REVIEW ARTICLE

Insects as alternative hosts for phytopathogenic bacteria

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Abstract

Phytopathogens have evolved specialized pathogenicity determinants that enable them to colonize their specific plant hosts and cause disease, but their intimate associations with plants also predispose them to frequent encounters with herbivorous insects, providing these phytopathogens with ample opportunity to colonize and eventually evolve alternative associations with insects. Decades of research have revealed that these associations have resulted in the formation of bacterial–vector relationships, in which the insect mediates dissemination of the plant pathogen. Emerging research, however, has highlighted the ability of plant pathogenic bacteria to use insects as alternative hosts, exploiting them as they would their primary plant host. The identification of specific bacterial genetic determinants that mediate the interaction between bacterium and insect suggests that these interactions are not incidental, but have likely arisen following the repeated association of microorganisms with particular insects over evolutionary time. This review will address the biology and ecology of phytopathogenic bacteria that interact with insects, including the traditional role of insects as vectors, as well as the newly emerging paradigm of insects serving as alternative primary hosts. Also discussed is one case where an insect serves as both host and vector, which may represent a transitionary stage in the evolution of insect–phytopathogen associations.

Introduction

Plant pathogenic bacteria are responsible for some of the most devastating losses of major agricultural crops and vital fruit trees, causing millions of dollars in damage annually. Their agricultural and economic impact has afforded them significant attention over the last 30 years, resulting in enormous strides in the exploration of their epidemiology and specialized disease strategies. Research of plant pathogenic bacteria has not only seeded our understanding of the genetics of disease, epidemiology, and the factors contributing to emerging infectious diseases, but has also led to the development of effective control and prevention measures for many plant diseases (Woolhouse *et al.*, 2002; Gardan *et al.*, 2003). More recently, however, there has been a shift in the exploration of the plant pathogens to a broader community level, which moves beyond the traditional single host–single pathogen model to a wider and more encompassing view of the evolution and ecology of plant pathogenic bacteria. Much of this research has expanded the field into a new direction, and has resulted in the unearthing of the hidden ecology and true pathogenic potential of many bacteria that have long been considered strict and very dedicated phytopathogens.

The exploration of phytopathogen life histories is often trumped by the striking and often contrasting disease symptomology that develops on host plants as a consequence of disease. Traditionally, this has resulted in an almost exclusive focus on the biology, ecology, and genetics of specific plant–phytopathogen relationships, often to the exclusion of other potentially relevant yet presumably less obvious associations. Even the most intimate association between pathogen and plant host in the natural environment, whether occurring at the interface of the phyllosphere or within plant tissues, is still subject to incursions by other ecological players. Phytophagous insects, in particular, which graze frequently and recurrently on plant tissues that may be colonized by epiphytic or plant pathogenic bacteria, are often neglected as key ecological players, despite the fact that they are most likely to have repeated encounters and associations with phytopathogenic bacteria that reside in or on their preferred host plants.

FEMS MICROBIOLOGY REVIEWS

There are numerous potential interactions that can result from the association between a microorganism and an insect, all of which are defined by the relative effects on the fitness of the individual organisms (referred to as symbionts). Mutualisms may form between the two organisms, where both derive a benefit from their interaction. Mutualisms may be defensive, where the microorganisms provides protection to the insect host (Wilkinson *et al.*, 2000), or nutritional, where the microorganism supplements the diet of the insect host with key nutrients (Barbosa & Letourneau, 1988). Parasitisms may also develop between microorganisms and insects, where the microorganism benefits by extracting nutrients from its host, but at a cost to its host. In the latter case, the microorganism may impair or disrupt the physiology and normal functioning of the insect host, resulting in specific disease symptomology. A commensalism describes the association between insect and microorganism where the microorganism benefits and the insect is unaffected. Commensalisms likely characterize many of the interactions that exist in the natural environment, but are most likely to go unnoticed. Both commensalistic and parasitic symbioses can range from highly specific to nonspecific, with the development of more specific interactions being favoured in cases where specialist microorganism encounter specialist insects recurrently over long periods of time, and more general interactions in cases where generalist or transient insects encounter specialist bacterial pathogens (or vice versa).

Phytopathogenic bacteria have evolved to harness insects as vectors to effect their dissemination and delivery directly onto or into their preferred plant hosts. These partnerships can either be commensalistic or slightly parasitic to the insect, but in either case, the insect performs as a living carrier that transmits the microorganism to its final (definitive) host. Many of these symbioses are highly specific, and are categorized by the ability of the bacterium to replicate in and move through its vector. The ability to replicate within the insect vector can be classified as either propagative or nonpropagative, and the ability of the microorganism to move through its vector can be classified as circulative or noncirculative (Blanc, 2004). In circulative nonpropagative transmission, the microorganism is ingested by its insect vector as it feeds on the host plant, after which it migrates into the midgut or the hindgut epithelium, and is then released into the haemolymph of the insect (Blanc, 2004). The microorganism then enters into the salivary glands, and can be inoculated to healthy plant hosts via the saliva while the insect feeds (List, 1939; Carter, 1950). In this case, the microorganism does not replicate in its host vector. In contrast, circulative propagative transmission occurs when a microorganism is able to replicate within its insect vector, and spread to other organs within the insect. The microorganism crosses the membrane, enters the haemolymph and then the saliva, and may be delivered into a new host plant when the insect feeds (Kwon *et al.*, 1999). Noncirculative and nonpropagative microorganisms are those that generally form a physical association with the insect and are subsequently mechanically transmitted to the plant host (infection by an insect stylet that is coated with a pathogen, for example) (James & Perry, 2004).

