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REVIEW

Burning questions for fire blight research: I. Genomics and evolution of *Erwinia amylovora* and analyses of host-pathogen interactions

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Abstract
Fire blight, caused by the bacterial pathogen *Erwinia amylovora*, continues to be a devastating disease affecting commercial apple and pear plantings in almost all areas of the world, with recent incursions into Korea and China. During the past two decades, significant gains in knowledge of *E. amylovora* and fire blight disease have been achieved, in topic areas such as genetic and genomic diversity, host-pathogen interactions, host resistance, and disease management. As we look forward to the next two decades and beyond of fire blight research, we summarize the current research knowledge in topics focused on *E. amylovora* pathogen and population biology and propose research questions that we hope can guide the field forward to gain the necessary understanding that will lead to sustainable management of this disease.

Keywords Biofilms · Blossom blight · CRISPR genotyping · pEA29 · Systemic acquired resistance

Introduction

Fire blight, caused by the bacterial pathogen *Erwinia amylovora* (Burrill; Winslow et al. 1920), is a significant disease affecting commercial pome fruit production and native Rosaceae trees. By 2023, fire blight has spread to most pome fruit-producing countries globally, with notable exceptions of Australia and countries in South America. The most recent incursions of fire blight have been into Korea and China (Myung et al. 2016; Sun et al. 2023), and fire blight is also threatening native *Malus sieversii* forests in central Asian countries such as Kazakhstan (Maltseva et al. 2023). Since 2003, one book and several important reviews have been published that address fire blight epidemiology and disease management, resistance breeding, host-pathogen interactions, and genomics (Norelli et al. 2003; Oh and Beer 2005; Smits et al. 2011, 2017; Malnoy et al. 2012; Van der Zwet et al. 2012; Vrancken et al. 2013; Pique et al. 2015; Emeriewen et al. 2018; Kharadi et al. 2021; Peil et al. 2021; Yuan et al. 2021a; Zeng et al. 2021; Pedroncelli and Puopolo 2023). In this perspectives paper, our objective is to summarize current research findings and provide context in topic areas including genome diversity, evolution, infection biology, and host-pathogen interactions of *E. amylovora* that lead us to propose research questions that we think will propel the field forward towards an ultimate goal of sustainable management of fire blight.

Genome diversity

Earlier studies examining the genetic diversity of *E. amylovora* conveyed the impression of a highly homogeneous species with nucleotide identities among the studied genomes often exceeding 99.99% (Smits et al. 2010a), corresponding to pairwise differences as

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low as 7.37E-05 (Parcey et al. 2020) (Box 1). We know now that these results were largely biased by the choice of the isolates that were analyzed, which was mostly restricted to the widely prevalent (WP) clade of Amygdaloideae-infecting *E. amylovora,* a group that originates from a genetic bottleneck caused by a limited number of worldwide dissemination events from the East Coast of the United States during the 20th century (Rezzonico et al. 2011; Kurz et al. 2021). When considering the overall variability of the species in North America, the center of origin of the disease, the genetic diversity revealed itself to be noticeably higher, with pairwise differences up to 1.43E-02 between *Rubus*-infecting (R-group) and Amygdaloideae-infecting isolates and 2.40E-03 within the B-Group superclade that contains strains infecting both types of hosts (Parcey et al. 2020). Within the WP clade, the natural mutation rate appears to be insignificant, leaving strains that were recovered several decades apart noticeably genetically unchanged. Taken together, the above observations suggest that the driving force for genetic diversity within *E. amylovora* is mainly host selection (Parcey et al. 2020), which largely occurred prior to the encounter with domesticated pomaceous species.

Why is the genomic diversity of *Erwinia amylovora* so narrow?

What does the current geographical distribution of the three major Amygdaloideae-infecting clades in North America tell us about the possible primeval host species for *E. amylovora*?

Why does *E. amylovora* appear to be a highly specialized pathogen if it evolved separately from its current host?

What factors are relevant for host specificity?

How and when did *E. amylovora* adapt to *Rubus* spp.?

Box 1 Burning questions—genetic diversity and host adaptation

Genetic diversity in North America

There are essentially two possibilities to explain the current geographical distribution of the three major Amygdaloideae-infecting clades of *E. amylovora* in North America. In the first scenario, domesticated apples imported from Europe were first infected with fire blight by the WP clade of *E. amylovora* on the East Coast, then the pathogen evolved into the Eastern North American (ENA) and Western North American (WNA) clades while traveling westward with the settlers and their newly planted orchards. Considering the low mutation rate of the species and the short time elapsed, this hypothesis is, however, highly improbable. A more plausible explanation is that, during the colonization of the North American continent, the domesticated apple trees were infected by the already genetically differentiated resident populations of *E. amylovora* that were adapted to the local plant species with which they were coevolving (Box 1).

It is particularly intriguing to notice here that the geographical distribution of the Amygdaloideae-infecting clades of *E. amylovora*, based on whole genome analysis and CRISPR typing (Parcey et al. 2020, 2022), roughly matches that of the main species of crabapples species that are native to North America (*Malus coronaria, Malus fusca, Malus ioensis* and *Malus angustifolia*) (Fig. 1) and it is thus tempting to speculate that such transfer (or from other potential host species such as mountain ash, hawthorn or *Amelanchier*) may have been the mechanism behind the current distribution of genetic diversity observable in *E. amylovora* isolated from domesticated apples. Considering the paucity of observations about the presence of fire blight in American crabapples and the possible lack of evident symptoms in infected trees, this hypothesis is not easy to test. However, phenotypic evaluation of a natural fire blight outbreak in the USDA *Malus* spp. collection caused by an isolate belonging to the WP clade of *E. amylovora*, which is prevalent in the eastern United States, showed that the disease severity in pacific crabapple (*M. fusca*) trees was higher than the severity recorded on *M. coronaria* and *M. angustifolia*, two species that are also native of the Atlantic area (Dougherty et al. 2021). Thus, extensive cross-testing of the susceptibility of the main crabapple species with representative isolates of the major clades of Amygdaloideae-infecting strains is proposed to provide interesting clues on a possible past coevolution between the different *Malus* host species and the *E. amylovora* pathogen.

