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Genetic Dissection of the *Erwinia amylovora* Disease Cycle

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Abstract

Fire blight, caused by the bacterial phytopathogen *Erwinia amylovora*, is an economically important and mechanistically complex disease that affects apple and pear production in most geographic production hubs worldwide. We compile, assess, and present a genetic outlook on the progression of an *E. amylovora* infection in the host. We discuss the key aspects of type III secretion–mediated infection and systemic movement, biofilm formation in xylem, and pathogen dispersal via ooze droplets, a concentrated suspension of bacteria and exopolysaccharide components. We present an overall outlook on the genetic elements contributing to *E. amylovora* pathogenesis, including an exploration of the impact of floral microbiomes on *E. amylovora* colonization, and summarize the current knowledge of host responses to an incursion and how this response stimulates further infection and systemic spread. We hope to facilitate the identification of new, unexplored areas of research in this pathosystem that can help identify evolutionarily susceptible genetic targets to ultimately aid in the design of sustainable strategies for fire blight disease mitigation.

INTRODUCTION: AN OVERALL ASSESSMENT OF THE CURRENT TRAJECTORIES IN *ERWINIA AMYLOVORA* RESEARCH

From the first report of fire blight disease in the late 1700s to the current explosion in research capabilities owing to the implementation of advanced microscopy, next-generation sequencing, and other high-throughput technologies, the *E. amylovora* disease cycle has been explored in detail (46, 61, 77, 140, 143). Early observational and pathogen-centric research by pathologists from the 1930s to the early 1990s (12, 15, 98, 106, 107, 119) has provided a strong foundation for hypotheses underlying current work that places recent genetic advances in the context of the disease cycle. In 2012, Malnoy et al. (77) reviewed new progress made in understanding the genomics of both *E. amylovora* and its hosts. With the first complete genome sequence of *E. amylovora* CFBP1430 released in 2010 (113), the extent of genomic research in *E. amylovora* was initially limited to the investigation of key pathogenicity or virulence factors such as the type III secretion system (T3SS) pathogenicity islands and T3SS effectors as well as the impact of exopolysaccharides (EPSs) such as amylovoran on phenotypic traits like biofilm formation. Since then, there has been a consistent shift acknowledging the complexity in each step of the infection process, through the systematic examination of multiple genetic and other stimulus factors at each stage. Key instances of this include evaluating multiple physical and physiochemical factors in the xylem affecting biofilm formation (61), decoding the multilevel genetic regulation of the T3SS (112, 140), and, more recently, exploring floral microbiomes and their influence on disease severity (34, 35, 114). In this review, we present a current analysis of each individual step of the *E. amylovora* infection process and highlight the critical factors affecting virulence within each step. We also explore the interconnectedness of the steps to each other and the embedded regulatory connections that modulate production of key virulence factors.

SUMMARY OF THE *ERWINIA AMYLOVORA* DISEASE CYCLE

E. amylovora infects the apple host predominantly through flowers or leaves at shoot tips. Three major pathogenicity factors are required for infection: the T3SS, the type III effector (T3E) DspA/E, and the EPS amylovoran (17, 22, 42, 86, 87). In *E. amylovora*, the hypersensitive response and pathogenicity (Hrp) T3SS is an essential pathogenicity factor because deletion of the T3SS-encoding genes results in the inability of the bacteria to induce fire blight in host plants or elicit the hypersensitive response in resistant plants (86, 87). Genomic and secretomic analyses of *E. amylovora* revealed that this bacterium secretes at least five effectors [DspA/E, Eop1, Eop3, Eop4 ($AvrRpt2_{Ea}$), and $HopPtoC_{Ea}$] two harpins (HrpN and HrpW), and other proteins such as Eop2, HrpJ, and HrpK (17, 18, 42, 62, 66, 84, 135, 140, 145, 146). Expression of the genes encoding the T3SS and T3Es is coordinately regulated and controlled by the alternate sigma factor HrpL (133); the HrpL regulon comprises a total of 39 genes (79).

The EPS amylovoran is a heteropolymer consisting of repeating subunits of glucose, galactose, glucuronic acid, and pyruvate substrates (14), and amylovoran is the major EPS component of *E. amylovora* biofilms (65). The biosynthesis of amylovoran is dependent on the 12 genes of the *ams* biosynthetic operon (25). The reason amylovoran is a pathogenicity factor of *E. amylovora* has not been definitively proven to date, with the most common speculation focusing on the potential of this EPS to protect *E. amylovora* cells from host defenses (43).

Other virulence factors contributing to *E. amylovora* pathogenesis include biofilm formation (65), the EPSs cellulose and levan (27, 44), the T3E $AvrRpt2_{Ea}$ (146), motility (9), the siderophore desferrioxamine (39), and sorbitol and sucrose utilization systems (1, 20). Major regulators of pathogenicity and virulence factors include two-component signal transduction systems, the second messenger molecule cyclic di-GMP (c-di-GMP), and small, noncoding RNAs (sRNAs)