Over the last decade, the exploration of phytopathogenic bacteria and their interactions with insects has expanded beyond the traditional phytopathogen–vector relationship to include cases where phytopathogens exhibit entomopathogenic associations. Most of these relationships have been characterized only recently, and represent a new paradigm in bacterial–insect interactions. Certainly, this has not lessened the focus on the traditional plant–microorganism or vector–microorganism association, or the use of genomic and high-throughput approaches for exploring these interactions. Instead, these studies have uncovered hidden alternative interactions for plant pathogenic bacteria, ultimately providing additional breadth to our understanding of their biology, ecology, and evolution. This review will examine the alternative associations of phytopathogenic bacteria with insects, focusing on the genetics and ecological relevance of those insects that can serve as either a transport host/vector, an insect that serves as a carrier of the pathogen, or a primary host, an insect that the pathogen can colonize, replicate in, and disperse from. Also examined is one special interaction where the microorganism exploits a single insect as both host and vector. This unusual association may represent a rare transitionary phase in the evolution of phytopathogen–insect associations.

Insects as vectors for phytopathogenic bacteria

The evolution of effective and stable phytopathogen–insect vector partnerships is dependent largely on the opportunity for the insect and the microorganism to encounter each other frequently. Generally, the dependence of many insects and phytopathogens on plants as their primary source of nutrition may lead to an overlap of ecological niche, providing the necessary conditions for insects to encounter, contact, or ingest phytopathogenic bacteria. In this section, we describe the best-characterized symbioses between insects and phytopathogens wherein the insect serves as a delivery vessel for the bacteria.

Xylella fastidiosa and the sharpshooter

Xylella fastidiosa is a xylem-restricted, fastidious phytopathogen that causes citrus variegated chlorosis and Pierce's disease of grape (Chang *et al.*, 1993; Chatterjee *et al.*, 2008). *Xylella fastidiosa* is transmitted between plant hosts by xylem-feeding sharpshooter leafhoppers (*Hemiptera*,

Cicadellidae) and spittlebugs (*Hemiptera*, *Cercopidae*) (Severin, 1949, 1950), which deliver the bacteria directly into the plant. Leafhoppers use their piercing and sucking mouthparts to penetrate the water-conducting xylem vessels of host plants to access the xylem sap, and if they carry the pathogen, extravasate *X. fastidiosa* through their food canal, injecting the bacteria directly into the xylem vessels of the plant (Wayadande *et al.*, 2005). Once inside, the bacteria multiply and spread from the site of infection to colonize the xylem and form a biofilm (Hopkins, 1989; Alves *et al.*, 2004; Fritschi *et al.*, 2007; Chatterjee *et al.*, 2008). From there, the bacteria spread to adjacent uncolonized xylem vessels, possibly through the pit membrane (Chatterjee *et al.*, 2008), resulting in the physical obstruction of water flow through plant tissues, and causing leaf, shoot, and eventually, plant death (Fogaça *et al.*, 2010).

The infiltration of key insect vectors into important grape and citrus farming areas of North America led to a drastic increase in the exploration of the epidemiology of *X. fastidiosa* and the role of insect vectors in pathogen dispersal (Hopkins, 1989; Purcell & Hopkins, 1996; Hopkins & Purcell, 2002; Almeida, 2007; Chatterjee *et al.*, 2008). The relatively recent introduction of the glassy-winged sharpshooter, *Homalodisca vitripennis*, and the blue-green sharpshooter, *Graphocephala atropunctata* (Signoret), into California resulted in Pierce's disease becoming a more aggressive and prevalent disease; however, because *X. fastidiosa* lacks vector-species specificity, as seen with many other phytopathogenic bacteria (Almeida *et al.*, 2005), nearly all sharpshooter species are able to transmit *X. fastidiosa*, albeit with differing transmission efficiencies (Chatterjee *et al.*, 2008). Although both insect vectors are capable of transmitting *X. fastidiosa*, the glassy-wing sharpshooter is often seen as a more efficient vector than the blue-green sharpshooter (Almeida, 2007). Transmission efficiency may be linked to feeding site preference because the blue-green sharpshooter is known to have a preference for feeding on young tissue and leaves, while the glassy-wing prefers both young tissue and mature woody parts of the plant (Hopkins & Purcell, 2002). Linked to this is the fact that *X. fastidiosa* is found to be disproportionately dispersed within symptomatic plants, an attribute that may influence the acquisition of the pathogen, depending on the tendency of specific insect species to feed on tissues that may have lower bacterial concentrations (Almeida, 2007). Acquisition efficiency was significantly higher from plants that had a higher bacterial load, thus implying a direct correlation between bacterial concentration and vector transmission efficiency (Hill & Purcell, 1997; Almeida, 2007).

Following ingestion, the bacteria become localized to the insect foregut, where they multiply and grow (Hill & Purcell, 1995). The pathogen can be transmitted immediately after acquisition (Purcell & Finlay, 1979; Wayadande *et al.*, 2005; Chatterjee *et al.*, 2008), indicating that bacterial multiplication in the foregut of the insect vector is not vital for pathogen transmission, and that *X. fastidiosa* is a noncirculative vectored phytopathogen (Purcell & Finlay, 1979; Wayadande *et al.*, 2005; Almeida, 2007). Although *X. fastidiosa* may propagate through noncirculative propagative transmission, the bacterium cannot be passed from parent to offspring, as neither transovarial (immature egg to adult) transmission nor trans-stadial (mature egg to adult) transmission has been observed for this bacterium (Freitag, 1951; Almeida & Purcell, 2003). In addition, infected newborn nymphs generally lose their infectivity after moulting their foregut cuticular lining (Purcell & Finlay, 1979).

The interaction of *X. fastidiosa* with its insect vectors appears to be influenced by the *rpf* locus (regulation of pathogenicity factor) (Newman *et al.*, 2004). *Xylella fastidiosa* uses cell-to-cell signalling mediated by a small diffusible signalling molecule known as diffusible signalling factor (DSF) (Chatterjee *et al.*, 2008). The production of DSF is dependent on the gene *rpfF*, which has characteristics similar to long-chain fatty acyl CoA ligases (Barber *et al.*, 1997; Chatterjee & Sonti, 2002; Fouhy *et al.*, 2007). Mutations in *rpfF* caused a deficiency in the ability of the bacteria to form a biofilm in the insect host, despite being taken up from the plant (Chatterjee *et al.*, 2008). Surprisingly, *rpfF* mutants are hypervirulent in grape plants (Newman *et al.*, 2004). Likewise, the mutation of a second locus, *rpfC*, does not impair the ability of *X. fastidiosa* to colonize the insect, but does alter its ability to be transmitted to new host plants (Chatterjee *et al.*, 2008). It has been proposed that this is due to *rpfC* mutants being stronger biofilm formers than the wild-type strain, which reduces the number of planktonic cells that can be released from the insect during feeding. For plant virulence, mutations in the *rpfC* gene cause *X. fastidiosa* to become deficient in longitudinal migration along the xylem vessel, resulting in lower growth and spread in grape stems than the wild-type strain (Chatterjee *et al.*, 2008).

