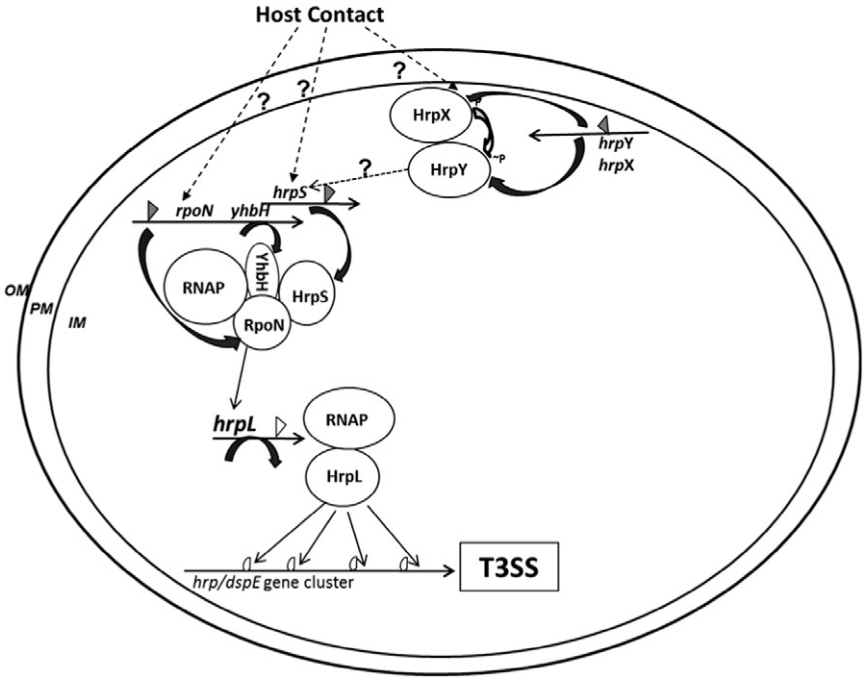

Fig. 5 A model of type III secretion system (T3SS) regulation in *Erwinia amylovora*. HrpL, an ECF (extracytoplasmic functions) sigma factor and master regulator of T3SS; HrpS, a sigma factor 54 (σ^{54})-dependent enhancer binding protein; HrpX/HrpY, two-component regulatory systems; RpoN, a σ^{54} alternative sigma factor; RNAP, RNA polymerase; YhbH, a σ^{54} modulation protein (ribosome-associated protein). OM, outer membrane; PM, plasma membrane; IM, inner membrane; P, phosphorylation; open triangle, σ^{54} promoter; filled triangle, σ^{70} promoter; circle open triangle, *hrp*-box promoter. Positive regulation is indicated by an arrow; '?' and broken line, unknown mechanism.

analyses of sequenced genomes indicated that the RpoN-YhbH locus is conserved among members of Enterobactericeae, such as *E. coli*, *Salmonella*, *Yersinia*, *Shigella*, *Vibrio*, *Pectobacterium*, *Erwinia*, *Pantoea* and *Dickeya*. Proteomic studies have shown that the YhbH protein is differentially expressed during the human macrophage response to *Francisella tularensis* infection (Carlson *et al*., 2007) and during biofilm formation in *Bacillus cereus* (Oosthuizen *et al*., 2002). However, most functional studies on YhbH so far have been limited to *E. coli*, and it has not been shown in any bacterium that YhbH is involved in virulence.

Bacteria usually slow down protein synthesis on entry into the stationary phase by converting ribosomes into translationally inactive 100S dimers or 70S monomers (Polikanov *et al*., 2012). In *E. coli*, 100S dimer formation is mediated by RMF and HPF, leading to 'ribosome hibernation' that aids cell survival, or, alternatively, another stationary phase protein YfiA promotes the formation of translationally inactive monomeric 70S ribosomes, which prevents the recycling of ribosomes for translation initiation (Ueta *et al*., 2005, 2008). In *E. coli*, all three proteins are under the control of RpoS, which is not involved in virulence in *E. amylovora* (Y. F. Zhao, unpublished data). It is believed that T3SS functions early in the infection cycle and that T3SS genes are expressed in actively growing bacteria, and thus it is reasonable to speculate that YhbH might have an alternative function by either interacting with the RpoN–RNAP complex as a σ^{54} modulation protein or promoting HrpS–RpoN interaction. However, the molecular mechanism responsible for why and how YhbH (HPF), but not YfiA and RMF, is involved in the mediation of σ^{54}-dependent *hrp*-T3SS gene expression in *E. amylovora* is still unknown.