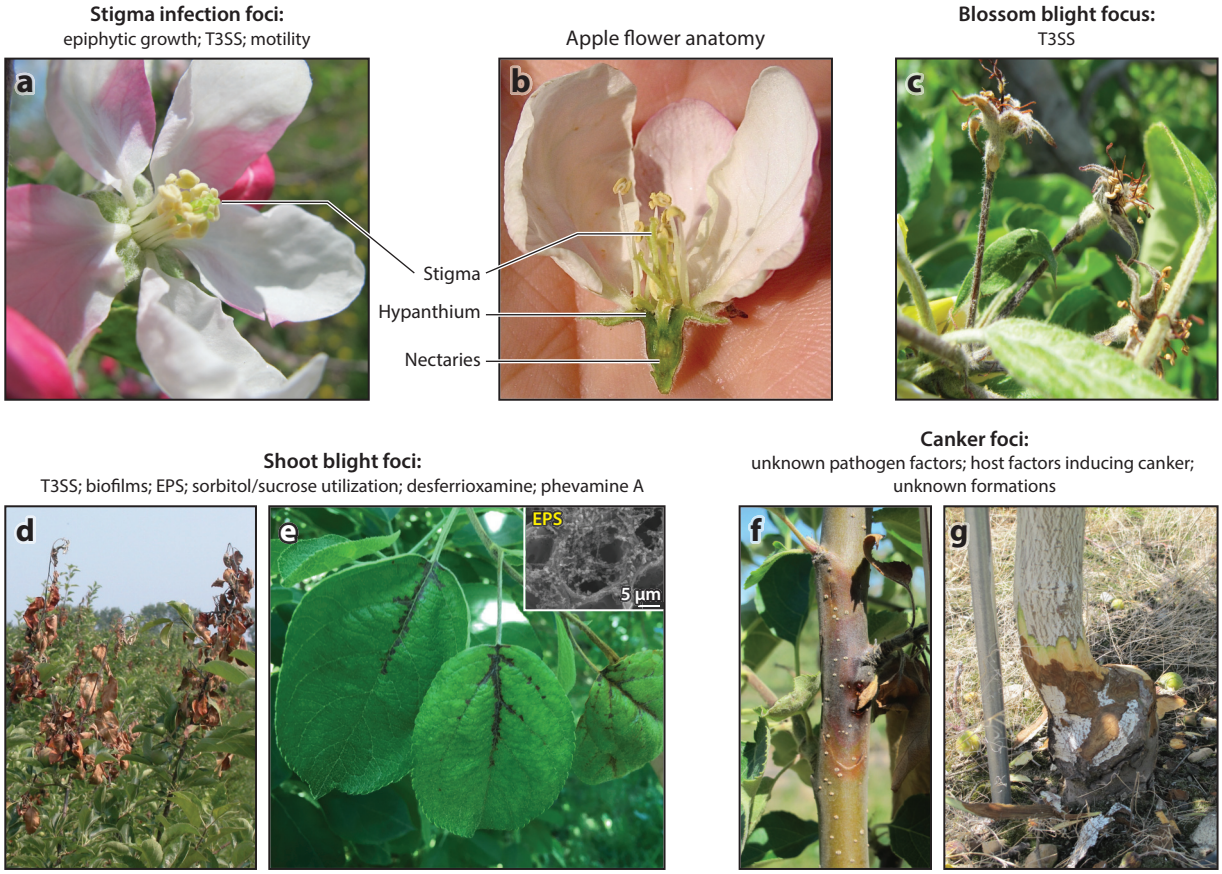

Figure 1

Major infection foci of the disease cycle of *Erwinia amylovora*, the causal agent of fire blight, and critical virulence and pathogenicity factors required at each step. (*a*,*b*) Apple flower. *E. amylovora* cells grow on stigmas prior to utilizing motility to migrate down the style to the hypanthium, initiating infection after entry into flower nectaries. (*c*) Blossom blight infection of flowers is mainly mediated by the type III secretion system (T3SS). (*d*) Shoot blight infection involves multiple phases, including T3SS-mediated infection and a biofilm phase showing (*e*) veinal necrosis in infected leaves. (*Inset*) a scanning electron micrograph shows an *E. amylovora* biofilm within leaf xylem. Cankers, forming on the trunk of an (*f*) apple tree or at the (*g*) scion–rootstock junction, represent the least-studied phase of the *E. amylovora* disease cycle. Abbreviation: EPS, exopolysaccharide.

that regulate at the posttranscriptional level (3, 40, 142, 144, 148). Lon protease and integration host factor (IHF) are two other global regulators that contribute to the regulation of some virulence factors (68, 69).

The *E. amylovora* fire blight disease cycle is relatively complex, especially when considering the various routes of secondary spread within and between infected hosts (85). However, for the purposes of this review, we focus on the active pathogenesis pathways within a single host tree, including flower infection, shoot infection, systemic spread, and canker formation. Flower infection combines an epiphytic growth phase on flower stigmas (**Figure 1*a***) with T3SS-mediated pathogenesis after *E. amylovora* cells enter the flower nectary (**Figure 1*b*,*c***). During shoot infection, *E. amylovora* cycles between T3SS-mediated pathogenesis in leaf cortical parenchyma cell layers and an active biofilm phase in leaf xylem (**Figure 1*d*,*e***). Thus, there is a need for precise on/off regulation of specific systems, e.g., T3SS or biofilm formation, and we later discuss the role of

the second messenger molecule c-di-GMP as a regulatory switch. Systemic spread of *E. amylovora* through the apple host relies predominantly on T3SS-mediated pathogenesis, with cells moving through foliar and stem tissues located close to the outer surface of the host, a location that can enable ooze emergence for secondary spread and can also facilitate the discovery of favorable sites for canker formation. Cankers can form on limbs or at the rootstock–crown junction and are the overwintering site of the pathogen (**Figure 1*f,g***), but this phase of the disease cycle has been the least characterized. As we fully review the *E. amylovora* disease cycle in the following sections, we describe the abovementioned critical pathogenicity and virulence factors and important regulatory pathways in more detail.

FLOWER INFECTION

Infection of flowers (blossom blight) is considered the primary mode of infection in the context of the overall *E. amylovora* disease cycle. Overwintering cankers harbor reserves of *E. amylovora* inoculum, which emerge in the form of ooze with warming temperatures in the spring (10). Ooze droplets are a viscous emulsion of high bacterial titers and the carbohydrate-rich EPS amylovoran (13); ooze often attracts insect vectors such as flies, which participate in the contact-based transfer of *E. amylovora* cells to floral tissue (primarily the stigmas) (111). Once on the stigma, *E. amylovora* is capable of rapid growth under conducive conditions, with populations capable of reaching the carrying capacity of approximately 10^7 per flower within a few days (95, 117, 121). Epiphytic growth of *E. amylovora* occurs in the intercellular spaces of the columnar papillae cells of the stigmas (77, 121); this microenvironment is characterized by high humidity and contains sufficient polysaccharides, simple sugars, and amino acids to enable rapid growth (96, 136).

From petal open to petal fall, the bacterial microbiome on the apple stigma undergoes a process of first establishing a diverse microbial community and then transitioning to a less diverse community with predictable members (35). When the petals first open, nutrients and space are readily available on the apple stigma, and competition between species is minimal. Correspondingly, there is an increase in taxonomic diversity of the stigma microbiome in the first 1–3 days after petals open (35). In the late stage of bloom (4–5 days after petals open), as the papillae cells burst and become degraded, the diversity of the stigma microbiome decreases drastically. Bacteria best adapted to the stigma environment, primarily Enterobacteriaceae and Pseudomonadaceae, outcompete other members and become the predominant species on apple stigmas (35, 114). The T3SS contributes to epiphytic growth on the flower stigma, as an *E. amylovora hrpL* (T3SS null) mutant showed a twofold decrease in growth when compared to the wild type (54). Johnson et al. (54) speculated that the activity of the *E. amylovora* T3SS has an effect on the stigma habitat, thereby increasing the availability of nutrients. Cui et al. (33) have recently demonstrated the translocation of T3E proteins into stigma papillae cells; however, it is not currently known whether the T3SS function on stigmas affects only the stigma habitat for epiphytic growth or also affects blossom blight infection potential. Having the T3SS genes induced on the stigma also helps *E. amylovora* to overcome a T3SS-repressive condition at the hypanthium (the basal receptacle of the flower that contains the nectary) and increases its blossom blight infection potential (33).

Flagellar Motility to Position *Erwinia amylovora* for Flower Infection

When *E. amylovora* is dispersed to uninfected flowers, the consensus is that those cells that arrive on the stigma are the most important, with the proximity of the anthers to the stigma and style seemingly preventing the direct deposit of *E. amylovora* to the nectary or hypanthium by pollinators or physical forces. Thus, *E. amylovora* must have a means of moving from the stigma to the hypanthium. Work to understand the environmental conditions important for bacterial

growth and disease incidence during bloom has provided strong evidence that moisture as rain, heavy dew, or high relative humidity is crucial for this migration to occur and lead to disease (94). However, specific evidence showing that the moisture physically redistributes *E. amylovora* cells is lacking. Indeed, evidence indicating strong chemotaxis of *E. amylovora* to nectar and specifically to organic acids (9, 97) suggests that the bacteria use flagellar-based motility to migrate toward the hypanthium and nectary.