There is early evidence that *X. fastidiosa* has developed a seemingly specific relationship with the xylem-feeding sharpshooters and spittlebugs. The identification of the *rpf* locus provides a promising beginning to understanding the specific genetic underpinnings of the interaction between *X. fastidiosa* and its insect vectors, but many aspects of this relationship still remain unexplored.

Pantoea stewartii and the flea beetle

Stewart's disease (or Stewart's wilt) of corn, caused by the bacterium *P. stewartii* (formerly *Erwinia stewartii*), causes significant yield loss in dent and sweet corn as a result of leaf blighting (Munkvold, 2001). The development of Stewart's wilt has two distinct symptomologies: wilt and leaf blight. In

both cases, the manifestation of disease initially begins once the bacterium has successfully invaded the leaf tissue through lesions produced by the flea beetle (Munkvold, 2001). Upon entry, *P. stewartii* multiplies within the leaves, producing yellowish, water-soaked lesions or streaks that eventually elongate and later coalesce along the leaf veins of corn leaves and soon become necrotic (Esker & Nutter, 2002, 2003). The bacteria colonize the xylem vessels, where their production of large amounts of bacterial exo/capsular polysaccharide (EPS), also known as stewartan, restricts the flow of free water, causing wilting, and this can be followed by a general browning and water soaking of the stalk tissue (Braun, 1982; Leigh & Coplin, 1992; Munkvold, 2001).

The successful infection of corn plants by *P. stewartii* appears to be dependent on the *hrp/wts* gene cluster, which directs the synthesis of a type III secretion system (T3SS) (Hueck, 1998; Frederick *et al*., 2001). Through transposon mutagenesis, Frederick *et al*. (2001) identified the *wtsE* gene, which encodes a 201-kDa protein that is strikingly similar to DspE in *Erwinia amylovora* and the protein AvrE found in *Pseudomonas syringae* pv. tomato, both of which have been implicated in virulence (Bogdanove *et al*., 1998a, b; Alfano *et al*., 2000). Additional work on *P. stewartii* pathogenesis identified the involvement of a quorum-sensing system, which allows bacteria to monitor their population density by utilizing small, diffusible signals and to orchestrate the expression of specialized gene systems for pathogenicity (Fuqua *et al*., 1996; Withers *et al*., 2001; Koutsoudis *et al*., 2006). Studies conducted by Koutsoudis *et al*. (2006) suggested a possible functional corollary between bacterial biofilm development and xylem colonization similar to that described for *X. fastidiosa* infections of grape vine. From their research, they recognized that the quorum-sensing system organized the timing and level of EPS produced, significantly affecting the degree of bacterial adhesion during *in vitro* biofilm formation and propagation within the plant host. Moreover, their microscopic studies revealed that *P. stewartii* colonizes the xylem of corn with spatial specificity rather than by arbitrary growth to fill the lumen of the xylem, as seen with *X. fastidiosa*.

Pantoea stewartii is disseminated among suitable host plants via a specific insect vector – the corn flea beetle, *Chaetocnema pulicularia* – which acquires the pathogen while feeding on infected corn plants (Esker & Nutter, 2003; Menelas *et al*., 2006). The pathogen becomes localized along the alimentary tract of adult corn flea beetles (Hogenhout *et al*., 2008), where it remains for the entire duration of the insect's life (Munkvold, 2001). The beetles overwinter in the soil of grassy areas near agricultural fields for the duration of the winter season, and although colder winter temperatures reduce beetle survivorship, many beetles still survive to transmit the disease (Munkvold, 2001; Esker & Nutter, 2002). With the spring thaw, the beetles exit their dormancy stage and begin to feed, and deposit the pathogen into the feeding wounds via their faeces, allowing *P. stewartii* to enter the veins of corn leaves and cause disease (Munkvold, 2001; Esker & Nutter, 2002).

Beetles that feed on infected tissue acquire the bacterium and promote the spread of the pathogen throughout the season (Munkvold, 2001). The colonization of corn flea beetles by *P. stewartii* appears to be mediated by a T3SS that is distinct from that used for colonizing the plant host (Coplin *et al*., 1992a). The pathogenicity of *P. stewartii* in plants depends on the *hrp/hrc* gene cluster, which encodes a T3SS that is essential for disease development (Coplin *et al*., 1992b). Recently, Correa *et al*. (2008) studied a mutant strain of *P. stewartii* DC283, which had a mutation in the *ysaN* gene, a component of the second T3SS apparatus in flea beetles. They discovered that the beetles were able to acquire both the mutant and the wild-type strains of *P. stewartii* equally well, but the *ysaN* mutant did not persist like the wild type, and declined in frequency 4 days following acquisition. Using confocal laser scanning microscopy, Correa *et al*. (2008) demonstrated that *P. stewartii* persists in the hindgut lumen of beetles, but did not invade the gut cells. *Pantoea stewartii* was capable, however, of invading cells of Malphigian tubules that protrude from the gut of beetles, which supported previous studies that indicate that the most likely route of bacterial transmission is through insect frass. This was also supported by observations that flea beetles cluster together in small groups on maize leaves under growth chamber conditions, which would result in plant wounds being contaminated more rapidly with frass, thereby promoting *P. stewartii* infiltration into plant tissues (Correa *et al*., 2008).