On the basis of our results and previously reported data, we propose the following model for the regulation of T3SS gene expression in *E. amylovora* (Fig. 5). RpoN, together with core RNAP, forms a σ^{54}–RNAP complex and binds to the −24 (GG) and −12 (TGC) positions from the HrpL transcription start, and remains transcriptionally silent. Meanwhile, HrpS, a σ^{54}-activating EBP, forms a hexamer and binds to upstream DNA activator sequences (UAS) of *hrpL*. Hexameric HrpS contacts the σ^{54}–RNAP–promoter complex via the consensus GAFTGA motif and the energy provided by ATP hydrolysis of the HrpS AAA+ domain triggers the opening of the σ^{54}–RNAP–promoter complex and DNA melting. In this process, the function of YhbH might also be required to start *hrpL* transcription. HrpL then recognizes a conserved '*hrp* box' at the promoter regions of HrpL-dependent operons or genes, and regulates other T3SS gene expression. However, in this model, how *E. amylovora* senses host or environmental signals or whether signals are sensed through HrpX/HrpY or HrpS/RpoN remain elusive. Future studies should focus on the detection of the signals activating T3SS and how these signals are sensed by the pathogen. The determination of the molecular mechanism of how YhbH is involved in the regulation of *hrpL* and other T3SS genes will also be critical.

EXPERIMENTAL PROCEDURES

Bacterial strain, plasmids and media

The bacterial strains and plasmids utilized in this study are listed in Table 1. Luria–Bertani (LB) broth was used for the routine growth of

Table 1 Bacterial strains and plasmids used in this study.

Strains, plasmids	Relevant characters or sequences (5'–3')*	Reference or source
Strains		
Erwinia amylovora		
Ea1189	Wild-type, isolated from apple	Burse *et al*. (2004)
ΔrpoN	*rpoN::Km;* Km^R-insertional mutant of *rpoN* of Ea1189, Km^R	This study
ΔyhbH	*yhbH::Cm;* Cm^R-insertional mutant of *yhbH* of Ea1189, Cm^R	This study
ΔhrpL	*hrpL::Km;* Km^R-insertional mutant of *hrpL* of Ea1189, Km^R	This study
ΔhrpS	*hrpS::Km;* Km^R-insertional mutant of *hrpS* of Ea1189, Km^R	This study
ΔyfiA (Eam_2617)	*yfiA::Cm*; Cm^R-insertional mutant of *yfiA* of Ea1189, Cm^R	This study
Δrmf3 (Eam_1373-74)	*rmf3::Cm;* Cm^R-insertional mutant of *rmf3* of Ea1189, Cm^R	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*Δ139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ–*rps*L (Str^R) *nup*G	Invitrogen, Carlsbad, CA, USA
Plasmids		
pKD46	Ap^R, P_{BAD} gam bet exo pSC101 oriTS	Datsenko and Wanner (2000)
pKD13	Km^R, FRT cat FRT PS1 PS2 oriR6K rgbN	Datsenko and Wanner (2000)
pKD3	Cm^R, FRT *cat* FRT PS1 PS2 oriR6K *rgbN*	Datsenko and Wanner (2000)
pWSK29	Ap^R; cloning vector, low copy number	Wang and Kushner (1991)
pBad30	Ap^R, arabinose-inducing promoter, cloning vector	Thao *et al*. (2010)
pRpoN	1.8-kb PCR fragment containing *rpoN* gene in pWSK29	This study
pYhbH	2.265-kb PCR fragment containing both *rpoN* and *yhbH* genes in pWSK29	This study
pHrpL	1.317-kb DNA fragment containing *hrpL* gene in pWSK29 vector	This study
pHrpS	1.81-kb DNA fragment containing *hrpS* gene in pWSK29 vector	This study
pHrpL-30	579-bp DNA fragment containing 30 nucleotides 5′ of the start codon of *hrpL* gene in pBAD30	This study

*Km^R, Cm^R, Ap^R, kanamycin, chloramphenicol and ampicillin resistance, respectively.

