INTRODUCTION

As one of the top 10 bacterial plant pathogens (Mansfield et al., 2012), *Erwinia amylovora*, the causal agent of fire blight of apple, pear, quince, blackberry, raspberry and other rosaceous plants, is of particular interest to plant pathologists because of its significant economic and political impact (Griffith et al., 2003; van der Zwet et al., 2012; Vanneste, 2000; Zhao, 2014). Identification and analysis of critical virulence traits laid the groundwork for our understanding of host-pathogen interactions in this system (Bugert and Geider, 1995; Gaudriault et al., 1997; Kim and Beer, 2000; Zhao et al., 2005; Koczan et al., 2009). Complete and draft genome sequences for more than a dozen strains of *E. amylovora* and related species have provided complete genetic information about the pathogen, and thus drastically increased our knowledge base and provided further resources to study its pathogenesis (Kube et al., 2008, 2010; Mann et al., 2013; Park et al., 2011; Powney et al., 2011; Sebaihia et al., 2010; Smits et al., 2010a, 2010b, 2013; Zhao and Qi, 2011). Additional information detailing plant physiological and genetic responses to *E. amylovora* infection as well as more recent genetic studies on plant resistance to fire blight infection have also appeared (Broggini et al., 2014; Emeriewen et al., 2015; Iakimova et al., 2013; Kamber et al., 2016; Vogt et al., 2013). Also, readers are referred to recent syntheses on fire blight and *E. amylovora* (Malnøy et al., 2012; Smits et al., 2017; Zhao, 2014).

Fire blight disease and geographic distribution. Fire blight disease is indigenous to North America. Historically, a severe outbreak of fire blight in Illinois led to the discovery of the first bacterial disease of plants by Thomas J. Burrill in the summer of 1880 (Griffith et al., 2003). The reported global spread of fire blight was from the United States to New Zealand in 1919, to the United Kingdom and Northern Europe in the late 1950s, and to the Mediterranean Region and Northern Africa in the 1960s. Since its discovery, fire blight has spread to more than 50 countries (Van der Zwet et al., 2012; EPPO, 2016). Fire blight is now widespread within North America, New Zealand, Europe, Northern Africa, and the Middle East, and has

SUMMARY

The hypersensitive response and pathogenicity (*brp*)-type III secretion system (T3SS) is a key pathogenicity factor in *Erwinia amylovora*, the causal agent of fire blight disease, which has recently reached Central/East Asia, the native origin of apple germplasm resources. It is well understood that the *brp*-T3SS in *E. amylovora* is transcriptionally regulated by the master regulator HrpL, an ECF alternative sigma factor. Recent genetic and biochemical evidence has further demonstrated that transcription of *hrp* is positively regulated by alternative sigma factor 54 (RpoN), its modulation protein (YhbH), a σ54-enhancer binding protein (HrpS), and integration host factor (IHF). However, the bacterial/host signals that activate the sigma factor cascade remain elusive. Biofilm formation also plays an important role in *E. amylovora* virulence and is the main contributing factor to population growth in xylem. Three exopolysaccharides, amylovoran, levan, and cellulose, are required for biofilm formation, and amylovoran and levan also contribute to virulence in other aspects of plant infection. In this short review, we summarized recent findings on the regulation of the T3SS and biofilm formation in *E. amylovora* by bacterial secondary messengers, including both linear and cyclic nucleotides. As an internal “switch”, the linear nucleotide second messengers (pppGpp mediates a stringent response under nutrient stress, which then activates RpoN-dependent regulation of the *hrpL* gene. Furthermore, cyclic di-GMP has been elucidated as a positive regulator of both amylovoran and cellulose biosynthesis and as a negative regulator of the T3SS. We present models for the T3SS and biofilm regulatory pathways in *E. amylovora* and discuss potential future research directions.

Keywords: fire blight, type three secretion, biofilm formation, virulence.