Host-pathogen coevolution

The evolution of host specificity in a pathogen usually involves a complex interplay of genetic and molecular factors that can only arise if the two organisms had the chance to coevolve. However, since North America is the center of origin of fire blight and domesticated apple species are native of Central Asia, such coevolution apparently did not have the opportunity to occur (Box 1). *E. amylovora* can infect and thrive only on a relatively narrow range of host plants and can be divided in two major groups that can be distinguished genetically (Rezzonico et al.

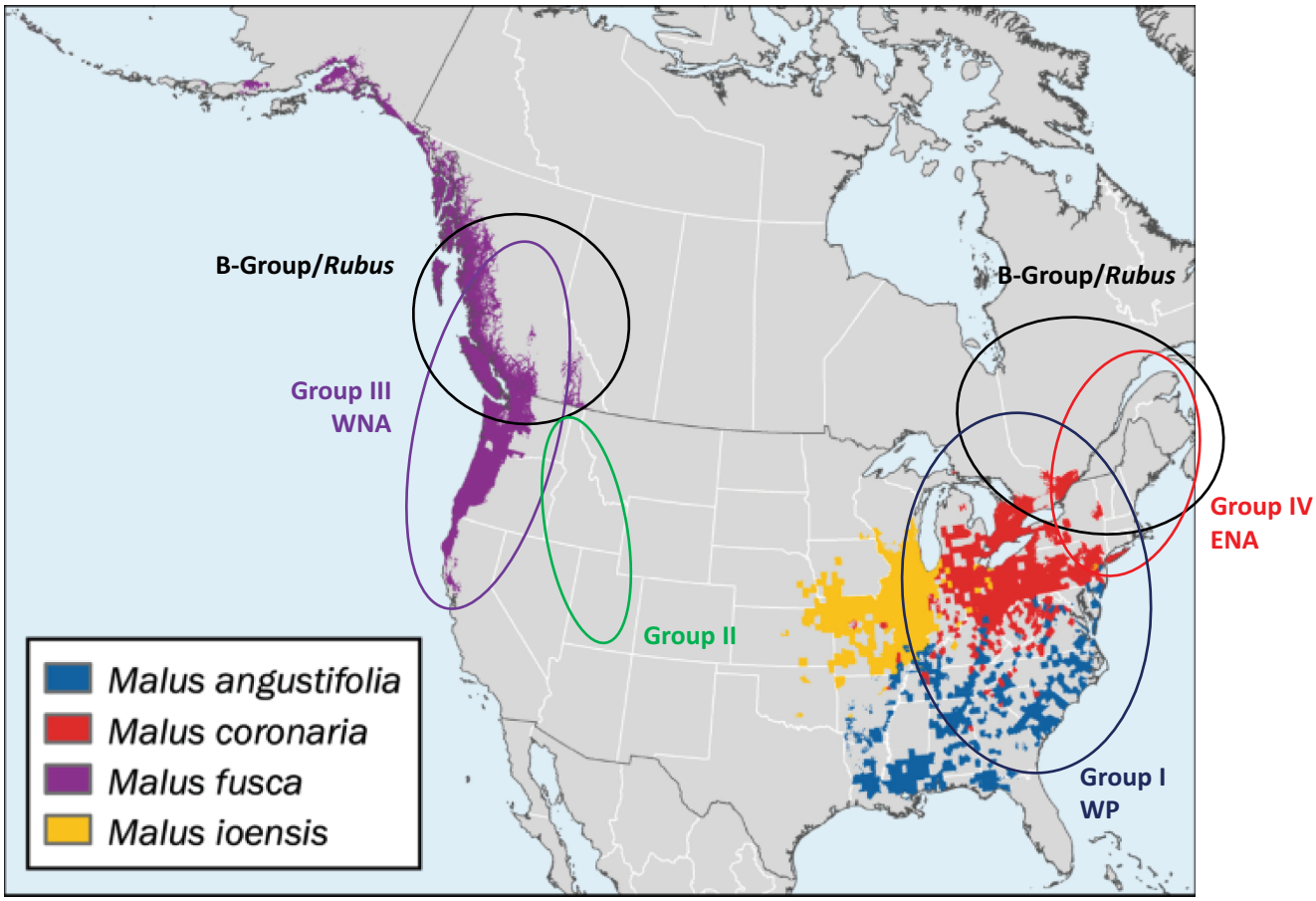

Fig. 1 Distribution of the main native crabapple species (Volk 2019) and presence of the main CRISPR genotypes of *E. amylovora* in North America. Genotype distribution is approximately displayed using data collected from several publications (Rezzonico et al. 2011; McGhee and Sundin 2012; Mann et al. 2013; Parcey et al. 2020). Figure adapted from Volk (2019)

2012). These groups are the Amygdaloideae-infecting clade, which is mainly found in apple, pear and quince but can also infect some other plants belonging to other genera within the Rosaceae family like *Sorbus, Crataegus, Amelanchier* or *Pyracantha* (Puławska and Sobiczewski 2012), and the *Rubus*-infecting clade that is specialized on blackberries, raspberries, and related species (Powney et al. 2011). Cross-pathogenicity between the two clades and their respective hosts appears to be extremely limited (Ries and Otterbacher 1977; Asselin et al. 2011) and host specialization on *Rubus* is mainly due to a series of different genetic adaptations by the corresponding isolates such as the acquisition of a different set of lipopolysaccharide biosynthesis genes (Rezzonico et al. 2012), or the loss of a number of gene clusters responsible for degradation of phenolic and sulfur compounds, and the metabolism of L-arabinose (Sprecher 2021).