Pantoea stewartii utilizes the corn flea beetle as a vector to disperse to corn plants, and this interaction appears to be facilitated at least in part by a T3SS. By extension, this would implicate the involvement of specific type III secreted effectors, which likely interact with host substrates to facilitate bacterial colonization of the insect. Although the actual mechanism of how *P. stewartii* colonizes its insect vector is not understood fully, it appears that the phytopathogen has acquired specific genetic determinants that allow it to associate with the beetle and promote its dissemination. *Pantoea stewartii* is not only able to utilize the corn flea beetle as a transport host to reach its primary plant host, but is also capable of exploiting the pea aphid as an alternative primary host. This relationship is discussed later.

Serratia marcescens and the squash bug

Serratia marcescens is a phloem-resident pathogen that causes cucurbit yellow vine disease of pumpkin (*Cucurbita moschata* L.) and squash (*Cucurbita pepo* L.), which are characterized by wilting, phloem discoloration, and

yellowing foliage (Bruton *et al*., 1998, 2001; Rascoe *et al*., 2003). Recent studies have shown that *S. marcescens* produces a biofilm along the sides of the phloem tissues of the plant once inside its host, blocking the transport of water and nutrients and eventually causing the plant to wilt and die (Labbate *et al*., 2004, 2007). A genetic screen to identify the genes that modulate biofilm formation in *S. marcescens* revealed the involvement of fimbrial genes, as well as an *oxyR* homologue, which is a conserved bacterial transcription factor that plays a primary role in the oxidative stress response (Shanks *et al*., 2007).

Serratia marcescens is transmitted by the squash bug, *Anasa tristis* (DeGreer), which is commonly found throughout the United States as well as between Canada and Central America (Alston & Barnhill, 2008). The squash bug feeds on its plant host using piercing–sucking mouthparts that penetrate intracellularly through the plant tissue toward the vascular bundles (Neal, 1993). The visible signs and extent at feeding damage to squash plants correlate with the number and size of bugs, as well as the amount of time each bug spends on the plant and at the feeding site (Neal, 1993). Long-term feeding on the fruit leads to fruit collapse, while leaf feeding induces isolated necrotic lesions (Neal, 1993). Early experiments revealed the presence of starch granules in the gut of *A. tristis*, which are only found in the cytoplasm of plants, suggesting that the squash bugs ingest the intracellular contents of plant cells (Breakey, 1936); however, experiments in which squash bugs were allowed to feed on plants having safranin-stained xylem fluid showed that red dye accumulated in the gut of the insects, suggesting that xylem is also a food source for the insects (Neal, 1993). Surprisingly, squash bug feeding damage extends beyond the xylem vessels and into the phloem. Areas adjacent to the spongy mesophyll of the leaf and the cells of the palisade and epidermal layers of leaves also exhibit signs of localized feeding-induced injury (Beard, 1940; Bonjour *et al*., 1991; Neal, 1993). Extensive feeding on the stem can damage the vascular tissue of the plant, thereby resulting in the wilt of the leaf apical to the feeding site or wilt of the entire plant if it is a seedling (Tower, 1914; Beard, 1940; Neal, 1993). Heavy feeding can cause leaves to turn black and soon become crisp (Alston & Barnhill, 2008).

Early studies examined the colonization of the squash bug by *S. marcescens*. Wayadande *et al*. (2005) initially hypothesized that *S. marcescens* shared a relationship with its insect vector similar to that seen between *X. fastidiosa* and sharpshooters, where the bacteria are localized to the foregut of the insect vector and are released through the food canal during successive feeding bouts; however, upon examination of the foregut of adult and nymph squash bugs allowed to feed on bacteria-infiltrated squash cubes, the foregut cibaria of the infected insects were found to be clear of any bacteria-like structures. From their results, Wayadande *et al*. (2005) concluded that the ability of *A. tristis* to transmit *S. marcescens* after moulting indicated that the haemocoel, and not the gut, acts as a possible site of retention for the infectious bacteria. This is in contrast to work showing that *S. marcescens* is pathogenic once introduced into the haemolymph of *A. tristis* (Bextine, 2001; Wayadande *et al*., 2005).

The incubation time (or latent period) of *S. marcescens* was shown to be very short, with some adults being capable of transmitting the bacterium 1–2 days after the initial acquisition (Bextine, 2001); however, adult squash bugs upon bacteria-infiltrated squash fruit cubes were noted to transmit the bacterium only sporadically to squash plants within a 21-day testing period (Wayadande *et al*., 2005). This short latent period coupled with an irregular transmission pattern are indicative of a noncirculative mode of transmission (Purcell & Finlay, 1979; Bextine, 2001). Despite its noncirculative association, *S. marcescens* overwinters in the dormant insect vector – a strategy that protects the pathogen against low winter temperatures – ensuring a high survival rate and thus successful transmission to plants in the following season (Pair *et al*., 2004).

Erwinia tracheiphila and the cucumber beetle

Bacterial wilt is a serious threat to commercial melon and cucumber production in some parts of the world, including North America. Bacterial wilt is caused by the bacterium, *E. tracheiphila*, which is transmitted by both the striped cucumber beetles (*Acalymma vittata*) and spotted cucumber beetles (*Diabrotica undecimpunctata*) (Ferreira & Boley, 1992). These beetles are attracted to their host by cucurbitacins, a group of secondary plant metabolites that are commonly found within the plant family *Cucurbitaceae* (Chambliss & Jones, 1966). Cucurbitacins are bitter toxic compounds (Metcalf *et al*., 1980), which are known to accumulate in cucumber beetles and confer protection against predation (Howe *et al*., 1976; Ferguson & Metcalf, 1985), but have detrimental effects on most invertebrate and vertebrate herbivores (David & Vallance, 1955; Nielson *et al*., 1977). The preferred plant hosts of *E. tracheiphila* are wild and cultivated cucurbits, including muskmelon, pumpkin, gourd, and squash, with cucumbers being the most susceptible hosts (Agrios, 1978).