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also reached Russia, Central/East Asia, the native origin of apple germplasm resources, including Kyrgyzstan, Kazakhstan and South Korea in 2015 (Myung et al., 2016). Interestingly, fire blight has not been reported in Australia (reported, but eradicated, Jock et al., 2000), China, South Africa and any country in South America so far.

Type III secretion system and current regulation model. *E. amylovora* is a highly virulent necrogenic vascular pathogen. Over the past several decades, genetic studies have extensively explored the molecular mechanism of *E. amylovora* pathogenesis (Khan et al., 2012; Zhao and Qi, 2011; Zhao, 2014). Identification of an essential virulence system, namely the hypersensitive response and pathogenicity (hrp)-type III secretion system (T3SS) is the highlight of those efforts (Oh and Beer, 2005; Oh et al., 2005). Since the discovery of the first cell free elicitor (HrpN, harpin) in *E. amylovora* in 1992 (Wei et al., 1992), *E. amylovora* has become a model pathogen for studying plant-microbe interactions (Malnoy et al., 2012; Zhao, 2014). The current consensus is that two virulence factors, i.e., the T3SS and its effectors, and the acidic exopolysaccharide (EPS) amylovoran, are strictly required for *E. amylovora* to cause disease (Zhao, 2014). In this review, we will only focus on regulation of the T3SS and biofilm formation. However, readers are referred to recent reviews detailing the function of effectors and regulation of amylovoran biosynthesis (Malnoy et al., 2012; McNally et al., 2015; Zhao, 2014).

In *E. amylovora*, the *hrp* gene cluster can be divided into three sub-regions: 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). Within the *hrp/hrc* region, four regulatory genes (*hrpL, hrpS* and *hrpXY*) control the expression of genes encoding structural components of the T3SS and effectors. It is well documented that in *E. amylovora*, transcription of the *hrp*-T3SS genes is activated by the master regulator HrpL, a member of the ECF 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. Therefore, most T3SS and effector genes are subject to direct HrpL regulation (McNally et al., 2012; Nissinen et al., 2007). A Hidden Markov model has identified about 30 *hrp* promoters in the genome of *E. amylovora* strain Ea273 (McNally et al., 2012).

It has been suggested that the expression of *hrpL* is activated by both HrpS and a two-component regulatory system HrpX (sensor) and HrpY (response regulator) (Wei et al., 2000). However, new evidence indicated that the role of HrpX/HrpY in regulation of the T3SS is still unclear as the *hrpX/hrpY* mutants remain virulent (Zhao et al., 2009b). It should be noted that domain structure analysis indicated that HrpX histidine kinase is a soluble cytoplasmic protein that may sense intracellular signals. Although an early report suggested that HrpS, a member of the NtrC family of $\sigma^{54}$ enhancer-binding proteins, only partially controls *hrpL* gene expression (Wei and Beer, 1995), recent genetic and biochemical evidence indicated that HrpS, along with alternative sigma factor RpoN ($\sigma^{54}$) and its modulation protein YhbH, are absolutely required for *hrpL* gene expression (Ancona et al., 2014; Lee and Zhao, 2016). The interaction between HrpS and RpoN also requires the function of the integration host factor (IHF), a nucleoid-associated protein, in *E. amylovora* (Lee et al., 2016). The *hrpL* gene contains a $\sigma^{54}$ consensus sequence in its promoter region, and bioinformatic analysis and electrophoretic mobility shift assays further determined the binding sites of HrpS and IHF on the *hrpL* promoter (Lee and Zhao, 2016; Lee et al., 2016). A 14 bp-dyad symmetry sequence (TGCAA-N$_{4}$-TTGCA) in the *hrpL* promoter is the upstream DNA activation site (UAS) for HrpS binding, which is conserved among enterobacterial plant pathogens (Lee et al., 2016).