Another relevant difference is the type three secretion system (T3SS) effector Eop1, whose gene nucleotide sequence in *Rubus*-infecting strains of *E. amylovora* clearly diverges from that of the Amygdaloideae-infecting clade or from other species that mainly infect Asian pear like *E. pyrifoliae* (Asselin et al. 2011; Mann et al. 2013; Smits et al. 2017) (Fig. 2). Taken together, these facts suggest that the primary adaptation event for these *Erwinia* species to the Rosaceae, and in particular to apple and pear, occurred during the pathoadaptation process of their common ancestor (Kamber et al. 2012; Smits et al. 2013) and that the genetic adaptation to *Rubus* plants was a stepwise process that subsequently affected only the R- and part of the B-group of *E. amylovora* through convergent evolution (Sprecher 2021). Another indication that *E. amylovora* coevolved in the past with *Malus* species is given by the fact that all four crabapple species native to North America exhibit strong resistance phenotypes against fire blight disease (Dougherty et al. 2021). A major quantitative trait locus (Emeriewen et al. 2014) and its related candidate genes for fire blight resistance (Emeriewen et al. 2018, 2022; Mansfeld et al. 2023) have been identified in pacific crabapple *M. fusca*, which was shown to be genetically more similar to *Malus* species of Asiatic origins than Eastern North American species based on chloroplast DNA analysis (Volk et al. 2015). One of such Asiatic species is *M. sieversii*, considered to be a progenitor of commercial apple (*Malus* × domestica)

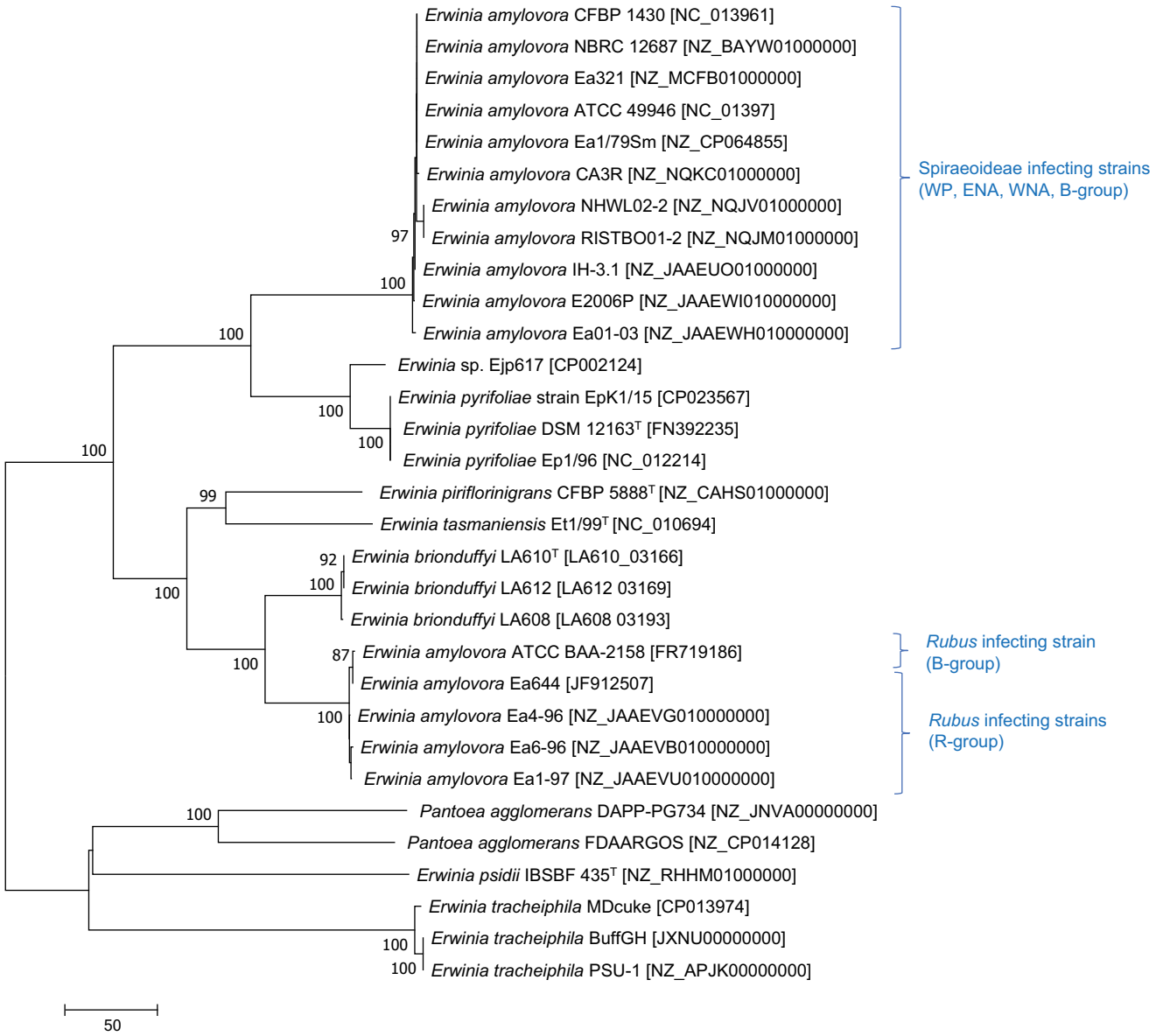

Fig. 2 Phylogenetic analyses of the type III secretion system gene *eop1* from strains of different species within the genus *Erwinia*. *Rubus*-infecting isolates cluster separately from their Amygdaloideae-infecting counterparts suggesting convergent evolution of some strains of the R- and B-group that lead to an adaptation process to this new host within *E. amylovora*. Taxonomy was inferred implementing the Neighbor-Joining method in MEGA-7 (1000 bootstrap replications; only values above 70% are shown)