The inability of *E. amylovora* to multiply under conditions mimicking high sugar concentrations found in nectar (52) also suggests that bacterial migration may be important in the nectary. Correlations between environmental relative humidity, sugar concentration in nectar, and blossom blight incidence reinforce the critical role for water availability (49). It is likely that rain or dew events may overcome a need for flagella-mediated chemotaxis and migration to the nectary and hypanthium, but disease incidence can still be high in the absence of such favorable weather events when relative humidity is high. This suggests that under high relative humidity, *E. amylovora* uses flagellar motility to migrate to the nectary, encounters reduced sugar concentration, and completes its journey to invade natural openings.

Interestingly, the transit from the stigmas to the nectary may be the only phase during the entire disease cycle that flagella are active. A previous study has shown that *E. amylovora* cells recovered from stem tissue of shoot blight infections are nonmotile (28). In addition, a recent proteomics analysis of an *E. amylovora* shoot infection showed that a highly virulent strain was not producing flagella proteins (50). One hypothesis for this observation is that invading cells minimize flagella production to minimize the production of the flg22 epitope, a conserved pathogen-associated molecular pattern (PAMP) that plants recognize, triggering PAMP-triggered immunity (PTI) (139).

Because of the very strict and highly limited requirement for flagellar motility during the disease cycle, regulation of motility is complex almost to an extreme. Several studies indicate careful and conserved regulation of flagellar motility in *E. amylovora*, suggesting selective pressure to maintain this trait (9, 97, 104, 142, 144, 147, 149). However, the motility trait has been demonstrated to be dispensable for infection in immature pear or apple shoot disease models (64, 147). Flagellar motility is primarily regulated by the cotranscribed FlhDC master regulator complex, and recent transcriptomic studies indicated that flagellar genes are often coexpressed with T3SS genes (93). The posttranscriptional regulatory system controlled by the conserved chaperone protein Hfq is also an important regulator of motility in *E. amylovora* (142). Hfq facilitates the base-pairing between a suite of sRNAs and their target mRNAs, resulting in translational activation or blockage (126). Three Hfq-dependent sRNAs, ArcZ, OmrAB, and RmaA, play roles in regulating flagellar motility in *E. amylovora* (104, 144). ArcZ directly interacts with *flhDC* mRNA, altering translational efficiency, and OmrAB also regulates *flhDC* posttranscriptionally, likely through a direct interaction (104). RmaA and ArcZ also regulate *flhDC* at the transcriptional level, the former through an unknown mechanism, and the latter indirectly through its regulation of the leucine regulatory protein Lrp (103, 104).

Additional regulators of motility include the global regulator RcsBCD, a two-component phosphorelay system, conserved in Enterobacteriaceae and essential for pathogenicity in *E. amylovora*, wherein RcsC and RcsD are two sensor kinase proteins and RcsB is a DNA binding response regulator (131). RcsB was found to be a suppressor of *flhDC* transcription (132). The RNA-binding protein CsrA (carbon storage regulator A), another global regulator of several targets in *E. amylovora*, was found to posttranscriptionally regulate RcsB and FlhD and positively regulate motility (3, 67). This represents the multifactorial network regulating flagellar motility. Upstream of this regulation driven by CsrA is the regulation of CsrA itself through the sRNA CsrB, which regulates CsrA through sequestration by binding (73). Integration host factor

(IHF), a nucleoid-associated protein, which mainly regulates T3SS was also found to positively regulate CsrB in *E. amylovora*, which has a downstream effect on CsrA functionality (69). Lon, an ATP-dependent protease, is partially suppressed by CsrA functionally, and positively affects *flhD* expression and phenotypic flagellar motility through elevated accumulation of RcsA/RcsB proteins resulting from the dampening degradative action of Lon toward these proteins (68). Finally, the two-component system EnvZ/OmpR positively regulates flagellar motility, and GrrS/GrrA was found to be antagonistic to the effect of EnvZ/OmpR (72). Identifying the environmental signals perceived in the stigma environment and determining how these signals are controlled by the various regulators of motility are critical to understanding how the motility trait is partitioned to such a temporally short part of the disease cycle.

Type III Secretion

Using quantitative real-time PCR analyses, Pester et al. (91) showed that several T3SS regulon genes were expressed in *E. amylovora* during the initial infection of apple flowers. In addition, Venisse et al. (125) demonstrated that wild-type *E. amylovora* induces a host defense response with the associated genesis of reactive oxygen species (ROS) during flower infection, whereas a *hrp* mutant defective in production of the T3SS does not. However, little information is available regarding the translocation and function of each *E. amylovora* T3SS effector and harpin in planta in a successful blossom blight infection. Thus, there is still not much clarity about the specific role of any of the *E. amylovora* T3Es in the context of flower infection.

SHOOT INFECTION AND SYSTEMIC SPREAD OF *ERWINIA AMYLOVORA* IN THE APPLE HOST

Mainly regarded as a secondary stage of infection, shoot infection, resulting in the classic shepherd's crook fire blight symptom, occurs in actively growing shoot tips and young leaf tissue (116). Environmental factors, including wind, blowing soil and sand, and hail, can mechanically damage young tissue and cause wounding (116). The wound sites, along with natural openings like hydathodes, provide a path of entry for *E. amylovora* cells, with ooze droplets being the predominant source of inoculum during this stage (111, 115, 116, 120). The initiation of a shoot blight infection can be triggered by as few as 100 cells (32). Upon entry into the leaf, unless cells gain direct access to the xylem through the damaged tissue, *E. amylovora* often gains access to the apoplast region within leaf tissue (45, 115, 116).

Type III Secretion in Shoot Infection

The deployment of T3Es is the critical virulence strategy employed by *E. amylovora* to initiate infection in the apoplast. The expression of most of the structural components and secreted proteins associated with the T3SS in *E. amylovora* is tightly coordinated by a complex network of regulatory elements (**Figure 2**). Many of these regulatory components have been previously reviewed (77, 86) and include the alternate sigma factor HrpL, the master regulator that recognizes and binds to a specific sequence (*hrp*-box) on its target gene promoters (79, 133). **Figure 2** graphically highlights that the complexity in the levels of regulatory control imposed on T3SS genes is mainly through their impact on *hrpL* transcription, which can result in the modulation of the HrpL regulon downstream. The expression of *hrpL* is modulated by several types of regulatory proteins, including the two-component transduction system HrpX/HrpY; HrpS, a member of the sigma 54 (σ^{54}) enhancer-binding protein family; and the σ^{54} protein RpoN and its modulators YhbH and IHF (4, 71, 79, 134). Upstream, the GrrS/GrrA and EnvZ/OmpR two-component systems both negatively