Mechanical wounding of the plant tissue is necessary for bacterial infection, because the bacterium cannot infect the cucumber plant through the normally found openings (stomates and hydathodes) of a plant (Ferreira & Boley, 1992). While feeding on infected cucurbits with their piercing and sucking mouthparts, the cucumber beetle acquires *E. tracheiphila*, which then migrates to the insect gut epithelium (Mitchell, 2004). While infected beetles feed on

healthy curcurbit plants, bacteria are deposited on the leaves via beetle faecal droppings, which leach into the lesions created by the feeding beetles (Yao *et al.*, 1996). *Erwinia tracheiphila* can only migrate toward a wound providing there is a sufficient aqueous film on the leaf surface (Ferreira & Boley, 1992), although the cucumber beetles' stylet can also become infected with the pathogen, providing a direct, mechanical method of infection (Yao *et al.*, 1996). Once inside the plant, *E. tracheiphila* spreads to the xylem vessels, multiplies, and infects all parts of the plant. As the bacterium multiplies in the xylem, the efficiency of water transport is reduced to less than one-fifth of the normal water flow, resulting in extensive plugging of the vessels and the subsequent wilt of the plant (Agrios, 1978).

Although little is known about the interaction between *E. tracheiphila* and the cucumber beetle, there appears to be some evidence of coevolution. The bacterium is able to overwinter in the digestive tract of its vector, and escape through the faecal droppings, without any apparent adverse impact on its insect vector. The precise coevolutionary processes leading to the formation of the interaction between *E. tracheiphila* and the cucumber beetle are still unknown.

Erwinia amylovora and pollinators

Erwinia amylovora is the causal agent of fire blight of apple and pear, a detrimental bacterial disease of rosaceous plants, infecting primarily significant pear and apple varieties (Eden-Green & Billing, 1974; Spinelli *et al.*, 2005). The effects of *E. amylovora* on apple and pear tress are catastrophic, as they cause the death of blossoms, shoots, limbs, and at times, entire trees (Johnson & Stockwell, 1998). The primary infection site of the pathogen in fire blight disease is through tree blossoms (Eden-Green & Billing, 1974; Wilson & Lindow, 1993; Johnson & Stockwell, 2000), which begins with bacterial colonization of the stigma, reproduction on the stigmatic surface, migration along the length of the style, and eruption into the host tissue via the nectarthodes (Thomson, 1986; Spinelli *et al.*, 2005). Stigmas, which are borne on the ends of the style, have been demonstrated to be the principal site of epiphytic colonization by *E. amylovora* (Hattingh *et al.*, 1986; Thomson, 1986; Wilson *et al.*, 1989, 1992; Wilson & Lindow, 1993; Johnson & Stockwell, 1998). Despite the generalization that aerial surfaces of plants like stigmatic surfaces are unreceptive to bacterial growth due to exposure to UV radiation and varying osmotic pressure, stigmas provide *E. amylovora* with a nutrient-rich, protected, and hydrated environment for growth (Johnson & Stockwell, 1998). Micrographs showing *E. amylovora* growing mostly within the large intracellular spaces between the secretory papillae of stigmas have reaffirmed this (Hattingh *et al.*, 1986; Wilson *et al.*, 1989). Disease development is dependent on a high-molecular-weight polysaccharide, designated amylovoran, which was shown to contribute to plugging of the vascular tissues, and leading to the wilting of shoots (Goodman *et al.*, 1974) (Oh & Beer, 2005). Other pathogenicity determinants include the polysaccharide levan (Gross *et al.*, 1992), and the *hrp*/*hrc* gene cluster, which encodes the T3SS (Oh & Beer, 2005).

Erwinia amylovora has a nonspecific association with pollinating insects that travel from tree to tree collecting nectar (Johnson & Stockwell, 1998), including honey bees, *Apis mellifera* (family *Apidae*), which have been shown to be extremely efficient vectors (Emmett & Baker, 1971). To investigate which species of insects were able to transmit *E. amylovora*, Emmett & Baker (1971) inoculated various insects with the bacteria and transferred the insects to apple and pear blossom trusses, and evaluated the rates of tree infection. Several insects were able to transmit the bacterium and induce infections in blossoms and shoots, although it appeared that larger species of insects, like bees, were more efficient in transmitting the pathogen to blossoms in comparison with smaller species of insects, such as anthomyiid flies. Larger insects were able to infect more trusses and more flowers per truss, possibly due to their ability to carry more inoculum, as well as their larger overall migration distances (Emmett & Baker, 1971).

There have been no conclusive studies demonstrating that the bacterium enters and colonizes insects; rather, there is overwhelming evidence that *E. amylovora* adheres to the external surfaces of its insect vectors and is subsequently transmitted to healthy plants mechanically. In one experiment by Hildebrand *et al.* (2000), *Aphis pomi* was surface contaminated with fluorescent *E. amylovora* through exposure of a thin lawn sprayed with the bacterium. Over several consecutive days, aphids were crushed, plated, aliquoted, and bacterial presence evaluated by PCR. The results revealed florescent bacteria on the legs, cornicles, proboscis, and antennae of the aphids (Hildebrand *et al.*, 2000). Persistence of bacteria on insect surfaces has been shown to be at least 72 h on *A. pomi* (Plurad *et al.*, 1967), 9 days on the flesh fly *Sarcophaga carnaria* (L Baker), 5 days on the green lacewing (*Chrysoperla carnea*) (Hildebrand *et al.*, 2000), and up to 12 days on some aphid species, likely facilitated by the exopolysaccharide capsule of the bacteria (Hildebrand *et al.*, 2000).

Because there is no evidence of bacterial internalization by insects, overwintering of *E. amylovora* appears to be within the canker on its host plant. Once spring emerges and temperatures are favourable, bacteria ooze from the cankers, and cause an infestation of the blossom (Rezzonico & Duffy, 2007). This process is contrary to *P. stewartii*, *S. marcescens*, and *E. tracheiphila*, which overwinter in their specific dormant insect vector and remain protected from harsh winters.