(Velasco et al. 2010). Thus, there is solid genomic evidence for a long history of coevolution between apples and the fire blight pathogen that even precedes the establishment of *E. amylovora* itself as a species. During the domestication process, however, these traits appear to have inadvertently been eliminated from the genome of the commercial apple.

Infection biology of *E. amylovora*

We started this perspectives article by discussing our current knowledge of topics relevant to *E. amylovora* (genetic diversity, geographical distribution, host-pathogen coevolution) that most likely arise from the fact that *E. amylovora* is a pathogen and that humans presented this pathogen with hosts that were more susceptible to fire blight disease than the hosts the bacterium evolved on, and on which likely orders of magnitude larger populations could be established. This has continued to the present day with the deployment of modern agricultural high-density planting systems that offers highly-susceptible tree hosts planted in large numbers (ca. 3700 trees per hectare) over wide geographic scales. Likewise, our knowledge of the infection biology and the genetics of host-pathogen interactions of *E. amylovora*, covered below, has also mostly been studied on modern highly-susceptible cultivars of domesticated

apple. We will also cover other distinct topics of recent interest in *E. amylovora* biology and virulence, including the ubiquitous plasmid pEA29, the occurrence of differential strain aggressiveness, *E. amylovora*-insect interactions, and the CRISPR-Cas system.

Flower infection

Infection of flowers resulting in the blossom blight symptom is a critical early phase of fire blight infection that can fuel significant disease epidemics. The stigma tip of Rosaceae flowers represents an optimal habitat sustaining rapid growth of *E. amylovora* under conducive environmental conditions (Thomson 1986; Slack et al. 2022) that provides cell populations that migrate to the flower hypanthium and infect through natural openings in nectaries (Farkas et al. 2012). Eliminating or suppressing *E. amylovora* growth on flower stigmas represents a central strategy for fire blight disease management. The flower stigma tip of Rosaceae plants is firstly an epiphytic habitat for *E. amylovora* and other microbes, but also represents an initial site for host-pathogen interactions and a site for microbe-microbe interactions within the floral microbiome. Growth of *E. amylovora* on stigmas is dependent on the availability of sugar exudates, environmental factors such as temperature and high relative humidity, and cell arrival to flowers that have been open for three or fewer days (Pusey 2000; Pusey and Smith 2008; Slack et al. 2022).

Although the flower stigma tip is an external plant habitat, a recent study indicated that approximately 50-70% of *E. amylovora* cells expressed type III secretion system (T3SS) genes and translocated the DspE effector into stigma papillae cells (Cui et al. 2021a). It was also previously shown that mutation of the T3SS regulator *hrpL* resulted in a 2-fold reduction in *E. amylovora* population size on flower stigmas (Johnson et al. 2009). A few other genetic studies have identified or examined specific genes that contribute to virulence during flower infection (Pester et al. 2012; Schachterle et al. 2022). It is also important to note that *E. amylovora* cells at stigma tips are also interacting with the apple flower microbiome, which could potentially affect disease outcome. Recent assessments of the apple flower microbiome have shown that the population size of *E. amylovora* on apple flower stigmas is not predictive of disease outcome, suggesting that the natural microbiome might be impacting pathogen activity (Cui et al. 2021b). This work was further substantiated with a study showing that the flower microbiome can be manipulated with inoculated microbes which may lead to fire blight disease suppression (Cui et al. 2021c).

The complexity of the flower phase of fire blight has been underappreciated, and expanded knowledge of the basic biology and ecology of flower infection will be critically important for the long-term sustainability of new non-antibiotic disease management interventions (Box 2).

What are the molecular bases of flower colonization and infection, and can this be manipulated from a disease management perspective?

What environmental signals does *Erwinia amylovora* perceive to regulate virulence genes on flower stigmas?

How does the flower microbiome impact *E. amylovora* growth on stigmas, movement to nectaries, and infection?

Can the flower (stigma) environment be manipulated to enhance the efficacy of biological control agents?

Box 2 Burning questions—flower infection

Shoot infection and canker formation

Systemic spread of *E. amylovora* through infected trees is a critical component of fire blight disease because: (i) *E. amylovora* kills branches as it moves through them; (ii) ultimately, *E. amylovora* migrates to rootstock crowns and can form cankers that kill the entire scion; (iii) ooze emergence from infected branches is very common, potentially furthering the spread of *E. amylovora* cells between trees (Slack et al. 2017); and, (iv) *E. amylovora* sometimes forms cankers, which are an overwintering site for the pathogen, and a source of primary inoculum from ooze emerging the following season (Van der Zwet et al. 2012). Some cankers also survive season-to-season, ultimately girdling and killing the branch they are associated with.