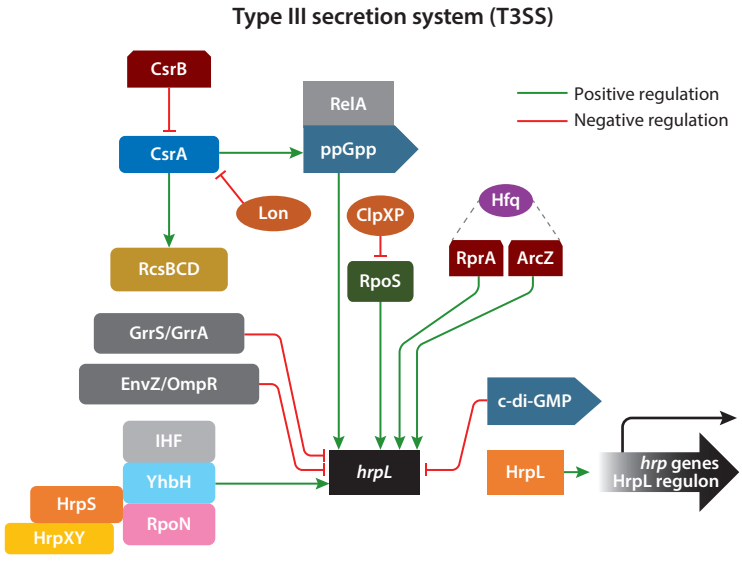

Figure 2

A graphical representation of the components that contribute to the regulation of the type III secretion system in *Erwinia amylovora*. Green and red lines represent positive and negative regulation, respectively. *hrpL* (alternate sigma factor) is transcriptionally regulated by HrpXY/HrpS in conjunction with the integration host factor (IHF), YhbH, and RpoN. The regulation imposed on the RcsBCD phosphorelay system through CsrA/B and the Lon protease feeds into the transcriptional regulation of *hrpS*. Other regulators that feed into the overall transcriptional regulation of T3SS genes include RpoS, ppGpp (stringent response), cyclic di-GMP (c-di-GMP), and Hfq-dependent sRNAs RprA and ArcZ.

regulate *hrpL* and *hrpN* expression in vitro (72). Lon protease is a negative regulator of virulence through its effect on *hrpS* transcription via RcsA/RcsB accumulation (68). RcsB is also posttranscriptionally regulated by CsrA, thus positively regulating the T3SS (67). The ATPase ClpXP positively regulates the T3SS through the suppression of the alternate sigma factor RpoS (70). The elimination of both ClpXP and Lon proteases resulted in an increase in T3SS expression (70). Recently, RprA, an Hfq-dependent sRNA, was found to positively regulate T3SS expression (90).

Early research on *E. amylovora* demonstrated that T3SS-associated genes are induced by environmental conditions mimicking apoplastic features such as nutrient limitation, low pH, and low temperature (135). Wei and collaborators (134) first demonstrated that the expression of both the *hrpX/hrpY* and *hrpS* promoters is enhanced in apoplast-like conditions, namely nutrient starvation in minimal media, when compared with their expression in a nutrient-rich environment. However, the signals recognized by *E. amylovora* to initiate transcriptional activation of the *hrp* genes have remained elusive. It has been demonstrated that the linear nucleotide alarmones guanosine 5′,3′ bispyrophosphate (ppGpp) and guanosine pentaphosphate (pppGpp), collectively known as ppGpp, are responsible for triggering the stringent response during nutritional starvation in bacteria (92). These alarmones act as internal messenger molecules that promote bacterial virulence via the transcriptional activation of T3SS structural and effector genes in *E. amylovora* after the bacterium encounters nutritional stresses such as those found in the apoplast (2, 71, 138).

Gene expression and transcriptomic analyses have demonstrated that the T3SS structural and effector genes are upregulated in inoculated apple shoots of susceptible and resistant cultivars as soon as 24 h after inoculation (93). The effector protein DspE, one of the main pathogenicity factors of *E. amylovora*, is involved in the suppression of salicylic acid (SA)-mediated innate defenses

such as callose deposition in apple and *Arabidopsis*, and the delay in the expression of the SA-induced defense marker *PR-1* gene, when transiently expressed in *Nicotiana tabacum* cv. *xanthi* leaf tissue (22, 37). Moreover, this effector protein also alters the expression of genes associated with jasmonic acid (JA)-dependent defense mechanisms in leaves of susceptible apple plants (36).

An analysis of the interactive landscape of DspE with apple proteins highlighted four leucine-rich repeat receptor-like serine/threonine kinases annotated as DIPM1–4 (80). The comparative homology of *DIPM* genes assessed across 93 *Malus* accessions indicated 30 single nucleotide polymorphisms associated with variations in host susceptibility (118). Correspondingly, the ERMRS motif located at the C-terminal end of DspE, generally conserved across the larger AvrE family of proteins, has been associated with translocation to the plant cell nucleus (48).

The effector protein $AvrRpt2_{Ea}$, a homolog of AvrRpt2 from *Pseudomonas syringae*, acts as a virulence factor, as a $\Delta avrRpt2_{Ea}$ mutant showed a reduction in symptom development in the immature pear infection model (146). Transient expression of $AvrRpt2_{Ea}$ in a susceptible apple host led to typical fire blight wilting and necrosis symptoms on growing shoots (108). In addition, this effector is involved in the activation of SA-mediated defense mechanisms, as the expression of $AvrRpt2_{Ea}$ results in increased levels of the *PR-1* gene and SA and its derivatives (108). The regulatory targets and functional analysis of a major subset of T3SS effectors (including Eop1–3, HrpK, and HrpW) remain relatively under-characterized.

During an analysis of a 62-kb sequence encompassing the *hrp* pathogenicity island of *E. amylovora*, Oh et al. (87) identified a three-gene operon downstream of *hrpU* that they ultimately demonstrated was required for full virulence and systemic spread during apple shoot pathogenesis. Because of their virulence function, the genes were named *hsvA* (*hrp*-associated systemic virulence), *hsvB*, and *hsvC*. *hsvA* was shown to have homology to an amidinotransferase family protein of *Pseudomonas syringae* pv. *tomato* (87). A *hrp* box promoter is located upstream of the *hsvABC* operon (87), and *hsvA* was later confirmed to be expressed as part of the HrpL regulon (79). Further functional studies of the amidinotransferase activity of HsvA were performed by Shanker et al. (109), but a real breakthrough occurred when O'Neill et al. (88), working with the related *hsv* operon in *P. syringae* pv. *tomato*, demonstrated that this operon encodes the production of the small molecule phevamine A. Phevamine A was then demonstrated to suppress the potentiation of the flg22-induced ROS generation (PTI response) in *Arabidopsis thaliana* by polyamines such as spermidine (88).

Induction of Host Defense Responses During Infection of Apple

During infection of apple flowers and shoots, *E. amylovora* elicits a defense response in apple, in a process that requires a functional T3SS (124, 125). This defense response includes the generation of ROS and expression of pathogenesis-related (PR) proteins, host responses that are typically associated with the hypersensitive response (124, 125). The T3SS and effectors DspA/E and HrpN are required to elicit this defense response (123). Expression of several defense response genes, e.g., chitinase, dihydroflavonol reductase, and flavonol synthase, expressed as part of the branches of the phenylpropanoid pathway (128), was initially induced but repressed between 48 and 72 h after infection (125). This repression was observed only in infections by wild-type *E. amylovora* and not when a *hrp* mutant was used, suggesting the involvement of a T3E(s) in dampening the resistance response.