Although in prokaryotes gene expression is primarily regulated at the level of transcription initiation, regulation at post-transcriptional and posttranslational levels also plays a pivotal role. Global regulators such as the GrrS/GrrA/GacS/GacA and EnvZ/OmpR two-component systems have been shown to negatively co-regulate T3SS gene expression (Li et al., 2014). It has only recently been revealed in *E. amylovora* that the GrrS/GrrA system negatively regulates the T3SS through positively controlling the expression of the non-coding small regulatory RNA csrB/rsmb, which acts as a “spoon” to antagonize the positive effect of the RNA-binding protein CsrA/RsmA on T3SS at the posttranscriptional level (Ancona et al., 2016; Lee and Zhao, 2016). These findings demonstrated that CsrA/RsmA plays a critical role in *E. amylovora* virulence (Ancona et al., 2016). In addition, the stabilities of HrpS and needle structural protein HrpA were found to be controlled by the Lon protease at the posttranslational level (Lee et al., 2017). These new findings suggest that the T3SS in *E. amylovora* is regulated at multiple levels.

Here we present a model for *E. amylovora* T3SS gene expression based on current knowledge (Fig. 1). Upon initiating plant infection, *E. amylovora* senses unknown host/environmental signals. This process activates a sigma factor cascade that regulates T3SS gene expression and at the same time, several two-component signal transduction systems regulate T3SS (Li et al., 2014). In the sigma factor cascade, HrpS forms a hexamer and binds to the UAS of the *hrpL* promoter. Meanwhile, RpoN and a core RNA polymerase (RNAP) form a $\sigma^{54}$-RNAP complex that binds to the $\sigma^{54}$ promoter of the *hrpL* gene, but remains transcriptionally silent. With the assistance of IHF, HrpS contacts the $\sigma^{54}$-RNAP-promoter complex via the consensus GAFTGA motif and by DNA looping, with the energy provided by ATP hydrolysis of the HrpS AAA + domain. This triggers the opening of the $\sigma^{54}$-RNAP-promoter complex and DNA melting. This process also requires a ribosome-associated protein YhbH with an unknown...
mechanism (Ancona et al., 2014; Lee et al., 2016). The HrpL/RNAP complex then recognizes the “hrp box” at the promoter regions of HrpL-dependent operons or genes (McNally et al., 2012). On the other hand, the GrrS/GrrA system along with IHF specifically regulates csrB/ rsmB, which stabilizes CsrA/RsmA to regulate the T3SS (Ancona et al., 2016; Lee and Zhao, 2016; Li et al., 2014). The most challenging questions remaining are (i) what are the target(s) of CsrA for positively regulating the T3SS, (ii) what are the environmental signals that regulate T3SS expression and (iii) how does the bacterium sense those signals to activate the T3SS through the sigma factor cascade?

BACTERIAL NUCLEOTIDE SECOND MESSENGERS

Nucleotide secondary messengers, including c-di-GMP, c-di-AMP, cGMP, cAMP, c-GMP-AMP (cGAMP), and (p)ppGpp, represent major regulatory players in signal transduction mechanisms in prokaryotes. These cyclic and linear nucleotides control diverse cellular processes in response to environmental cues (Gomelsky, 2011; Kalia et al., 2012; Potrykus and Cashel, 2008; Pesavento and Hengge, 2009). In bacteria, cAMP, c-di-GMP and (p)ppGpp are among the most comprehensively studied nucleotide-based second messengers. Cyclic di-GMP, in particular, has attracted tremendous interest since it regulates both biofilm formation and virulence factors in many bacteria. Only recently, cGMP, c-di-AMP and cGAMP were found to act as signaling molecules involved in cyst formation of Rhodospirillum centenum act as signaling molecules involved in cyst formation and virulence factors in many bacteria. Only recently, cGMP, c-di-AMP and cGAMP were found to act as signaling molecules involved in cyst formation of Rhodospirillum centenum, sporulation of Bacillus subtilis, and chemotaxis regulation in Vibrio cholerae, respectively (Dong et al., 2012; Gao et al., 2016). In addition, cyclic di-nucleotides are recognized as important pathogen-associated molecular patterns (PAMP) for the host innate immune system (Gao et al., 2016). In this review, we will focus on (p)ppGpp and c-di-GMP for their role in E. amylovora virulence and biofilm formation.