E. amylovora and *E. coli* strains. An *hrp*-inducing minimal medium (HMM) [1 g $(NH_4)_2SO_4$, 0.246 g $MgCl_2.6H_2O$, 0.099 g NaCl, 8.708 g K_2HPO_4, 6.804 g KH_2PO_4] containing 10 mM galactose was used to induce T3SS gene expression (Wei *et al*., 1992). When required, antibiotics were used at the following concentrations: 20 μg/mL kanamycin (Km), 100 μg/mL ampicillin (Ap) and 10 μg/mL chloramphenicol (Cm). The oligonucleotide primers used for mutant construction, mutant confirmation, qRT-PCR and cloning in this study are listed in Table S1 (see Supporting Information).

DNA manipulation and the construction of plasmids

Plasmid DNA purification, PCR amplification of genes, isolation of fragments from agarose gels, cloning, restriction enzyme digestion and T4 DNA ligation were performed using standard molecular procedures (Sambrook and Russel, 2001).

Construction of mutants in *E. amylovora* by Lamda-Red recombinase cloning

E. amylovora mutant strains were generated using the λ phage recombinase method, as described previously (Datsenko and Wanner, 2000; Zhao *et al*. 2009a, b). Briefly, overnight cultures of *E. amylovora* strains harbouring pKD46 were inoculated in LB broth containing 0.1% arabinose and grown to exponential phase [optical density at 600 nm (OD_{600}) = 0.8]. Cells were harvested, made electrocompetent and stored at −80 °C. Recombination fragments consisting of a kanamycin (Km^r) or chloramphenicol (Cm^r) resistance gene with its own promoter, flanked by a 50-nucleotide homology arm, were generated by PCR from pKD13 and pKD32 plasmids, respectively. To confirm the deletion mutations, PCR amplifications from internal resistance gene primers to the external region of the target genes were performed (Table S1). For the resulting mutants, the majority of the coding region of each gene was replaced by the marker gene, except for the first and last 50 nucleotides.

Cloning of genes for complementation

For the complementation of the mutants, the regulatory and gene sequences of *hrpL*, *hrpS*, *rpoN* and *yhbH* genes were used to design primers to amplify fragments of the gene and their promoter sequences (Table S1). The PCR fragments were cloned into low-copy-number vector pWSK29 with *Kpn*I–*Sac*I restriction sites. The final plasmids were designated pHrpL, pHrpS, pRpoN and pYhbH, respectively.

The primer pair hrpL-30F/hrpL-30R containing *EcoR*I and *Xba*I restriction sites was used to amplify the 579-bp promoterless in-frame *hrpL* gene, which was then cloned into pBAD30 with an arabinose-inducing promoter. Following amplification, DNA fragments and the vector were both digested by *EcoR*I and *Xba*I, and ligated together. The final plasmid was designated as pHrpL-30. All plasmids were introduced into the *E. amylovora* strain by electroporation. Transformants were selected on LB plates supplemented with Km and Ap. Their genotypes were confirmed by both enzymatic digestion and sequencing.

Virulence assays on apple shoots and immature pear fruits

Virulence assays on apple shoots were performed as described previously (Wang *et al*., 2010). Briefly, overnight cultures of *E. amylovora* WT and mutant strains were harvested by centrifugation and suspended in 0.5 × phosphate-buffered saline (PBS). Cell suspensions were adjusted to OD_{600} = 0.1 in PBS and inoculated onto seven actively growing 'Gala' apple shoots by pricking the tip with a sterile needle. Five microlitres of bacterial suspension were added to the wounding site by pipetting.

Symptom development (progression of necrosis) was recorded at 7 days post-inoculation and the length of necrotic tissue was measured. The experiment was performed at least twice.

Immature fruits of pear (*Pyrus communis* L. cv. Bartlett) were surface sterilized and pricked with a sterile needle (Zhao *et al*., 2005, 2006). Two microlitres of cell suspension (OD_{600} = 0.1, 100 × dilution) were added to the wounded tissue and the pears were incubated in a humidified chamber at 28 °C. Symptoms were recorded at 4 and 8 days post-inoculation. For each strain tested, fruits were assayed in triplicate, and each experiment was performed three times.

HR assay on tobacco

Overnight cultures of *E. amylovora* WT and mutant strains were harvested by centrifugation and cells were resuspended in PBS to OD_{600} = 0.1. Bacterial suspension was infiltrated into tobacco leaves (*Nicotiana tabacum*) by needleless syringe. Infiltrated plants were kept in a humid growth chamber and HR symptoms were recorded at 24 h post-infiltration. For inducible-*hrpL* complementation HR assay, arabinose was added to the bacterial suspension to a final concentration of 0.1% immediately before infiltration. All experiments were repeated three times.