Candidatus Liberibacter and citrus psyllid

Candidatus Liberibacter is a phloem-limited phytopathogenic bacterium that causes huanglongbing disease (HLB) or citrus greening on citrus fruits around the world (Teixeira *et al*., 2005; Manjunath *et al*., 2008). *Candidatus* Liberibacter has a semi-specific symbiotic relationship with two different psyllid insect vectors: *Diaphorina citri* (Kuwayama) (Capoor *et al*., 1967) and *Trioza erytreae* (del Guercio) (McClean & Oberholzer, 1965). *Diaphorina citri* is the principal vector in Asia, Brazil, and Florida, while *T. erytreae* transmits *Ca*. Liberibacter in Africa (Manjunath *et al*., 2008). *Diaphorina citri* has been in existence in Brazil for over 60 years (Lima, 1942; Bové, 2006) and in Florida since 1998 (Halbert *et al*., 2002); however, HLB appeared in both locations simultaneously. The psyllid has also been reported in areas of Texas in 2001 (French *et al*., 2001) as well as in several other countries in the Caribbean basin (Halbert & Nunez, 2004).

HLB has been divided into Asian and African strains based on the influence of temperature and host symptoms. In Asia, the HLB bacterium has been identified as *Candidatus* Liberibacter asiaticus (*Las*), which infects the majority of citrus cultivars and causes extensive economic loss by limiting the lifespan of infected trees (Miyakawa, 1980; Jagoueix *et al*., 1997; Garnier *et al*., 2000; Hung *et al*., 2004). *Las* is heat tolerant, and can produce HLB symptoms at temperatures above 30 °C (Bové *et al*., 1974; Hung *et al*., 2004). In contrast, the African species, *Candidatus* Liberibacter africanus, is heat-sensitive and does not cause symptoms above 30 °C (Bové *et al*., 1974; Hung *et al*., 2004). Recently, a new species of *C*. Liberibacter was identified, which was unique from the other two species because it caused disease in solanaceous plants, and was vectored by a different psyllid species, *Bactericera cockerelli* (Hansen *et al*., 2008). *Bactericera cockerelli* is a polyphagous phloem feeder that can reproduce on a wide variety of host plant species, but is predominantly a pest of potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicon* L.) (Pletsch, 1947; Wallis, 1955; Hansen *et al*., 2008).

Trioza erytreae and *D. citri* psyllids are efficient vectors of HLB, which carry the bacteria in the haemolymph and salivary glands (Moll & Martin, 1973; Xu *et al*., 1988). Work by Hung *et al*. (2004) demonstrated that infected nymphs, which are barely mobile, quickly develop into *Las*-carrying adults with the capability to fly and transmit the pathogen to other citrus plants. They show that *Las* cannot be detected at all in first instars, suggesting that first instars are incapable of carrying the pathogen. Second instars, however, were shown to carry the pathogen, but at an extremely low titre. Psyllids are therefore able to bear the bacterium in either adult or nymphal stages, but not as first instars. Hung *et al*. (2004) also demonstrate that bacterial titre increases with each instar, suggesting that the pathogen replicates during vector metamorphosis (Hung *et al*., 2004), and can therefore be considered propagative (Manjunath *et al*., 2008). In a separate study, the bacteria were found to be present at a higher infection frequency in eggs, first instars, and second instars isolated from potato host plants than from those isolated from tomato (Hansen *et al*., 2008). Psyllids from potato were found to have a fixed concentration of bacteria from the first instar stage to the adult phase, whereas those isolated from tomato had very low titres at the egg and first instar phase, which increases considerably in the second instar stage and becomes fixed at the third instar period (Hansen *et al*., 2008). This suggested that the bacteria are transmitted vertically but this transmission rate is dependent on the host plant from which it was isolated. This is in direct conflict to previous reports that *Las* persists in the adult insect vector for 12 weeks and is not passed directly to the offspring (Hung *et al*., 2004).

The interaction between *C*. Liberibacter and its insect allows the pathogen to reach and gain entry into its plant host. The ability of *C*. Liberibacter to be transmitted by both sharpshooters and spittlebugs suggests that its interaction with these sap-feeding insects may be semi-specific. Although the genetics of the interaction have yet to be explored, *C*. Liberibacter may have specific genetic factors that enable insect association, colonization, and persistence, with the extent of any adaptation or coevolution with its insect vectors having yet to be determined.

Pectobacterium and the fruit fly

Pectobacterium carotovorum (formerly *Erwinia carotovora*) is a member of the *Enterobacteriaceae* (Molina *et al*., 1974) and the causal agent of the tuber-borne lethal potato blackleg disease (De Boer, 2002). The pathogen produces pectolytic enzymes, which break down plant cell walls (Pirhonen *et al*., 1993). The production of these exoenzymes is controlled by a global regulatory mechanism, and more specifically, the *expI* gene (Pirhonen *et al*., 1991). *expI* mutants are deficient in exoenzyme production, and are completely avirulent as they can neither break down the plant tissue nor multiply within potato plants (Pirhonen *et al*., 1991, 1993; Palva *et al*., 1993). *expI* has a general signalling function, and directs the synthesis of a signal molecule that is involved in cell density-dependent control of exoenzyme genes in *P. carotovorum*. Pirhonen *et al*. (1993) demonstrated this through extracellular complementation of the defect in exoenzyme production, where the diffusible signal molecule produced by ExpI-proficient cells can be recognized by the mutant and subsequently used to activate exoenzyme gene expression.

In addition to causing disease in potato, *P. carotovorum* also has another suitable host–the fruit fly, *Drosophila* – which it uses as a vector. Using a genetic screen, Basset *et al*.