(p)ppGpp and its role in bacterial virulence. Guanosine tetraphosphate (pppGpp) and guanosine pentaphosphate (ppppGpp) [collectively known as (p)ppGpp], two unusual linear nucleotides, were first discovered in 1969 as bacterial “alarmone” compounds produced in response to nutrient starvation (e.g. amino acids, phosphates, fatty acids, carbon, or iron) (Dalebroux and Swanson, 2012). RelA and SpoT homologous (RSH) proteins, which are present in almost all bacteria, are responsible for the synthesis and degradation of (p)ppGpp. In Proteobacteria, the ribosome-associated RelA protein is a monofunctional synthase and synthesizes (p)ppGpp using ATP and GTP in response to amino acid starvation. The cytoplasmic SpoT protein is a bifunctional hydrolase-synthase enzyme, and activates (p)ppGpp biosynthesis in response to a lack of fatty acid, carbon, phosphorous, and iron, as well as hyper-osmotic shock and oxidative stress (Fig. 2) (Atkinson et al., 2011; Battesti and Bouveret, 2009; Jain et al., 2006; Potrykus et al., 2011). In E. coli, direct interactions between RNAP, (p)ppGpp, and its partner transcription factor DksA lead to down-regulation of highly expressed stable RNA, DNA replication, ribosome and protein synthesis, and simultaneous up-regulation of stress and starvation genes, as well as virulence gene expression (Balsalobre, 2011; Kanjee et al., 2012; Magnusson et al., 2005). This so-called “stringent response” ultimately redirect the global transcriptional capacity of the cell from genes for growth and reproduction towards those for survival (Paul et al., 2005; Shyp et al., 2012; Srivatsan and Wang, 2008). However, inhibition of bacterial growth by (p)ppGpp is transient and reversible (Dalebroux and Swanson, 2012). In addition, (p)ppGpp and DksA indirectly promote alternative sigma factor-dependent gene regulation by competing for limiting amounts of core RNAP in the cell (Jishage et al., 2002). Furthermore, (p)ppGpp has been found to have a major impact on RpoN-dependent transcription (Laurie et al., 2003) due to that many RpoN-controlled processes (e.g. nitrogen starvation) are counteracting stresses.

![Fig. 1. A working model illustrated the regulation of T3SS in Erwinia amylovora in response to plant and environmental stimuli. This model is based on findings reported in previously studies (Ancona et al., 2014, 2015, 2016; Lee and Zhao, 2016; Lee et al., 2016; Li et al., 2014; Zhao et al., 2009a). GacS/GacA and EnvZ/OmpR: two-component regulatory systems; HrpS: a σ^54-dependent enhancer binding protein; IHFαβ: integration host factor σβ; (p)ppGpp: linear nucleotide second messengers, i.e. guanosine tetraphosphate (pppGpp) and guanosine pentaphosphate (ppppGpp); RelA/SpoT/paSpo: (p)ppGpp synthesis/hydrolysis enzymes; RpoN: a σ^54 alternative sigma factor; RNAP: RNA polymerase; CsrA: RNA-binding protein A; csrB: small non-coding regulatory RNA; T3SS: type III secretion system; YhbH: σ^54 modulation protein (ribosome-associated protein). Symbols: ↑, positive effect; ↓, negative effect; ?, unknown; P, phosphorylation; OM: outer membrane; IM: inner membrane.](image-url)
biofilm formation, hydrogen peroxide and antibiotic tolerance, as well as nodulation (Moris et al., 2005; Khakimova et al., 2013; Takeuchi et al., 2012; Vercruysse et al., 2011). In plant pathogenic bacteria, (p)ppGpp is required for cell-wall degrading enzyme production, quorum sensing signal degradation, Ti plasmid transfer and more recently T3SS (Bowden et al., 2013; Chatnaparat et al., 2015a, 2015b; Wang et al., 2007; Zhang et al., 2004).