In general, the defense responses induced within the host by a pathogen can be classified by the process of the elicitation being dependent on a broad range of nonspecific epitopes (PTI) or an evolutionarily narrow set of T3SS effectors [effector-triggered immunity (ETI)] (139). Both ETI and PTI can trigger a range of host defense responses, including the generation of ROS and

callose deposition. Observations show that during *E. amylovora* infection of apple, callose deposition is poorly elicited by PAMPS such as flg22 or others that exist in a T3SS mutant; instead, the T3E HrpN is required, and another T3E HrpW is involved in eliciting callose deposition (23). However, no callose deposition ultimately occurs in a compatible apple infection, presumably due to suppression by DspE (37). *E. amylovora* also induces the expression of some JA pathway genes and suppresses others, and likewise induces the expression of some SA pathway genes (21, 36, 38, 125). What remains unclear is an understanding of the complete suite of JA and SA pathway genes that are induced, how those genes are specifically induced, and how induction of these genes contributes to *E. amylovora* pathogenesis. Notably, SA pathways were induced when *E. amylovora* was inoculated onto susceptible and resistant apple cultivars, but JA pathway genes were downregulated only in the susceptible host (36). Treatment of apple with methyl-jasmonate increases resistance to fire blight disease, confirming the importance of JA pathway genes to resistance (36) and emphasizing the importance of downregulation of the JA pathway in *E. amylovora* infection. However, increasing expression of SA pathway genes, either in a transgenic apple host constitutively expressing the *NPR1* gene or through application of the systemic acquired resistance inducer compound acibenzolar-*S*-methyl, can also decrease the severity of fire blight (24, 76). Thus, the impact of the timing of the SA pathway, i.e., induction during infection versus prepriming prior to *E. amylovora* cells arriving at a host, seems to significantly affect the outcome of the interaction.

Complicating these findings is the knowledge of two gene-for-gene interactions in *E. amylovora–Malus* interactions, with T3Es serving as *avr* (avirulence) genes that correspond to host resistance genes (*R* genes). These interactions between Eop4 ($AvrRpt2_{Ea}$) and *Malus* × *robusta* Mr5 and between Eop1 and an unknown *Malus R* gene trigger ETI, and both confer host resistance to fire blight and prevent infection (127, 137). Thus, some effectors can elicit an ETI response that abrogates disease, whereas others, notably DspE, elicit a response that fuels disease. These observations also bring up the possibility that both of the Eop1 and Eop4 effectors targeted in gene-for-gene interactions conferring host resistance are required together to suppress JA pathway defense responses that enable continued infection by wild-type *E. amylovora* cells. Eop1 is a member of the YopJ/AvrRxv family (5) that includes effectors such as AvrBst1 that are known to suppress ETI (75). Likewise, the Eop4 homolog AvrRpt2 from *P. syringae* can interfere with defense by cleaving RIN4 and can block activation of the mitogen-activated protein kinases MPK4 and MPK11 in *A. thaliana* (41).

Venisse et al. (125) hypothesized that the host oxidative burst and ROS kill plant cells in the locality of the infection, thereby supplying the pathogen with nutrients providing energy for subsequent infection. The oxidative burst elicited in apple leaves generates approximately 4-mM concentrations of H_2O_2; *E. amylovora* cells exhibit a minimal inhibitory concentration of H_2O_2 of 5 mM (102) and utilize various strategies to survive in the presence of elevated ROS. For example, survival in the presence of elevated ROS is dependent on detoxifying enzymes such as catalase and thiol peroxidase (100, 102) and production of the siderophore desferrioxamine, as iron scavenging can prevent the synthesis of hydroxyl radicals by the host (123). In addition, it can be hypothesized that phevamine A (detailed above) could contribute to dampening the ROS response. Ultimately, the killing of host cells, elicitation of host defenses, suppression of JA pathway genes, and tolerance of elevated ROS conditions suggest that *E. amylovora* has evolved a coordinated, T3E-dependent strategy driving pathogenesis and that the development of an effective population size is critical for shoot infection by *E. amylovora*.

Biofilm Formation in Leaf Xylem

As stated above, the initiation of shoot blight infection by *E. amylovora* in apple hosts involves T3SS-mediated pathogenesis in the apoplast of the cortical parenchyma cell layer. *E. amylovora*

cells then quickly move toward xylem vessels located in the main and axillary leaf veins, gain entry into the xylem, and form biofilms. *E. amylovora* produces three EPSs, amylovoran, the most abundantly produced EPS, levan, and cellulose. Similar to the T3SS, amylovoran is a pathogenicity factor of *E. amylovora* because the absence of amylovoran abolishes fire blight infection in host plants (11, 86, 147). Amylovoran is the most important EPS component of *E. amylovora* biofilms, but levan and cellulose are both required for the complete structural integrity of the biofilm (26, 27, 65).

Biofilm formation by *E. amylovora* occurs in infected leaves at shoot tips, and our current hypothesis is that biofilms serve to increase the population size of the pathogen, which then contributes to further systemic infection. However, the exact nature of the evolutionary advantage in this regard, as it pertains to the suitability of the growth environment within a biofilm, has not been established. *E. amylovora* can gain access to the xylem in leaves either directly through damaged shoot tips/leaves or, more commonly, via movement through the parenchyma cell layers into the xylem (45, 115, 116). Although the mechanical/enzymatic process of entry into the xylem is not entirely clear, it has been hypothesized that following a brief expansion of population and EPS production (amylovoran and levan) in the parenchymal tissue, *E. amylovora* cells might use nutrient/osmotic signals as well as the microfluidic pressure differential from the xylem sap to chemotactically move toward and into the xylem (19, 115, 116). However, it has not been proven yet whether *E. amylovora* cells are indeed motile at this stage of the infection process.

Following entry into the xylem, attachment to the xylem vessels and establishment of biofilms are important processes in the initiation of systemic infection in the *E. amylovora* disease cycle. Several mechanochemical factors characteristic of xylem structure and function can impact the ability of *E. amylovora* to attach and form biofilms within the xylem vessels. The microfluidic flow of xylem sap through the vessel channels can inflict shear force on the walls of the vessels (55) (**Figure 3**). Biofilm formation in *E. amylovora* was found to be positively regulated under conditions in which fluidic flow was present compared to when flow was entirely absent (59, 65). Although this is also a common denominator in factors affecting biofilm formation in other xylem-dwelling phytopathogens such as *Ralstonia solanacearum* and *Xylella fastidiosa* (74, 81), a spatiotemporal understanding of how variations in the flow of xylem sap, resulting from changes in the transpiration output, can impact the dynamics of biofilm formation is lacking in phytopathogenic systems.