(2003) identified two genes that are required by *P. carotovorum* to colonize *Drosophila*. One gene, *evf*, enabled persistence in the host, and was controlled by the *hor* gene – a key regulator capable of conveying signals from various environments to effectors involved in both plant pathogenesis and *Drosophila* colonization (Thomson *et al*., 1997; Basset *et al*., 2003). Transfer of the *evf* gene to noninfectious *Pectobacterium* strains or to other enterobacteria was found to improve the ability of the bacterium to survive in the gut of *Drosophila* and trigger an immune response, and the fact that the gene *evf* was found in only a few *P. carotovorum* strains was suggested to indicate that this gene had been acquired recently through horizontal gene transfer. When the *evf* gene was overexpressed in *P. carotovorum*, bacteria were able to colonize the apical side of the gut epithelium and at times to spread to the body cavity. Furthermore, Basset *et al*. (2000) identified one strain of *P. carotovorum*, *Ecc*15, which induced a systemic immune response in *Drosophila* larvae following natural ingestion (Basset *et al*., 2000; Williamson *et al*., 2010). Feeding of larvae with living *Ecc*15 resulted in them having a high expression of antimicrobial peptide genes in their fat body, which is functionally analogous to mammalian liver (Hoffman & Reichhart, 1997). Although this bacterial strain did not appear to be pathogenic to its insect vector, its ability to induce a systemic immune response implied that it may have infectious properties that can be recognized by the *Drosophila* innate immune system (Basset *et al*., 2003). Out of the 16 *Ecc* strains tested, only three were found to have the ability to infect *Drosophila* larvae by natural infection. Based on these results, they hypothesized that there may be specific genes that allowed *Ecc*15 to associate with its insect vector.

The expression of the *evf* gene results in the accretion of bacteria in the anterior midgut and radically influences gut physiology (Acosta Muniz *et al*., 2007). It was suggested that *evf* could disrupt the peritrophic membrane, which is a chitinous membrane that outlines the insect vector's gut and prevents bacteria from entering the gut cells (Basset *et al*., 2003). It was also proposed that *evf* could allow the propagation of bacteria in this environment or produce a toxin that could disrupt the physiology of the gut cells (Basset *et al*., 2003). Recent crystal structure data of Evf show it to be an α/β protein having a novel fold and intricate topology, with evidence for a palmitoic acid being covalently linked to the 209 cysteine residue of the Evf protein through an association with a thioester linkage, and suggesting that Evf may be targeted to membranes (Quevillon-Cheruel *et al*., 2009). Palmitoylation, a post-translational modification that increases the affinity of soluble proteins for lipid membranes (Dunphy & Linder, 1998; Smotrys & Linder, 2004), is necessary for biological activity as shown by the abolishment of Evf function following the mutation of the key cysteine residue required for palmitoylation (Quevillon-Cheruel *et al*., 2009). Surprisingly, Evf was found to be present in the cytoplasm, not in the periplasm (Acosta Muniz *et al*., 2007), but was shown to bind to model membranes and promote aggregation. In subsequent studies, Quevillon-Cheruel *et al*. (2009) showed that the overexpression of the Evf protein promoted bacterial accumulation in the gut in an arrangement typical of an organized community, as seen in a biofilm, and suggest that the ability of the Evf protein to be able to amass bacteria may be due to its capacity to interact with and promote the aggregation of vesicles. Quevillon-Cheruel *et al*. (2009) concluded that the function of the Evf protein must be related to post-translational modification, where the biological function of the *evf* gene may be more directed towards membrane anchoring of the protein. *Pectobacterium carotovorum* can effectively spread from plant to plant via *Drosophila*, and although *Drosophila* may not be its intended carrier, there is evidence for adaptation of the bacterium to this host that results in efficient bacterial association, retention, and ultimately, dispersal.

Insects as primary hosts for phytopathogenic bacteria

New research has highlighted several instances of phytopathogenic bacteria exploiting insects as primary hosts, with experimental evidence pointing to the ability of many phytopathogens to invade and colonize insects as they would their plant hosts. These interactions exhibit pathologies similar to those seen between phytopathogens and their plant hosts, including rapid bacterial growth and the manifestation of disease. In this section, we describe three distinct cases involving three phytopathogens exploiting an insect host. Interestingly, the pea aphid, *Acyrthosiphon pisum*, is the target insect host in all three cases.

Dickeya dadantii and the pea aphid

Dickeya dadantii (formerly *Erwinia chrysanthemi*) is a member of the *Enterobacteriaceae* and the agent of soft rot disease of a wide range of economically important crops, including potatoes and maize (Bing *et al*., 2007). Disease develops following the movement of the pathogen from the stem base throughout the tissues, producing a brown staining of the vascular tissues, and occasionally, necrosis and hallowing of the stem (Tsror *et al*., 2009). *Dickeya dadantii* causes the rapid disruption of parenchymatous tissues, principally induced by the use of its pectic enzymes, and accelerates the disease process with cellulases, iron assimilation, a T3SS, EPS, and proteins involved in resistance to plant defences (Hugouvieux-Cotte-Pattat *et al*., 1996; Thomson & Gouk, 2003; Grenier *et al*., 2006; Yang *et al*., 2008; Antunez-Lamas *et al*., 2009). Despite its long history as a typified plant pathogen, *D. dadantii* strain 3937

was shown to be a pathogen of the pea aphid, *A. pisum* (Grenier *et al*., 2006). Grenier *et al*. (2006) determined that *A. pisum* aphids that had ingested *D. dadantii* eventually succumbed to their infection, with the minimum infectious dose of *D. dadantii* being calculated as fewer than 10 bacterial cells. Recent genome sequencing of the *D. dadantii* strain 3937 revealed the presence of four genes encoding homologues of insecticidal toxins, which were hypothesized to contribute to the pathogenicity of the bacterium in the aphid (Grenier *et al*., 2006). These homologues were later found to be able to complement the *cyt* family of genes from *Bacillus thuringiensis*, which encode haemolytic toxins (Crickmore *et al*., 1998). Gut proteases were hypothesized to cleave and activate the *D. dadantii* Cyt toxins in the aphid, resulting in pore formation in the insect gut membrane, and leading to bacterial invasion of the aphid and eventual death (Promdonkoy & Ellar, 2000; Grenier *et al*., 2006); however, the Δ*cyt* mutant retained virulence, suggesting that other virulence genes or factors are involved. The *cyt*-like toxins may therefore be involved in the early colonization of the aphid digestive tract, which is consistent with what is known for the *B. thuringiensis* homologues (Chattopadhyay *et al*., 2004).