RNA isolation

Bacterial strains grown overnight in LB medium with appropriate antibiotics were harvested by centrifugation and washed twice with PBS before inoculating into 5 mL HMM medium at 18 °C. After 6 h in HMM medium, 2 mL of RNA protect reagent (Qiagen, Hilden, Germany) were added to 1 mL of bacterial cell cultures. For *in vivo* conditions, overnight bacterial cultures were harvested by centrifugation, resuspended in PBS and adjusted to an OD_{600} of 0.2–0.3. Immature pear fruits were cut in half and inoculated with bacterial suspension. After 6 h at 28 °C in a moist chamber, bacterial cells were collected by washing pear surfaces with RNA protect reagent (Qiagen), 2:1 with water, as described previously (Wang *et al*., 2012). Cells were harvested by centrifugation for 10 min at 4000 ***g*** and RNA was extracted using a Qiagen Bacterial RNA Protect Mini Kit, as recommended by the manufacturer (Qiagen). On-column DNA digestion was performed using DNase I. RNA was quantified using a Nano-Drop ND-100 spectrophotometer (Nano-Drop Technologies; Wilmington, DE, USA), and RNA quality was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

qRT-PCR

One microgram of total RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. qRT-PCR was performed using an ABI 7300 System (Applied Biosystems, Foster City, CA, USA). Power SYBR® Green PCR master mix (Applied Biosystems) was used to detect the expression of selected genes with primers designed using Primer3 software. One microlitre of cDNA (2 ng/reaction) or water (no-template control) was used as template for qRT-PCRs with Fast SYBR Green PCR Master Mix (Applied Biosystems) and primers at a final concentration of 500 nM. qRT-PCR amplifications were carried out at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and a final dissociation curve analysis step from 65 to 95 °C. Technical replicate experiments were performed for each biological triplicate sample. Gene expression levels were analysed using the relative quantification (ΔΔCt) method. A 16S rDNA (*rrsA*) gene was used as a control. A relative quantification value was calculated for each gene with the control group as a reference. A *P* value was computed using a moderated *t*-test to measure the significance associated with each relative quantification value. Variations were considered to be statistically significant when $P < 0.05$.

ACKNOWLEDGEMENTS

This project was supported by the Agriculture and Food Research Initiative Competitive Grants Program grant no. 2010-65110-20497 from the USDA National Institute of Food and Agriculture (YFZ). We would like to thank Dr P. Lawrence Pusey at the USDA Agricultural Research Service, Wenatchee, WA, USA for providing the immature pear fruits used in this study. The authors have no conflicts of interest to declare.

REFERENCES

Alarcón-Chaidez, F.J., Keith, L., Zhao, Y.F. and Bender, C.L. (2003) RpoN (σ54) is required for plasmid-encoded coronatine biosynthesis in *Pseudomonas syringae*. *Plasmid*. **49**, 106–117.

Barrios, H., Valderrama, B. and Morett, E. (1999) Compilation and analysis of σ54-dependent promoter sequences. *Nucleic Acids Res.* **27**, 4305–4313.

Bogdanove, A.J., Kim, J.F., We, Z.M., Kolchinsky, I., Charkowski, A.O., Conlin, A.K., Collmer, A. and Beer, S.V. (1998) Homology and functional similarity of an *hrp*-linked pathogenicity locus, *dspEF*, of *Erwinia amylovora* and the avirulence locus *avrE* of *Pseudomonas syringae* pathovar tomato. *Proc. Natl. Acad. Sci. USA*. **95**, 1325–1330.

Burse, A., Weingart, H. and Ullrich, M.S. (2004) NorM, an *Erwinia amylovora* multidrug efflux pump involved in *in vitro* competition with other epiphytic bacteria. *Appl. Environ. Microbiol*. **70**, 693–703.

Bush, M. and Dixon, R. (2012) The role of bacterial enhancer binding proteins as specialized activators of σ^{54}-dependent transcription. *Microbiol. Mol. Biol. Rev.* **76**, 497–529.

Carlson, P.E. Jr, Carroll, J.A., O'Dee, D.M. and Nau, G.J. (2007) Modulation of virulence factors in *Francisella tularensis* determines human macrophage responses. *Microb. Pathog.* **42**, 204–214.