Surface sensing and interaction with a surface are the key first steps to enable attachment, which is required to initiate bacterial biofilm formation. In other bacterial systems, such as *Pectobacterium carotovorum* and *Pseudomonas aeruginosa*, the transition to biofilm formation is preceded by motile bacteria (often flagella driven) approaching a surface to make initial contact (8, 51). Initial findings suggested that in *E. amylovora*, the flagellar regulator FlhDC and the structural components FlgA–N were overall positive regulators of surface attachment in vitro (64). Since then, the flagellar filament protein FliC was found to be required for initial surface sensing that led to eventual attachment and biofilm formation in a flow-based system (58). Although the flagella–surface interaction is often relatively weak, secondary surface anchoring with other extracellular appendages can strengthen this interaction (83). In addition to the requirement of FliC for initial surface sensing, type IV pilus-mediated surface anchoring is also critical for the initiation of biofilm formation (58) (**Figure 3**).

Following surface attachment, the formation of robust biofilms is heavily dependent on the production of EPSs, which form the bulk of the biofilm matrix. Amylovoran is the most important biofilm EPS, as mutants defective in amylovoran production are unable to aggregate and form biofilms in vitro (65). Levan, a homopolymer of fructose, is synthesized from sucrose by the levansucrase enzyme, encoded by the *lsc* gene (44, 47). Similar to amylovoran, levan is an important component of biofilms formed in vitro, as Δ*lsc* mutants were not detected in leaf xylem during

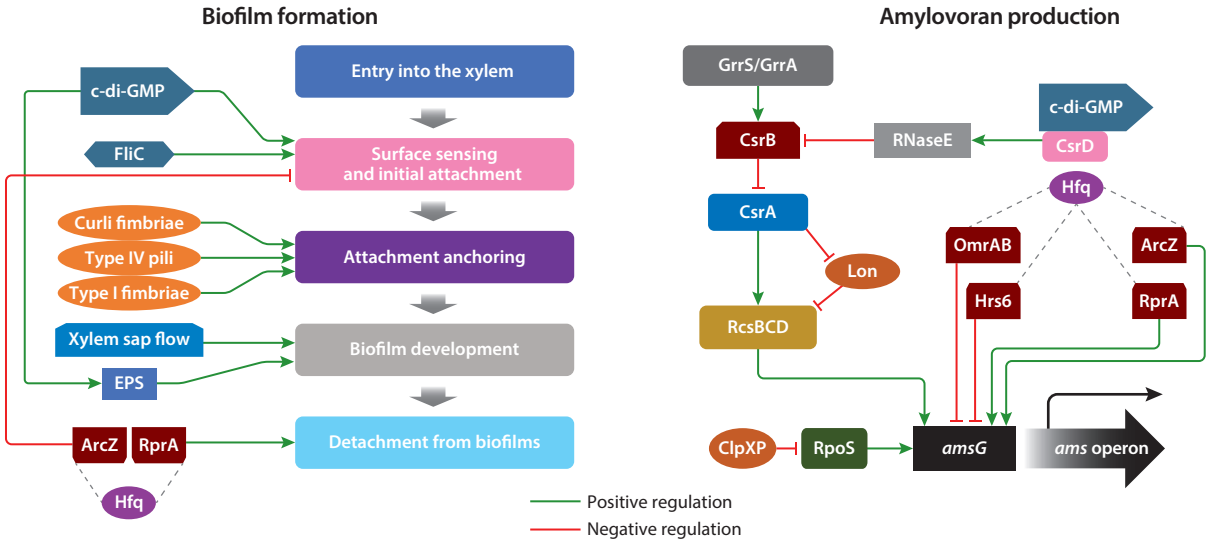

Figure 3

Biofilm formation in *Erwinia amylovora* is a multistage process involving entry into the xylem, followed by initial surface sensing and attachment, which require cyclic di-GMP (c-di-GMP) and the flagellar filament (FliC). Type IV pili in addition to curli and type I fimbriae are the appendages that contribute to permanent surface anchoring, which aids in the development of mature biofilms within the xylem through the additive effects of microfluidic xylem sap flow and exopolysaccharide (EPS) generation. Hfq-dependent sRNA RprA was found to regulate detachment of cells from a mature biofilm, whereas the sRNA ArcZ negatively regulates surface attachment in *E. amylovora*. Several factors contribute to the transcriptional regulation of the *ams* operon (primarily measured through the expression of *amsG*), leading to an impact on amylovoran production. C-di-GMP bound to CsrD selectively regulates the degradation specificity of RNaseE toward the sRNA CsrB (also independently regulated by the two-component system GrrS/GrrA). CsrB, through its sequestration of CsrA, asserts a transcriptional effect on *amsG* through the RcsBCD phosphorelay. Lon and ClpXP proteases can affect *amsG* transcription through the suppression of RcsB and RpoS, respectively. Hfq-dependent sRNA subgroups OmrAB/Hrs6 and ArcZ/RprA are involved in the negative and positive regulation of the *ams* operon, respectively.

apple shoot infection experiments (65). More recently, Castiblanco & Sundin (27) demonstrated that cellulose, synthesized by a cellulose synthase multiprotein complex encoded by seven genes contained in the *bcs* operon, is also a critical component of biofilms formed by *E. amylovora*. A deletion of *bcsA*, the gene encoding the catalytic unit of the cellulose synthase complex, rendered *E. amylovora* cells unable to form a mature biofilm structure in vitro; the Δ*bcsA* mutant was also reduced in virulence in the apple shoot infection model (27).

Current understanding of the regulation of EPS biosynthesis in *E. amylovora* is that regulation of the amylovoran biosynthetic operon *ams* is highly complex (**Figure 3**) and that some information is available on the regulation of the levansucrase *lsc* gene and the cellulose-biosynthetic operon *bcs*. Initial findings with *ams* operon regulation revealed that c-di-GMP positively regulated amylovoran production and *amsG* (the first gene in the *ams* operon) in vitro (40, 57), as discussed below. The RcsBCD phosphorelay system is a critical regulator of amylovoran, marked by the presence of an RcsB binding box in the promoter region of *amsG*. RcsB is a positive transcriptional regulator of the *ams* operon (129). Additionally, Lon protease, which affects the accumulation of RcsA/RcsB proteins, was found to be a negative regulator of amylovoran production, the activity of which is potentially suppressed by CsrA to induce positive regulation (68). ClpXP positively regulates *amsG* expression through the selective degradation of the alternative sigma factor RpoS. However, the deletion of both *clpXP* and *lon* led to increased amylovoran production (70). Additionally, among the described Hfq-dependent sRNAs, ArcZ and RprA are positive

regulators, whereas Hrs6 and OmrAB are negative regulators of amylovoran production (90, 144) (**Figure 3**). Finally, AmyR, a member of the YbjN family in enterobacteria, has been identified as a negative regulator of amylovoran production, which acts via an unknown mechanism (130). Collectively, these studies present evidence of the complex regulatory network involved in the transcriptional and phenotypic regulation of amylovoran production in *E. amylovora* and possibly reflect the necessity for some level of amylovoran to be present at all times, as amylovoran is a pathogenicity factor, and that increased levels of amylovoran are required during biofilm formation. The chemical composition of the xylem, including both organic and inorganic substrates, has a direct impact on biofilm development within the xylem vasculature. This has been reviewed in detail by Kharadi & Sundin (61).