While *E. amylovora* does form biofilms in leaves at shoot tips during shoot blight infection (Koczan et al. 2009; Castiblanco and Sundin 2018), systemic spread of *E. amylovora* in branches is almost entirely accomplished by spreading through cortical parenchyma cell layers, a location where virulence is mediated by the T3SS (Billing 2011; Kharadi et al. 2021). As movement through infected branches is enabled by T3SS-mediated pathogenesis, management of shoot blight infection can be accomplished via application of acibenzolar-*S*-methyl, an inducer of systemic acquired resistance (Johnson and Temple 2016; Yuan et al. 2023), or with prohexadione-calcium (ProCa), a growth inhibitor that results in thickened plant parenchyma cell walls that inhibit T3SS-mediated infection (McGrath et al. 2009). ProCa has also recently

been shown to induce a SAR response in apple (Yuan et al. 2023).

The canker phase represents the least studied and least understood component of fire blight disease. Cankers are formed on woody tissue of infected branches, trunks of trees, and rootstocks (Van der Zwet et al. 2012) and consist of infected tissue that becomes surrounded by a suberized layer of host cortical parenchyma cells (Biggs 1994; Santander et al. 2022). Cankers formed at the scion-rootstock junction of apple will typically kill trees within 1–2 years (Norelli et al. 2003; Acimovic et al. 2023).

An increased understanding of the genetic bases contributing to the systemic movement of *E. amylovora* through infected shoots is expected to help optimize the deployment of SAR-based management and could identify new targets for novel disease management strategies (Box 3). In addition, studying the physical aspects of the movement of *E. amylovora* through branches and between younger and older branches on trees and the underlying aspect of host effects on movement is expected to provide practical information that will feed into optimized management strategies. The importance of cankers to the disease cycle of *E. amylovora* is clear, but we lack a mechanistic understanding of the contribution of pathogen and host to canker formation. Thus, studies of host and environmental factors that contribute to canker formation are needed and may yield information that can be used in canker-inhibition strategies in the future (Box 3).

What are the environmental, genetic, and host factors that are required for systemic movement through shoots, and between branches that differ in age?

What is the mechanistic basis for reductions in systemic spread of *Erwinia amylovora* in shoots of fire blight-tolerant cultivars? Can this knowledge be exploited for management in highly susceptible cultivars?

How are *E. amylovora* cells partitioned between active infection and ooze emergence in infected shoots?

What are the environmental, genetic, and host factors that stimulate canker formation?

What are the environmental, genetic, and host factors that stimulate the emergence of *E. amylovora* ooze from cankers in the spring?

Box 3 Burning questions—shoot infection and canker formation

Genetics and regulation of virulence during *E. amylovora*-host interactions

The amylovoran exopolysaccharide (EPS) capsule and the T3SS are essential pathogenicity factors required by *E. amylovora* to cause fire blight disease (Bugert and Geider 1995; Oh and Beer 2005; Oh et al. 2005; Kharadi et al. 2021). The T3SS effector DspA/E is also a pathogenicity factor, as Δ*dspA/E* mutants are nonpathogenic (Boureau et al. 2006). As described above, T3SS-mediated pathogenesis is essential for the infection of flowers and shoots. DspA/E is the major effector with both virulence function and a role in the suppression of host defense (Gaudriault et al. 1997; Debroy et al. 2004). At least five other type three effectors are translocated into host cells however, to date, only $avrRpt2_{EA}$ has been shown to have a role in virulence (Schröpfer et al. 2018; Zhao et al. 2006). The precise reason why amylovoran is required for pathogenesis is more unclear with the most prominent hypothesis being that the EPS capsule protects cells against recognition by the host plant and plant defense responses (Bugert and Geider 1995). All other studied phenotypes requiring amylovoran including biofilm formation, ooze production, protection against desiccation, nutrient acquisition, and sliding motility are virulence factors and are not required for pathogenicity (Geider 2000; Koczan et al. 2009; Slack et al. 2017; Yuan et al. 2022). Other virulence traits, although not directly required for pathogenesis per se, are involved in bacterial survival and metabolism during host colonization. For example, nutritional interactions also contribute to pathogenesis and host colonization by *E. amylovora*. For example, the ability to utilize sorbitol and production of the iron siderophore desferrioxamine are both required for full virulence, and the synthesis of arginine is absolutely required for pathogenesis (Aldridge et al. 1997; Delaggi et al. 1998; Ramos et al. 2014). Lastly, protection from reactive oxygen species (ROS) produced by host plants is critically important to pathogenesis, since *E. amylovora* cells induce a host defense response during the initiation of pathogenesis. Catalase enzymes, the lipopolysaccharide cell layer, and amylovoran EPS all contribute to protection from ROS toxicity (Berry et al. 2009; Santander et al. 2018; Schachterle et al. 2019a).

The regulation of virulence in *E. amylovora* is amazingly complex, most significantly because the deployment of specific virulence factors will differ based on physical location within the host. Like most bacterial plant pathogens, *E. amylovora* possesses multiple mechanisms for controlling the expression of individual virulence determinants including the utilization of an alternate sigma factor (Wei and Beer 1995), two-component signal transduction systems (TCSTs; Zhao