Cyclic di-GMP and its role in bacterial virulence. Cyclic di-GMP is a second messenger molecule that is a widely-conserved bacterial signaling molecule with important regulatory roles of both virulence and housekeeping genes (Romling et al., 2013). Cyclic di-GMP has been intensively studied over the past 25 years as a regulator of the transition from a motile to a sessile bacterial lifestyle, i.e., from a planktonic phase to a biofilm phase. Cyclic di-GMP levels within bacterial cells are maintained via the synthesis and degradation of the molecule; c-di-GMP is synthesized by diguanylate cyclase (DGC) enzymes and degraded either to 5'-phosphoguanylyl-(3', 5')-guanosine by phosphodiesterase (PDE) enzymes or to GTP by enzymes containing an HD-GYP domain (Romling et al., 2013; Orr et al., 2015). The functional activity of c-di-GMP as a regulator of transcription is conferred through binding to proteins whose regulatory function is altered upon c-di-GMP binding. In other situations, c-di-GMP can bind directly to promoter elements termed riboswitches, resulting in structural alterations that induce or repress gene expression. Finally, c-di-GMP can also bind to proteins and activate their function.

Fueled by comparative genomic analyses and genetic examinations of biofilm formation and bacterial virulence, it has become clear that c-di-GMP is a universal signaling molecule in bacteria, with many species encoding more than 10 to as many as 100 enzymes involved in c-di-GMP synthesis and degradation (Chou and Galperin, 2016; also, see https://www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html). The plant pathogen Xanthomonas campestris was one of the first organisms in which the regulation of virulence by c-di-GMP was extensively studied. In this organism, perception of the quorum sensing signaling molecule diffusible signal factor (DSF) by the two component signal transduction proteins RpfC/G leads to alteration in c-di-GMP levels that in turn regulates levels of extracellular enzymes and exopolysaccharide virulence factors (Dow et al., 2006). Ryan et al. (2006) further demonstrated that the HD-GYP domain of the X. campestris protein RpfG degraded c-di-GMP. The rpf/DSF system is conserved in other Xanthomonas spp. and in Xylella fastidiosa, and knowledge of the regulatory role of c-di-GMP in controlling virulence in these organisms continues to expand (Chatterjee et al., 2010; Yang et al., 2015; Wei et al., 2016). In other plant pathogens, c-di-GMP regulates motility and the levels of secreted enzymes such as cellulases and pectinases in Pectobacterium atrosepticum, an

### Fig. 2. A schematic diagram showing the pathway for (p)ppGpp synthesis and its major role in response to nutrient starvation. The ribosome-bound RelA protein is activated via amino acid starvation, while the cytoplasmic SpoT protein is activated in response to carbon starvation. RelA/SpoT use GTP or GDP and ATP to generate (p)ppGpp, which is degraded by hydrolysis to either GDP or GTP and PP_i (pyrophosphate) by SpoT. (p)ppGpp directly binds to RNA polymerase and, along with transcription factor DksA to activate/suppress alternative sigma factor-mediated global gene expression. Symbols: ↓, positive effect; ┴, negative effect.