Chatterjee, A., Cui, Y. and Chatterjee, A.K. (2002) Regulation of *Erwinia carotovora hrpL*, which encodes an extracytoplasmic function subfamily of sigma factors required for expression of the HRP regulon. *Mol. Plant–Microbe Interact.* **15**, 971–980.

Datsenko, K.A. and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA*. **97**, 6640–6645.

DeLisa, M.P., Wu, C.F., Wang, L., Valdes, J.J. and Bentley, W.E. (2001) DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in *Escherichia coli*. *J. Bacteriol.* **183**, 5239–5247.

Dong, T.G. and Mekalanos, J.J. (2012) Characterization of the RpoN regulon reveals differential regulation of T6SS and new flagellar operons in *Vibrio cholerae* O37 strain V52. *Nucleic Acids Res.* **40**, 7766–7775.

Frederick, R.D., Majerczak, D.R. and Coplin, D.L. (1993) *Erwinia stewartii* WtsA, a positive regulator of pathogenicity gene expression, is similar to *Pseudomonas syringae* pv. *phaseolicola* HrpS. *Mol. Microbiol*. **9**, 477–485.

Ghosh, T., Bose, D. and Zhang, X. (2010) Mechanisms for activating bacterial RNA polymerase. *FEMS Microbiol. Rev.* **34**, 611–627.

Grimm, C., Aufsatz, W. and Panopoulos, N.J. (1995) The HrpRS locus of *Pseudomonas syringae pv. phaseolicola* constitutes a complex regulatory unit. *Mol. Microbiol*. **15**, 155–165.

Gruber, T.M. and Gross, C.A. (2003) Multiple sigma subunits and the partitioning of bacterial transcription space. *Annu. Rev. Microbiol.* **57**, 441–466.

He, S.Y., Nomura, K. and Whittam, T.S. (2004) Type III protein secretion mechanism in mammalian and plant pathogens. *Biochim. Biophys. Acta.* **1694**, 181–206.

Hendrickson, E.L., Guevera, P. and Ausubel, F.M. (2000a) The alternative sigma factor RpoN is required for hrp activity in *Pseudomonas syringae pv. maculicola* and acts at the level of *hrpL* transcription. *J. Bacteriol.* **182**, 3508–3516.

Hendrickson, E.L., Guevera, P., Peñaloza-Vàzquez, A., Shao, J., Bender, C. and Ausubel, F.M. (2000b) Virulence of the phytopathogen *Pseudomonas syringae pv. maculicola* is RpoN dependent. *J. Bacteriol.* **182**, 3498–3507.

Hendrickson, E.L., Plotnikova, J., Mahajan-Miklos, S., Rahme, L.G. and Ausubel, F.M. (2000c) Differential roles of the *Pseudomonas aeruginosa* PA14 rpoN gene in pathogenicity in plants, nematodes, insects, and mice. *J. Bacteriol.* **183**, 7126–7134.

Hutcheson, S.W., Bretz, J., Sussan, T., Jin, S. and Pak, K. (2001) Enhancer-binding proteins HrpR and HrpS interact to regulate *hrp*-encoded type III protein secretion in *Pseudomonas syringae* strains. *J. Bacteriol.* **183**, 5589–5598.

Jovanovic, M., James, E.H., Burrows, P.C., Rego, F.G., Buck, M. and Schumacher, J. (2011) Regulation of the co-evolved HrpR and HrpS AAA+ proteins required for *Pseudomonas syringae* pathogenicity. *Nat. Commun.* **2**, 177.

Kazmierczak, M.J., Wiedmann, M. and Boor, K.J. (2005) Alternative sigma factors and their roles in bacterial virulence. *Microbiol. Mol. Biol. Rev.* **69**, 527–543.

Khan, M.A., Zhao, Y.F. and Korban, S.S. (2012) Molecular mechanisms of pathogenesis and resistance to the bacterial pathogen *Erwinia amylovora*, causal agent of fire blight disease in Rosaceae. *Plant Mol. Biol. Rep.* **30**, 247–260.

Koczan, J.M., McGrath, M.J., Zhao, Y. and Sundin, G.W. (2009) Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: implications in pathogenicity. *Phytopathology.* **99**, 1237–1244.