et al. 2009b), signaling systems based on the regulatory compound cyclic di-GMP (Kharadi et al. 2019, 2021, 2022), and post-transcriptional regulation by non-translated small RNAs (sRNAs; Zeng and Sundin 2014). In addition, virulence regulation can involve regulators that are multi-functional in the cell and regulate housekeeping-related functions. For example, the Rcs phosphorelay system regulates amylovoran exopolysaccharide biosynthesis and hundreds of other *E. amylovora* genes (Wang et al. 2012b), and the leucine-responsive regulatory protein Lrp regulates motility but is also a global regulator of amino acid biosynthesis (Schachterle and Sundin 2019). Finally, the complexity of virulence regulation is enabled because the different regulatory systems typically function collectively in controlling traits that are differentially required based on physical location in the plant host. For example, understanding the role of cellular motility in fire blight infection is notable because of the importance of motility for flower infection (Bayot and Ries 1986); however, *E. amylovora* loses flagella once inside the plant (Raymundo and Ries 1981; Bayot and Ries 1986; Cesbron et al. 2006; Holtappels et al. 2018), and systemic movement is accomplished by sliding motility, a phenotypic trait that requires the exopolysaccharides amylovoran and levan, but not flagella (Yuan et al. 2022). Currently known motility regulators in *E. amylovora* include the TCST FlhDC, the Hfq-dependent sRNAs ArcZ, OmrAB, and RmaA (Schachterle et al. 2019b), Lrp (Schachterle and Sundin 2019), and the IHF (integration host factor protein (Lee and Zhao 2016)), but it is not yet clear which of these regulators or others accomplish the switching between flagella on and off stages. Likewise, amylovoran exopolysaccharide biosynthesis is currently known to be regulated by the Rcs phosphorelay and potentially other TCSTs (Zhao et al. 2009b; Wang et al. 2012b), the cyclic di-GMP system (Edmunds et al. 2013), multiple Hfq-dependent sRNAs including ArcZ and RprA (Zeng and Sundin 2014; Peng et al. 2021), the Csr sRNA-based regulatory system (Ancona et al. 2016; Kharadi and Sundin 2022), Lon protease (Ancona et al. 2016), and the proteins AmyR and Hns (Hildebrand et al. 2006; Wang et al. 2012a).

The sheer number of different regulators converging on the control of a single trait suggests that the various regulators are responding to different environmental inputs and are likely functioning at different times and different locations in the host plant. Our ability to identify and understand how regulators perceive and respond to environmental signals may provide opportunities to manipulate the abundance of a perceived signal(s) that could reduce *E. amylovora* virulence and disease severity (Box 4).

What are the key environmental signals encountered by *Erwinia amylovora* during systemic infection, and can these signals be manipulated for disease management?

Why does *E. amylovora* switch to a non-motile state during shoot infection and how is this accomplished?

How does *E. amylovora* transition between T3SS-mediated infection and biofim formation during shoot infection?

What are the molecular bases of differential aggressiveness between *E. amylovora* strains if the genomic homogeneity is so high?

What is the ecological significance of differential aggressiveness in the *E. amylovora* population?

Box 4 Burning questions—molecular host-pathogen interactions, differential aggressiveness

The ubiquitous pEA29 plasmid

Whereas its circular chromosome is highly conserved with only a low number of nucleotide changes per genome, the pan-genome of *E. amylovora* is still considered to be open, mainly based on the presence of a diverse set of plasmids (Llop et al. 2011; Mann et al. 2013; Ismail et al. 2014; Smits et al. 2017; Parcey et al. 2020). Many of them are cryptic, highly related to plasmids present in other Enterobacteriaceae and, based on phenotypic tests, do not contribute to the pathogenicity of the species (Llop et al. 2011; Ismail et al. 2014). While their sequences do not allow the identification of known virulence factors, most of these plasmids are mobilizable as they contain *mob* and *tra* regions (Garcillán-Barcia et al. 2011), indicating that these plasmids can be transferred from the plasmid pool in the environment to *E. amylovora* and vice versa.

In contrast, the plasmid pEA29 is nearly ubiquitous (Smits et al. 2011; Parcey et al. 2020). Currently, only a very low number of strains are known that do not contain this plasmid, yet these strains retain full virulence (Llop et al. 2006). pEA29 is only ~29 kb, and contains an IncF-type replicon, which renders it not mobilizable, as both the *mob* and *tra* regions are lacking. The pEA29 plasmid contains two repeat regions, which are commonly used in the VNTR scheme that can be used for population studies (Schnabel and Jones 1998; Kim and Geider 1999; Bühlmann et al. 2014). The only function that was so far identified on the pEA29 plasmid is the *thiOSGF* gene

cluster that is potentially involved in thiamine biosynthesis (McGhee and Jones 2000) (Box 5). This trait is shared not only with similar plasmids in other pathoadapted *Erwinia* spp. (Kamber et al. 2012; Barbé et al. 2013; Smits et al. 2013), but also with other members of the Erwiniaceae, where the corresponding plasmid LPP-1 can be up to 750 kb (Gantotti and Beer 1982; De Maayer et al. 2012; Rezzonico et al. 2016). Although natural loss of the large LPP-1 in *Pantoea vagans* C9-1 and in *Pantoea agglomerans* was reported when strains are placed under stress (Lindh et al. 1991; Smits et al. 2010b), this phenomenon has not been described for *E. amylovora* strains. However, it cannot be excluded that the loss of pEA29 in *E. amylovora* UPN527 and related strains is an artefact caused by laboratory conditions. A major reason for maintaining the plasmid may thus be that it allows the biosynthesis of thiamine. However, as the strains lacking this plasmid are still viable and pathogenic (Llop et al. 2006), it can be presumed that they are able to take up sufficient thiamine from the plant host. It has recently been shown that a complete thiamine biosynthetic pathway, including the *thioGSF* operon on pEA29, is required for full virulence of *E. amylovora*, and that thiamine enhanced the activity of the tricarboxylic acid cycle and bacterial respiration which provides the energetic requirements for the biosynthesis of the amylovoran EPS (Yuan et al. 2021b). Furthermore, it was observed from the number of reads generated during sequencing that the copy number of pEA29 must be between one and four copies per cell (Mann et al. 2013). An increased copy number of the *thiOSGF* cluster may enhance the transcriptional level of the gene cluster, thus enabling *E. amylovora* to produce the required amount of thiamine during host infection.

What is the role of the two inv/spa T3SS systems in *Erwinia amylovora*?