Dispersal from Biofilms

Biofilms are formed by *E. amylovora* in xylem vessels of leaves located at shoot tips, i.e., at the origination point of a shoot blight infection (65). However, further systemic spread of the pathogen in apple trees appears to be through cortical parenchyma cell layers in most cases (16). This is evidenced by the emergence of ooze droplets from stem tissue as infections progress (111) and by the formation of cankers in some cases, particularly at stem–trunk branch junctions. There are a few reports of movement of *E. amylovora* through symptomless trees; in these cases, the bacteria may remain in xylem vessels (56, 82).

Following the buildup and maturation of a biofilm, dispersal is the culmination step that allows cells to be detached from the biofilm matrix and either resume the planktonic mode of growth or subsequently initiate formation of a new biofilm (99). Several lines of evidence indicate that biofilm dispersal is a critical step for the systemic movement of *E. amylovora* cells within the hosts. Although development of a matrix-enmeshed population within the host xylem vessels is advantageous to protect *E. amylovora* cells from the shear force of sap flow, host defense responses, and nutrient scarcity (64, 65), dispersal movement of biofilm cells enables the translocation of *E. amylovora* cells to cause systemic infection. Through scanning electron microscopy analyses of the longitudinal dissections of apple shoots inoculated with *E. amylovora* cells, progressing biofilms as discontinuous aggregates have been observed (55). This indicates that *E. amylovora* cells constantly transit between the sessile mode and the planktonic mode to allow establishment and reestablishment of biofilms for systemic infection of *E. amylovora* in xylem tissue of apple leaves. Another example of biofilm dispersal during *E. amylovora* pathogenesis is the observation of *E. amylovora* cells that break out from xylem vessels to the intercellular spaces of the surrounding cortical parenchyma tissue (19); this method of dispersal requires that dispersing cells also genetically reprogram back to the T3SS-mediated planktonic mode of infection.

Recently, the varied modulatory roles of an Hfq-dependent small RNA, RprA, in affecting virulence factors of *E. amylovora* were characterized (90). It was further determined that RprA activation promotes the dispersal of *E. amylovora* cells from biofilms in vitro, suggesting the importance of this sRNA in biofilm control and systemic infection of *E. amylovora* (90). Although involvement of sRNAs in regulating biofilm formation has been described in a wide range of bacterial species, e.g., *E. coli* and *P. aeruginosa* (6, 29), their direct relevance to biofilm dispersal remains largely enigmatic. In *P. aeruginosa*, expression levels of *rsmY* and *rsmZ*, which function to sequester RsmA (also known as CsrA), are significantly reduced in biofilm-dispersed cells compared with their corresponding planktonic counterparts (31), suggesting a role of these sRNAs in facilitating biofilm dispersal in *P. aeruginosa*. Similarly, quorum sensing–regulated sRNAs Qrr1–4 were transcriptionally activated at low cell density and ceased at high cell density, contributing to biofilm development in *V. cholerae* (7). Given that multiple *E. amylovora* sRNAs, except RprA, contribute

to biofilm formation or affect determinants of biofilms (104, 142, 144), it is likely that multiple sRNAs function collaboratively during dispersal of *E. amylovora* biofilm cells to contribute to further systemic infection within hosts.

Overall, biofilm formation requires a successful initial transition to the attached lifestyle, the subsequent management and maturation of robust multilayered biofilms within the xylem vessels through the regulation of EPS production, and a final step of detachment from a biofilm to proliferate into newer biofilms, thus advancing infection within the host.

CYCLIC DI-GMP: A REGULATORY SWITCH COMPOUND CRITICAL FOR *ERWINIA AMYLOVORA* VIRULENCE

An important bacterial regulatory factor that drives the transition from a motile to a sessile lifestyle is the second messenger c-di-GMP (53). C-di-GMP is the primary positive regulator of biofilm formation in *E. amylovora* and an important negative regulator of the T3SS (40, 57). The synthesis of c-di-GMP, enzymatically characterized by the dimerization of two GTP subunits, is mediated by diguanylate cyclase enzymes that contain a conserved GGDEF domain necessary for the biochemical process of dimerization (53). Intracellular reserves of c-di-GMP can be enzymatically hydrolyzed into either the linearized molecule 5′-phosphoguanylyl-(3′,5′)-guanosine (pGpG) or GTP by specific phosphodiesterase (Pde) enzymes that encode an EAL domain or an HD-GYP domain, respectively (53). pGpG is then further degraded into compositional GTP units by the action of oligoribonucleases (89). The *E. amylovora* genome encodes five diguanylate cyclases (EdcA–E) and three Pdes (PdeA–C) and does not encode any HD-GYP proteins. Most of the Edc and Pde proteins encode additional sensory domains that are predicted to respond to external stimuli that then activate enzymatic function (40, 57). The counteractive use of the formative and degradative enzymes enables the regulation of intracellular c-di-GMP levels in response to signaling stimuli (53). Evidence in other bacterial systems has suggested the presence of cellular localization of c-di-GMP and a compartmentalized regulatory model downstream of c-di-GMP production (30).

A c-di-GMP null strain of *E. amylovora* (Ea1189Δ12), in which all 12 enzymatically active and degenerate proteins related to c-di-GMP metabolism were deleted, was unable to attach to surfaces and could not colonize the xylem in apple shoots (58). Thus, c-di-GMP is indispensable for surface attachment that initiates biofilm formation in *E. amylovora* (58) (**Figure 3**). Additional regulatory determinants affecting attachment in *E. amylovora* have been identified, including the chaperone protein Hfq and sRNA ArcZ (142, 144). The deletion of either *hfq* or *arcZ* resulted in a hyperattachment phenotype under in vitro static conditions, suggesting that ArcZ negatively regulates attachment during biofilm formation (144).

C-di-GMP is a negative regulator of the T3SS in *E. amylovora* and negatively regulates the transcription of *hrpL* (40, 57) (**Figure 2**). Elevated intracellular levels of c-di-GMP also led to a decrease in the translocation of the effector DspE in a tobacco leaf model (57). A total absence of all c-di-GMP metabolic components resulting in a lack of any intracellular c-di-GMP production led to elevated *hrpL* expression in vitro and increased infection in the immature pear model (58). The observation that c-di-GMP is a negative regulator of the T3SS in *E. amylovora* is similar to that found in many other bacterial pathogen systems; however, the mechanisms behind this negative regulation have not been elucidated to date in most of these systems.

Genetic reductionist studies involving *edc* and *pde* genes involved in c-di-GMP metabolism have highlighted some specific regulatory effects of the enzymes encoded by these genes. EdcC and EdcE were found to be strong positive regulators of amylovoran and cellulose production, which in turn impacted the overall virulence in planta and biofilm formation in vitro (27, 40).