Mann, R.A., Smits, T.H.M., Buhlmann, A., Blom, J., Goesmann, A., Frey, J.E., Plummer, K.M., Beer, S.V., Luck, J.E., Duffy, B. and Rodoni, B. (2013) Comparative genomics of 12 strains of *Erwinia amylovora* identifies a pan-genome with a large conserved core. *PLoS ONE.* **8**, e55644.

McNally, R.R., Toth, I.K., Cock, P.J., Pritchard, L., Hedley, P.E., Morris, J.A., Zhao, Y.F. and Sundin, G.W. (2012) Genetic characterization of the HrpL regulon of the fire blight pathogen *Erwinia amylovora* reveals novel virulence factors. *Mol. Plant Pathol.* **13**, 160–173.

Nissinen, R.M., Ytterberg, A.J., Bogdanove, A.J., van Wijk, K. and Beer, S.V. (2007) Analyses of the secretomes of *Erwinia amylovora* and selected *hrp* mutants reveal novel type III secreted proteins and an effect of HrpJ on extracellular harpin levels. *Mol. Plant Pathol.* **8**, 55–67.

O'Toole, R., Milton, D.L., Hörstedt, P. and Wolf-Watz, H. (1997) RpoN of the fish pathogen *Vibrio* (*Listonella*) *anguillarum* is essential for flagellum production and virulence by the water-borne but not intraperitoneal route of inoculation. *Microbiology.* **143**, 3849–3859.

Oh, C.-S. and Beer, S.V. (2005) Molecular genetics of *Erwinia amylovora* involved in the development of fire blight. *FEMS Microbiol. Lett.* **253**, 185–192.

Oh, C.S., Kim, J.F. and Beer, S.V. (2005) The Hrp pathogenicity island of *Erwinia amylovora* and identification of three novel genes required for systemic infection. *Mol. Plant Pathol.* **6**, 125–138.

Okuda, S., Katayama, T., Kawashima, S., Goto, S. and Knehisa, M. (2006) ODB: a database of operons accumulating known operons across multiple genomes. *Nucleic Acids Res.* **34**, D358–D362.

Oosthuizen, M.C., Steyn, B., Theron, J., Cosette, P., Lindsay, D., Holy, A. and Brözel, V.S. (2002) Proteomic analysis reveals differential protein expression by *Bacillus cereus* during biofilm formation. *Appl. Environ. Microbiol.* **68**, 2770–2780.

Österberg, S., del Peso-Santos, T. and Shingler, V. (2011) Regulation of alternative sigma factor use. *Annu. Rev. Microbiol.* **65**, 37–55.

Peñaloza-Vázquez, A., Fakhr, M.K., Bailey, A.M. and Bender, C.L. (2004) AlgR functions in algC expression and virulence in *Pseudomonas syringae pv. syringae*. *Microbiology.* **150**, 2727–2737.

Polikanov, Y.S., Blaha, G.M. and Steitz, T.A. (2012) How hibernation factors RMF, HPF, and YfiA turn off protein synthesis. *Science.* **336**, 915–918.

Preston, G., Deng, W.L., Huang, H.C. and Collmer, A. (1998) Negative regulation of *hrp* genes in *Pseudomonas syringae* by HrpV. *J. Bacteriol.* **180**, 4532–4537.

Sambrook, J. and Russel, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schumacher, J., Joly, N., Rappas, M., Zhang, X. and Buck, M. (2006) Structures and organisation of AAA+ enhancer binding proteins in transcriptional activation. *J. Struct. Biol.* **156**, 190–199.

Shingler, V. (2011) Signal sensory systems that impact σ54-dependent transcription. *FEMS Microbiol. Rev.* **35**, 425–440.

Smits, T.H.M., Rezzonico, F., Kamber, T., Blom, J., Goesmann, A., Frey, J.E. and Duffy, B. (2010) Complete genome sequence of the fire blight pathogen *Erwinia amylovora* CFBP 1430 and comparison to other *Erwinia* spp. *Mol. Plant–Microbe Interact.* **23**, 384–393.

Sperandeo, P., Pozzi, C., Deho, G. and Polissi, A. (2006) Non-essential KDO biosynthesis and new essential cell envelope biogenesis genes in the *Escherichia coli yrbG-yhbG* locus. *Res. Microbiol.* **157**, 547–558.