Why is pEA29 nearly ubiquitous and what is its ultimate function(s)?

Is the CRISPR/Cas system still functional and what are its roles in *E. amylovora*?

Box 5 Burning questions—life cycle

Molecular bases of differential aggressiveness of *E. amylovora*

Genomic analyses have revealed an extremely low level of nucleotide sequence differentiation among Amygdaloideae-infecting strains of *E. amylovora* (Parcey et al. 2020; Singh and Khan 2019; Zeng et al. 2018). In addition, although the pan-genome of *E. amylovora* contains numerous plasmids, besides the ubiquitous plasmid pEA29, the other sequenced plasmids from *E. amylovora* remain cryptic and are not known to encode any genes that affect virulence or ecological fitness (Llop et al. 2012). Despite the close genomic similarity of global *E. amylovora* strains, strains are known that differ greatly in virulence, and there are some instances of strains known that differ in the quantity of production of specific exopolysaccharides (Bereswill et al. 1997; Roach et al. 2013).

Factors contributing to differences in virulence have been investigated, but this topic remain an open question. A phenotypic analysis of virulence differences among six *E. amylovora* strains indicated that characters such as amylovoran production, biofilm formation, elicitation of the hypersensitive response, sorbitol utilization, and growth in immature apple fruit accounted for > 75% of the variation in disease severity observed in a shoot blight test on apple cv. Gala (Lee et al. 2010). In another study, Wang et al. (2010) observed some differences in expression of amylovoran and T3SS genes in *E. amylovora* strains differing in virulence, but did not identify the genetic bases of differences. Zeng et al. (2018) demonstrated that the low virulence *E. amylovora* strain CTBT1-1 had a single nucleotide polymorphism (SNP) conferring an amino acid substitution in the *hfq* gene, which was shown to impact virulence, stressing the importance of the Hfq sRNA system in impacting virulence. The occurrence of SNPs in type III effector genes and in the pEA29 plasmid have also been postulated to affect strain virulence (Vogt et al. 2013; Singh and Khan 2019). Below, we present one instance (Mendes et al. 2021) in which *E. amylovora* own CRISPR/Cas system may have been involved in the regulation of pathogenicity levels. The maintenance of low virulence strains in populations could be through complementation during infection. It has been demonstrated that co-inoculation of apple shoots with two nonpathogenic *E. amylovora* mutants (amylovoran and T3SS deletion mutants) resulted in infection and systemic movement by both of the mutant strains (Zhao et al. 2009a). However, it remains an open question how/if differential virulence contributes to the environmental fitness of *E. amylovora* (Box 4).

Role of insects within the life cycle of *E. amylovora*

Genomic analysis has revealed three T3SSs in *E. amylovora*. One of the bacterial key plant virulence factors is delivered by the hypersensitive response and pathogenicity (Hrp) T3SS, which translocates the DspA/E effector protein into plant-host cells, where it suppresses cell wall-based defenses (Boureau et al. 2006).

Two additional T3SSs (Inv/Spa-1, Inv/Spa-2) display a significantly lower mol% G+C content with respect to the surrounding genome and were probably acquired during pathoadaptation by the common ancestor of *E. amylovora, E. pyrifoliae, E. piriflorinigrans* and *E. tasmaniensis* (Smits et al. 2011, 2013). Phylogeny analysis demonstrated high sequence identity of these T3SSs with those of *Pantoea stewartii* subsp. *stewartii* (Correa et al. 2012) and of the insect and animal pathogens *Sodalis glossinidius* and *Yersinia enterocolitica*, respectively (Zhao et al. 2009a; Smits et al. 2010a). Analysis of deletion mutants of both Inv/Spa-1 and Inv/Spa-2 in immature pear fruits and apple seedlings indicated that neither of those systems is involved in direct interaction with the host plant or has a beneficial effect on the growth of *E. amylovora* (Zhao et al. 2009a) (Box 5). On the other hand, in the *S. glossinidius*/tse-tse fly system, the corresponding Inv/Spa-1 and Inv/Spa-2 are required to establish infection (Dale et al. 2001) and to replicate once established intracellularly in the host, respectively (Dale and Moran 2006). It is thus reasonable to assume that the same two T3SSs are likewise required by *E. amylovora* for interaction with insect hosts that are used as vectors to move from one host plant to the other. This part of the bacterial lifecycle remains, however, largely unexplored. It seems highly probable that *E. amylovora*-insect interactions may have been important in natural systems where hosts were not located in close proximity. The association of *E. amylovora* with different types of insects such as honeybees, aphids, leafhoppers (van der Zwet and Keil 1979) or fruit flies (Ark and Thomas 1936; Ordax et al. 2015) has repeatedly been demonstrated, although the involvement of either of the two Inv/Spa T3SS has never been directly evaluated. Although this question may apparently seem purely academic, understanding and possibly weakening the interaction of *E. amylovora* with its insect vectors could help reduce the spread of fire blight by limiting the impact of secondary infections.