As one of the global regulatory systems in bacteria, the stringent response often results in swift and massive transcriptional reprogramming in response to various nutrient limitation conditions (Balsalobre, 2011; Kanjee et al., 2012). It has been demonstrated in numerous studies that (p)ppGpp is involved in processes related to stress, starvation, survival and virulence from a wide range of bacterial species, including adherence, quorum sensing, biofilm formation, persistence, sporulation, antibiotic tolerance, invasion and intercellular survival (Dalebroux et al., 2010). For example, (p)ppGpp accumulation in Salmonella enterica induces HiiA, a master regulator of Salmonella pathogenicity island 1 (SPI1) involved in invasion, and directly interacts with SlyA, a transcriptional activator of pathogenicity island 2 (SPI2), to facilitate intracellular replication (Pizarro-Cerdá and Tedin, 2004; Ramachandran et al., 2014). In E. coli, (p)ppGpp activates LEE gene expression and increases bacterial adherence (Nakanishi et al., 2006). However, (p)ppGpp-related studies in plant-associated bacteria are very limited. In plant-associated pseudomonads and rhizobia, (p)ppGpp affects epiphytic fitness, biocontrol activity,
organism that encodes 23 proteins with GGDEF, EAL, or HD-GYP domains (Tan et al., 2014). In addition, a high degree of complexity and interconnectivity of c-di-GMP with other global regulatory pathways was recently revealed in the regulation of the T3SS in Dickeya dadantii (Yuan et al., 2015).

THE ROLE OF (P)PPGPP IN REGULATING THE T3SS IN ERWINIA AMYLOVORA

T3SS genes are believed to be induced when bacteria are in contact with plant tissue or in minimal media thought to mimic conditions in plant apoplast, including low nutrients, low pH, and relatively low temperature; and to be repressed in rich media (Stauber et al., 2012). These early observations suggest that nutrient limitation stress may be one of the primary factors that activate the RpoN-HrpL sigma factor cascade and trigger the expression of T3SS genes. Recent reports have provided indisputable evidence that (p)ppGpp accumulation under nutrient starvation conditions is the trigger for T3SS gene expression in both E. amylovora and Pseudomonas syringae (Ancona et al., 2015; Chatnaparat et al., 2015a, 2015b) (Fig. 1). Furthermore, both relA and spoT mutants caused reduced disease symptoms in apple, suggesting that both RelA and SpoT are required for full virulence of E. amylovora. Although a linear relationship between ppGpp levels and T3SS gene expression was observed in E. amylovora, HrpA protein accumulation and the abilities to cause disease on apple shoots in the spoT and relA mutants was not significantly different. The discrepancy between T3SS gene expression, protein accumulation, and disease-causing ability could be due to that T3SS in E. amylovora are also regulated at the post-transcriptional or translational levels. In addition to T3SS, deficiency in ppGpp resulted in loss of swarming motility, reduction of pyoverdine production, increased sensitivity to oxidative stress and antibiotic tolerance, as well as reduced ability to utilize γ-amino butyric acid (GABA) in Pseudomonas. Interestingly, most ppGpp0 mutant cells were not viable on plant surfaces, suggesting that (p) ppGpp-mediated stringent response temporarily limits cell growth, and might control cell survival on plants. These findings suggest that plant bacterial pathogens might utilize intracellular (p)ppGpp to sense environmental signals for rapid, precise, and reversible control of their pathogenesis and survival.

Unlike in E. coli, both E. amylovora and P. syringae genomes encode three or even four RSH proteins. Besides RelA and SpoT, E. amylovora and most plant pathogenic P. syringae, such as P. s. pv. syringae B728a, contain a mono-functional small RSH protein paSpo/pbcSpo2 with a single hydrolase domain, whereas a few P. syringae, such as P. syringae pv. tomato DC3000 (PstDC3000) contain fpRel with a single synthase domain (Ancona et al., 2015; Chatnaparat et al., 2015a, 2015b). The recovery of a spoT null mutant in E. amylovora, P. fluorescens, and P. syringae suggested that single ppGpp-hydrolase-domain-containing proteins such as paSpo and pbcSpo2 are functional (Chatnaparat et al., 2015a, 2015b), as null mutation of the spoT gene in the relA+ background in E. coli and in PstDC3000 is lethal, further suggesting that high levels of ppGpp may be toxic. Interestingly, the spoT null mutant in E. amylovora, P. fluorescens and P. syringae B728a exhibited very unique phenotypes, including reduced antibiotic activity and elongated cells.