Stauber, J.L., Loginicheva, E. and Schechter, L.M. (2012) Carbon source and cell density-dependent regulation of type III secretion system gene expression in *Pseudomonas syringae* pathovar *tomato* DC3000. *Res. Microbiol.* **163**, 531–539.

Thao, S., Chen, C.S., Zhu, H. and Escalante-Semerena, J.C. (2010) N^{ε}-lysine acetylation of a bacterial transcription factor inhibits its DNA-binding activity. *PLoS ONE.* **5**, e15123.

Ueta, M., Yoshida, H., Wada, C., Baba, T., Mori, H. and Wada, A. (2005) Ribosome binding proteins YhbH and YfiA have opposite functions during 100S formation in the stationary phase of *Escherichia coli*. *Genes Cells.* **10**, 1103–1112.

Ueta, M., Ohniwa, R.L., Yoshida, H., Maki, Y., Wada, C. and Wada, A. (2008) Role of HPF (hibernation promoting factor) in translational activity in *Escherichia coli*. *J. Biochem.* **143**, 425–433.

Vanneste, J. (2000) *Fire Blight: The Disease and Its Causative Agent,* Erwinia Amylovora. Wallingford, Oxfordshire: CABI Publishing.

Wang, D., Korban, S.S. and Zhao, Y.F. (2009) The Rcs phosphorelay system is essential for pathogenicity in *Erwinia amylovora*. *Mol. Plant Pathol.* **10**, 277–290.

Wang, D., Korban, S.S. and Zhao, Y.F. (2010) Molecular signature of differential virulence in natural isolates of *Erwinia amylovora*. *Phytopathology.* **100**, 192–198.

Wang, D., Qi, M., Calla, B., Korban, S.S., Clough, S.J., Cock, P.J., Sundin, G.W., Toth, I. and Zhao, Y. (2012) Genome-wide identification of genes regulated by the Rcs phosphorelay system in *Erwinia amylovora*. *Mol. Plant–Microbe Interact.* **25**, 6–17.

Wang, R.F. and Kushner, S.R. (1991) Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene.* **100**, 195–199.

Wei, C.F., Deng, W.L. and Huang, H.C. (2005) A chaperone-like HrpG protein acts as a suppressor of HrpV in regulation of the *Pseudomonas syringae pv. syringae* type III secretion system. *Mol. Microbiol.* **57**, 520–536.

Wei, Z., Kim, J.F. and Beer, S.V. (2000) Regulation of hrp genes and type III protein secretion in *Erwinia amylovora* by HrpX/HrpY, a novel two-component system, and HrpS. *Mol. Plant–Microbe Interact.* **13**, 1251–1262.

Wei, Z.M. and Beer, S.V. (1995) *hrpL* activates *Erwinia amylovora hrp* gene transcription and is a member of the ECF subfamily of sigma factors. *J. Bacteriol.* **177**, 6201–6210.

Wei, Z.M., Sneath, B.J. and Beer, S.V. (1992) Expression of *Erwinia amylovora hrp* genes in response to environmental stimuli. *J. Bacteriol.* **174**, 1875–1882.

Zhao, Y.F. and Qi, M.S. (2011) Comparative genomics of *Erwinia amylovora* and related *Erwinia* species – what do we learn? *Genes.* **2**, 627–639.

Zhao, Y.F., Blumer, S.E. and Sundin, G.W. (2005) Identification of *Erwinia amylovora* genes induced during infection of immature pear tissue. *J. Bacteriol.* **187**, 8088–8103.

Zhao, Y.F., He, S.Y. and Sundin, G.W. (2006) The *Erwinia amylovora avrRpt2*$_{EA}$ gene contributes to virulence on pear and AvrRpt2$_{EA}$ is recognized by *Arabidopsis* RPS2 when expressed in *Pseudomonas syringae*. *Mol. Plant–Microbe Interact.* **19**, 644–654.

Zhao, Y.F., Sundin, G.W. and Wang, D. (2009a) Construction and analysis of pathogenicity island deletion mutants of *Erwinia amylovora*. *Can. J. Microbiol.* **55**, 457–464.

Zhao, Y.F., Wang, D., Nakka, S., Sundin, G.W. and Korban, S.S. (2009b) Systems level analysis of two-component signal transduction systems in *Erwinia amylovora*: role in virulence, regulation of amylovoran biosynthesis and swarming motility. *BMC Genomics.* **10**, 245.

SUPPORTING INFORMATION

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

Table S1 Primers used in this study.