The CRISPR/Cas system of *E. amylovora*

The CRISPR/Cas system confers acquired heritable immunity against invasive mobile genetic element (IMGEs) in prokaryotes, restraining horizontal gene transfer of plasmids and phage infections (Rezzonico et al. 2011). The level of CRISPR-Cas activity within *E. amylovora* needs to be evaluated to determine the risk of resistance or immunity to phage biocontrol. Genome sequencing disclosed the presence of three CRISPR repeat regions (CRRs) and one Type I-E CRISPR-associated (Cas) gene cluster in *E. amylovora*. Comparative genomics revealed that CRR4 is a essentially inactive remnant that only displays a limited number (≤7) of mostly invariable spacers and that was originally associated to a now deleted Type I-F Cas gene cluster, which is still present in cognate species *E. pyrifoliae, E. tasmaniensis* and *E. piriflorinigrans* (Rezzonico et al. 2011; Smits et al. 2013; Parcey et al. 2022). In the last decade, CRISPR typing has been widely applied for population genetics studies within *E. amylovora* at various geographical levels, as CRRs display greater variability compared to the coding regions of the genome (Rezzonico et al. 2011; McGhee and Sundin 2012; Tancos and Cox 2016; Mendes et al. 2021; Kurz et al. 2021; Parcey et al. 2022), thus allowing a resolution level that can be surpassed only by whole-genome sequencing.

Functions of CRISPR/Cas system

There are two main mechanisms that contribute to the plasticity of the CRRs: acquisition of new spacers at the 3′-end of the array following the encounter with a new IMGE such a virus or a plasmid, or the deletions/duplications of internal spacers that are functional to the regulation of the array length, preventing an excessive elongation that may be detrimental to the fitness of the cell (Garrett 2021). Almost the totality of the CRRs variability so far found in *E. amylovora* is to be attributed to the latter mechanism. This is particularly true when analyzing the sequence of isolates of the WP clade retrieved outside North America: over a time span of almost seven decades only two isolates were recovered that showed the incorporation novel spacers next to the leader sequence of the array (Rezzonico et al. 2011; Mendes et al. 2021) (Box 5).

A new spacer was found in the CRR2 of strain Ea680, which was isolated in 2015 from Rocha pear in Portugal. Surprisingly, this spacer targeted an intergenic region within the genome of *E. amylovora* itself (Mendes et al. 2021). Although uncommon, and often reported as toxic, self-targeting spacers have been hypothesized to regulate the expression of endogenous genes through a RNA interference mechanism (Devi et al. 2022). In the case of Ea680, the protospacer is situated in antisense orientation 124 bp upstream of start codon of the *ybaL* gene (CFBP1430, Eamy_1029) (Smits et al. 2010a), which is coding for a Kef family K^+ monovalent cation-proton antiporter, a gene that is 2.5x upregulated during plant infection after 24 h compared to in vitro growth in TY culture media (Puławska et al. 2017). Considering the position of the targeted sequence, a negative regulatory function of this spacer on *ybaL* expression cannot be excluded. This hypothesis

is reinforced by the fact that, among all thirty-six highly clonal isolates associated with the 2010–2017 outbreaks in Portugal, Ea680 caused the weakest symptoms development in an immature pear slices assay seven days after infection (Mendes et al. 2021). Strain Ea263, retrieved in 1997 from *Cydonia oblonga* in Israel, displayed the acquisition of one spacer each in CRR1 and CRR2, which were both targeted against a conserved 38-Kb plasmid found across several Enterobacteriaceae species (i.e., *E. coli*, "*Mixta hanseatica", Klebsiella pneumoniae*). The correlation between the presence of a spacer and the absence of the related plasmid was previously demonstrated in WP strains Ea273/pEA72 (CRISPR group I) and UTFer2/pEU30 (CRISPR group II), in which the loss of spacers 2004 and 1022, respectively, seems to have allowed the acquisition of the associated plasmids (Rezzonico et al. 2011). On the other hand, in strains from the WNA clade (CRISPR group III), an accumulation of more than 30 spacers distributed between CRR1 and CRR2 and directed against pEU30 was not sufficient to oust the plasmid from the cell. A non-silent mutation Q20H in Cas8 protein, which plays a crucial role in the interference stage of the Type I-E CRISPR/Cas system, was initially hypothesized to be responsible for this disagreement (Rezzonico et al. 2011). However, through the use of two plasmid-borne artificial CRISPR arrays, Parcey et al. (2022) demonstrated that the failure of inducing CRISPR-Cas mediated interference in WNA isolates was not dependent from the Q20H mutation but from the presence of pEU30 itself, thus suggesting that the latter plasmid harbors one or more genes that encode for a factor that counteracts the CRISPR/Cas system.

In vitro challenging of *E. amylovora* with different phages resulted in resistant phenotypes but neither in an increased level of expression of the *cas* genes (Yagubi 2016) nor in the incorporation of new spacers in the affected strains, thus implying that other mechanisms than the CRISPR/Cas system may be prevalent in conferring phage resistance in the fire blight pathogen (Knecht et al. 2022; Parcey et al. 2022). Nonetheless, while the repertoire of spacers in Amygdaloideae-infecting strains is largely directed against plasmids, the fraction of spacers directed against phages is considerably higher in the *Rubus*-infecting clade, a fact that may be ascribed to the differences in niches and hosts that these isolates occupy (Parcey et al. 2022). It is thus clear, that despite the apparent relative low incorporation rate of new spacers, the Type I-E CRISPR/Cas system of *E. amylovora* is operational and absolves several functions such as protection from IMGE (mainly plasmids) and even gene regulation, but it is probably not the main source of phage resistance.

Conclusions and future directions

Fire blight remains a devastating disease that is exacerbated by the high susceptibility of most commercial pome fruit cultivars and modern high-density planting systems. These planting systems emphasize maximizing vigor which consequently also results in an increase in susceptibility to shoot infection and internal systemic spread of the pathogen. Our hope is that new cultivars with fire blight resistance genes and newer approaches to fire blight management will provide growers with effective and sustainable solutions for disease protection. Meanwhile, continued genetic and genomic research addressing host-pathogen interactions and *E. amylovora* evolution (Boxes 1–4) is expected to fill in gaps and potentially provide significant leaps in understanding that ultimately result in better management strategies and tactics.

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Declarations

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