Elevated intracellular c-di-GMP levels resulting from the deletion of multiple *pde* genes had a positive impact on biofilm formation in vitro (57). However, a severe increase in c-di-GMP levels as those observed in a complete *pde* knockout in *E. amylovora* (Ea1189Δ*pdeABC*) resulted in an autoaggregation phenotype in which *E. amylovora* cells were tightly bound in clusters (59). Autoaggregated cells showed some reduction in overall biofilm formation under static and flow-based conditions. Autoaggregation was found to be partially dependent on amylovoran and cellulose production, the EPSs that grant the aggregates structural integrity (59). Additionally, EagA, a peptidoglycan hydrolase of the metallopeptidase class, was recently shown to regulate autoaggregation potentially through its effect on cell membrane dynamics and peptidoglycan distribution (60). An additional outcome of the work with *eagA* was the discovery that high levels of c-di-GMP transcriptionally regulate *znuAeagA* and *znuBC* operons; ZnuABC functions in zinc uptake (60). The functional impact of c-di-GMP on zinc uptake in the context of infection within the xylem is yet to be explored.

One of the mechanisms by which c-di-GMP is involved in the transcriptional regulation of the *ams* operon is through the effect of c-di-GMP on the Csr system. CsrD (RNaseE specificity factor) binds c-di-GMP when the intracellular levels of the second messenger were high (R.R. Kharadi & G.W. Sundin, unpublished results). This c-di-GMP–bound CsrD degrades the sRNA CsrB more efficiently, potentially resulting in an increased proportion of unbound CsrA, which would otherwise be sequestered by CsrB binding to it. This ultimately leads to increased *amsG* transcription (**Figure 3**). CsrA was previously shown to be a positive regulator of amylovoran production (3), which corroborates this result. The two-component system GrrS/GrrA also regulates amylovoran production through CsrB regulation (3).

PATHOGEN DISPERSAL AND OVERWINTERING SURVIVAL

Although the systemic spread of *E. amylovora* in the form of biofilms developed in the xylem vasculature (with roots and shoots) is critical for the internal proliferation and spread of infection within an already infected host, extrusion from the host to enable spread within and between hosts is a critical factor determining the overall extent of an outbreak in the field mediated by secondary infection. Ooze (emerging either from cankers or shoot tissue) is the predominant source of inoculum that affects the spread of *E. amylovora* infections during the growing season (111).

Bacterial Ooze

There is direct and model evidence that EPSs produced by *E. amylovora* absorb water and cause swelling (105–107). This swelling leads to the bacteria moving through internal spaces without the physical force of the vascular system. There is evidence that this pressure is responsible for the exudation of ooze droplets in asymptomatic tissue (106, 111, 141). The pressure hypothesis is dependent on certain weather conditions, mainly surrounding ambient temperature and relative humidity, and when the water potential of the host increases (78, 107, 122).

Slack et al. (111) investigated the dynamics of ooze formation in the context of a field-wide outlook as well as the mechanobiology of ooze emergence within shoot tissue. Ooze emerging from the shoot shows gradation in coloration and droplet size. Although the average bacterial concentration within ooze droplets was $\sim 10^8$ CFU/μL, darker-colored ooze droplets typically had higher than average cell counts and a smaller volume. Within infected shoot tissue, ooze was found to be exuded through tears in the shoot tissue that formed localized bulges due to the internal pressure differential based on the presence of yet to emerge ooze fluid. These wounded sites were the main site of ooze exit in shoots. EPS, mainly in the form of amylovoran, is the other major component of ooze droplets, aside from the bacterial cell load (13). Ooze fluid trapped

within the shoots just upstream of the site of emergence showed elevated transcription of *hrpL*, *dspE*, and *amsK*, indicating a generalized positive regulation of virulence factors coinciding with ooze emergence, relative to in vitro conditions (111). Kharadi et al. (57) found that c-di-GMP was a positive determinant of ooze development in infected immature pear fruit. The deletion of each of the individual *pde* genes resulted in an elevation of ooze volume and the amylovoran content of the ooze droplets in immature pear (57). Although the regulatory pathway of c-di-GMP–mediated regulation of amylovoran production is known to be mediated through the CsrA/CsrB system, regulation has not been specifically validated in the context of an in planta/fruit model (58). Overall, considering that cells contained in ooze are not representative of cells actively in a biofilm or recently detached from a biofilm, several unanswered questions remain about the dynamics of internal formation of ooze, movement of ooze through shoot tissue, and the relative temporal synchronicity with the formation and spread of biofilms within the xylem.

Cankers

Cankers are a relatively underexplored area of the fire blight disease cycle. Cankers represent the primary overwintering location for *E. amylovora*, but there has been little progress since the late 1970s in understanding the dynamics of pathogen survival within cankers and the transition to the infective phase (10). This partially results from inconsistencies in the ability of cankers to remain active overwinter and still maintain viable *E. amylovora* inoculum in the spring. The genetics of canker formation and the host and environmental cues that trigger canker formation are completely unknown. Recently, Santander et al. (101) developed a digital viability PCR approach that utilized propidium monoazide to enable live/dead screening of cells acquired from cankers. This method has the potential to aid in determining the relative levels of inoculum prevalence and exploring the etiological factors that impact the survivability of *E. amylovora* cells in cankers. *E. amylovora* cells persisting in cankers face multiple physical environmental pressures over an extended period of time. This raises several interesting questions about the physiological status of *E. amylovora* cells over extended time periods in cankers and the regulatory networks that enable their viability over time.

CONCLUSIONS: THE PATH AHEAD TO DISEASE MITIGATION

Over the past decade, the vast expansion in research pertaining to the specific nature of the *E. amylovora* disease cycle has revealed several virulence determinants beyond the well-established factors of T3SS-mediated infection and effector biology. Landmarks during this time period have included the exploration of new regulatory virulence determinants, including sRNAs, c-di-GMP, and several two-component signaling systems (40, 57, 144, 148). Several new fitness determinants that aid in successful infection were identified, including amino acid auxotrophy, the Csr regulatory system, and toxin–antitoxin systems (3, 63, 110). Through the use of advanced microscopy, biomarkers, and layers of genetic reductionist studies, greater mechanistic clarity has been achieved in understanding the processes of floral colonization and biofilm development (35, 58). Through the use of next-gen omics approaches, the link of the state of the floral microbiome to the initial establishment of infection has been highlighted (33, 34). Also, the use of transcriptomic and proteomic approaches during the infection process helped decipher the regulatory complexity underlying a successful infection (50, 93).

With this newly acquired vast body of knowledge, we are left with several unanswered questions to address with future research. In the immediate future, there is a need to integrate and generate a regulatory hierarchy map for the multiple inputs that feed into regulating critical steps of the infection process, including floral infection, apoplastic infection, and biofilm formation.

Furthermore, a greater emphasis on including environmental conditions both inside and outside the host, including ambient weather measures as well as the substrate/ionome composition of the flower apoplast, xylem, root, and fruit tissues during the infection process, will help to provide some context to the temporal dynamics of regulatory variability. Complementary to the targeted genetic studies, the concurrent use of transcriptomic, proteomic, and metabolomic approaches in the context of both the host and the pathogen can potentially become a high-throughput method to highlight several regulatory pathways with any potential overlap, leading to the creation of several new avenues of investigation, and this can also expose any evolutionary limitations in host–pathogen interaction that can be exploited to enable disease mitigation in the future.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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