Many studies have also reported that nutrient availability adversely affects cell size (Hill et al., 2013; Monier and Lindow, 2003; Traxler et al., 2008; Yao et al., 2012). Upon inoculation on bean leaf surface, the length of P. syringae cells was rapidly reduced, suggesting that the leaf surface is a habitat with limited nutrients (Monier and Lindow, 2003). Similarly, the ppGpp mutant cells of E. coli were considerably longer when cultured under starvation condition (Traxler et al., 2008). It has also been reported that cells of smaller sizes become increasingly resistant to abiotic stresses, including osmotic and oxidative stresses, thus enhancing their abilities to survive under harsh environmental conditions (Jubair et al., 2012; Steinberger et al., 2002; Vercruyssse et al., 2011). Recent studies showed that increased levels of ppGpp resulted in reduced cell size in both E. amylovora and Pseudomonas (Ancona et al., 2015; Chatnaparat et al., 2015a, 2015b). These findings suggest that E. amylovora and Pseudomonas might require ppGpp to regulate cell size and resistance to environmental stresses, thus contributing to overall survival.

THE ROLE OF C-DI-GMP IN REGULATING VIRULENCE AND BIOFILM FORMATION IN ERWINIA AMYLOVORA

In E. amylovora, c-di-GMP regulates all of the major pathogenicity and virulence determinants including the T3SS, amylovoran biosynthesis, biofilm formation, and motility (Edmunds et al., 2013). The synthesis and breakdown of c-di-GMP in E. amylovora is controlled by five DGCs and three PDEs, and four of the DGCs (edcB, edcC, edcD, edcE) are known to regulate virulence (Edmunds et al., 2013). In general, when cellular levels of c-di-GMP are elevated, amylovoran biosynthesis and biofilm formation are both increased, and this is correlated with a significant increase in transcription of the promoter of the ams operon upstream of amsG (Edmunds et al., 2013). In contrast, elevated cellular levels of c-di-GMP have a negative effect on the T3SS and result in a significant reduction in the expression of hrpA (Edmunds et al., 2013). Deletion of individual genes encoding DGCs such as edcC or edcE did not affect virulence; however, the edcCE double deletion mutant and the edcACE triple deletion mutant were significantly more virulent in both the immature pear and apple shoot infection models (Edmunds et al., 2013).
In addition to influencing biofilm formation in *E. amylovora* through positively affecting amylovoran biosynthesis, cyclic di-GMP also directly modulates biofilm formation by activating the synthesis of cellulose through allosteric binding to the cellulose catalytic subunit BcsA (Castiblanco and Sundin, 2017). The PilZ domain present in BcsA is one of the best-characterized c-di-GMP-binding domain; PilZ domain proteins play major roles in virulence regulation in a variety of bacterial pathogens (Ryan *et al.*, 2012). In *E. amylovora*, cellulose affects biofilm architecture both *in vitro* and *in planta*, and does affect bacterial virulence in the apple shoot blight infection model (Castiblanco and Sundin, 2017). Regulation of amylovoran biosynthesis and biofilm formation by c-di-GMP in *E. amylovora* also reveals the specificity of individual DGC enzymes; for example, overexpression of edcB in *E. amylovora* Ea1189 results in significantly-increased biofilm formation, but has no effect on amylovoran biosynthesis, suggesting that the c-di-GMP produced by EdcB is specifically involved in the activation of BcsA for cellulose synthesis (Castiblanco and Sundin, 2017).

**INFECTION MODEL WITH C-DI-GMP**

The inverse regulation of the T3SS and biofilm formation is critical to *E. amylovora* pathogenesis during fire blight infection. During initial infection of apple shoots through leaves at shoot tips, *E. amylovora* cells utilize the T3SS during the infection process to deliver effector proteins such as DspA/E and AvrKpt2EA to plant cells resulting in the initial symptoms of shoot blight (Fig. 3A; Oh and Beer, 2005; Zhao *et al.*, 2006). Thus, during this initial infection event, c-di-GMP levels in cells are expected to be low, enabling expression of T3SS genes. As the infection progresses in leaves at shoot tips, *E. amylovora* cells preferentially track to the veins of leaves, and ultimately to the main central leaf vein. Biofilms are formed by *E. amylovora* in xylem vessels in leaf veins, enabling growth to large population size (Fig. 3B; Koczan *et al.*, 2009). The formation of biofilms in xylem is a strategy used by many plant pathogens including *Pantoea stewartii* and *X. fastidiosa* that allows cells to grow to large population size (Chatterjee *et al.*, 2010; Koutsoudis *et al.*, 2006). For
biofilm formation to occur, there must be a reprogramming of virulence gene expression, as T3SS genes and biofilm genes are inversely regulated (Ahmad et al., 2011; Kuchma et al., 2005; Romling et al., 2013). We hypothesize that part of this genetic reprogramming is controlled by c-di-GMP signaling, and that an increase in c-di-GMP levels, possibly triggered by attachment of cells to xylem vessels, affects a transition from planktonic E. amylovora cells infecting plant cells and congregating in the apoplast, to cells developing biofilms within xylem vessels. Biofilm development also requires EPS production (amylorovan, cellulose, levan), requiring c-di-GMP regulation to work together with other regulatory systems controlling EPS biosynthesis (Wang et al., 2009, 2010, 2012a, 2012b).

After attaining sufficient population size in xylem vessels, E. amylovora cells can escape through direct disruption of xylem vessels (Bogs et al., 1998). Following this redeployment out of xylem and into cortical parenchyma, E. amylovora cells are again in an apoplast location, and must again reprogram virulence by turning the T3SS genes back on. Active expression of the T3SS then enables the most common form of systemic spread by E. amylovora through the cortical parenchyma during advancement through host stems. Systemic infection in this manner enables E. amylovora cells to be located closer to the external surface of stems where cankers can be formed (Fig. 3C) and where ooze can emerge (Fig. 3D). We have recently shown that ooze emergence in apple stems occurs through wounds that we hypothesized resulted from internal pressure buildup caused by the dense packing of E. amylovora cells within the confined location of the plant intercellular spaces (Slack et al., 2017). We also determined that E. amylovora cells either within stem sections adjacent to ooze droplets or within the ooze droplets themselves were actively expressing T3SS genes (Slack et al., 2017).

CONCLUSIONS AND PERSPECTIVES

These findings suggest that both ppGpp and c-di-GMP are required for virulence, bacterial growth, T3SS gene expression and thus survival in E. amylovora. The T3SS has become the main target in drug discovery by finding inhibitors that disable the function of T3SS (Yang et al., 2014). These inhibitors could possibly be used as a novel class of antibacterial agents. Furthermore, considering the vital role of ppGpp in bacterial virulence, targeting the stringent response would be the next crucial step for the development of a novel class of antibacterial agents (Gaca et al., 2015; Muller et al., 2012; Wexselblatt et al., 2012). Given that the ppGpp-deficient strain is unable to survive and cause disease, further research on targeting ppGpp in E. amylovora for development of control strategies is warranted. In addition, research should focus on investigating the global effect of ppGpp both in vitro and in planta, and the signals that activate ppGpp accumulation and T3SS.

Regarding c-di-GMP, it will also be critical to identify the environmental signals that regulate the synthesis and degradation of this molecule during E. amylovora infection in planta. In particular, as E. amylovora cells migrate within leaves, regulatory triggers of biofilm formation within xylem are still unknown. Attachment factors of E. amylovora involved in biofilm formation have been studied (Koczan et al., 2011), and it is known in Pseudomonas aeruginosa, for example, that surface sensing regulates cAMP and c-di-GMP production to coordinate surface behaviors (Luo et al., 2015). Similar mechanisms could be in place in E. amylovora. Likewise, research should also focus on factors influencing dispersal from biofilms and re-induction of the T3SS enabling systemic migration in planta.

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