In a later study, Costechareyre *et al*. (2010) found that the four coregulated *cyt* genes are expressed in response to high osmolarity. They suggest that this is because *D. dadantii* is commonly found in the low-osmolarity intercellular fluids of its host plant, where toxin synthesis is likely not necessary; however, a high concentration of sucrose is prevalent in the phloem sap, which would trigger toxin production if bacteria are internalized in a phloem-feeding insect gut, like that of aphids. Further exploration revealed that *cyt* gene expression is repressed by both *hns* (histone-like nucleoid structuring protein) (Costechareyre *et al*., 2010) and *vfmE*, a regulator of plant cell wall-degrading enzymes (Reverchon *et al*., 1994), because both *hns* and *vfmE* mutants retained the pathogenicity of the wild type. PecS, a regulator of pectinases and cellulases (Reverchon *et al*., 1994), appeared to regulate *cyt* gene expression because *pecS* mutants were found to be nonpathogenic when ingested by the aphid. Mutants of the GacA, OmpR, and PhoP regulators, which are involved in plant pathogenesis (Nasser *et al*., 2001; Llama-Palacios *et al*., 2005; Lebeau *et al*., 2008) and do not appear to affect Cyt toxin production, had reduced virulence in the aphid. The Cyt toxins, which are expressed under very specific conditions, are therefore only part of the suite of virulence factors used by *D. dadantii* to cause disease in aphids.

The relatively low minimum infectious dose of *D. dadantii* required for aphid infection could suggest high infectious rates for aphids overall, as this low density can be easily acquired from plant surfaces or from feeding on contaminated vascular tissues (Toth *et al*., 2003; Stavrinides *et al*., 2009; Costechareyre *et al*., 2010). It is unclear whether *D. dadantii* is transmitted readily to healthy plants by the aphids or whether this represents a more opportunistic or generalized association.

Erwinia aphidicola and the pea aphid

Erwinia aphidicola, a member of the *Enterobacteriaceae*, has been identified as the causal agent of leaf spot disease of common bean (*Phaseolus vulgaris*) and chlorosis and necrosis of pea (*Pisum sativum* cv. Tirabeque) (Gonzalez *et al*., 2005; Santos *et al*., 2009). In addition to causing plant disease, *E. aphidicola* also exhibits pathogenicity toward the pea aphid, *A. pisum*. Harada *et al*. (1997) initiated the study of a mysterious bacterium, called bacterium X, which was found to infect the gut of insects that had been kept aseptically. The bacterium, which was later identified as *E. aphidicola*, could grow productively in the aphid gut, inhibiting post-final ecdysis and resulting in mortality of the adult insect. Harada & Ishikawa (1997) observed that the bacteria produced EPS when they were left to grow in a medium containing sucrose, trehalose, or their component monosaccharides. This capsule may not be essential to cellular function, but it may allow certain saprophytes to attach to areas where there is an abundance of nutrients, allow certain pathogens to avoid engulfment by phagocytes, or contribute to the attachment and colonization of the pathogen in the aphid gut (Harada & Ishikawa, 1997). The cause of aphid mortality following colonization was proposed to be due to the aseptic conditions of the aphid gut, because normal gut colonizers would help maintain *E. aphidicola* densities in check (Harada & Ishikawa, 1997). Still, there are likely many genetic factors that contribute to the colonization of the gut, but these remain unexplored.

Pantoea stewartii and the pea aphid

Pantoea stewartii, the Stewart's wilt pathogen, which normally associates with its flea beetle vector, was recently found to exploit the pea aphid as a host. A study by Stavrinides *et al*. (2010) showed that *P. stewartii* DC283 (DC283) was pathogenic toward the pea aphid, as aphids fed a single dose of DC283 began to accumulate bacteria in their gut, with titres reaching 5×10^8 CFU, and aphid death following within 72 h. To identify the specific genetic determinants that were involved in pathogenesis, and more specifically, those that contributed to the lethality of the aphid, transposon mutagenesis screen was conducted. A single locus was identified, termed *ucp1* (you cannot pass), which appeared to be essential for the aggregation and pathogenicity of DC283. *ucp1*-proficient bacteria formed aggregates in the crop and hindgut, whereas the *ucp1* mutant did not. Aggregates of *ucp1*-expressing bacteria were suggested to be more resilient, accumulating in the crop and hindgut until the point of barricading the flow of honeydew.

This result coincides with the structural and functional features of *ucp1*, which included several predicted transmembrane domains, suggesting membrane localization and possible substrate or matrix-binding capabilities.

Six potential homologues of Ucp1 were identified in the draft genome of DC283, all of which were found to share a highly conserved N terminus, but an entirely nonhomologous C terminus (Stavrinides *et al.*, 2010). The conserved N terminus contains the transmembrane domains, and the prediction of protein localization places the hypervariable C terminus facing the extracellular environment. Based on this, *ucp1* was proposed to function as a microbial surface component recognizing adhesive matrix molecules (MSCRAMM) – a family of adhesion proteins utilized by animal pathogens to bind to proteinaceous components of the eukaryotic host cell to allow pathogenesis (Patti *et al.*, 1994). To lend credence to this hypothesis, all seven related genes were expressed in *Escherichia coli*, and each line fed to aphids. Only *ucp1* was necessary and sufficient for pathogenicity in the aphid, whereas the other lines were avirulent like control lines. In addition, *E. coli* lines expressing the protein exhibited the same aggregation phenotype as that seen for wild-type DC283, suggesting that this protein was necessary and sufficient for this phenotype. It was unclear, however, whether this protein was involved in direct binding to an aphid gut receptor and whether the other six related proteins could bind to other matrix molecules in different hosts. The drastic variability seen in the potentially exposed C terminus of all seven proteins could be the direct result of genetic shuffling or pathoadaptation imposed by host immune pressures (Stavrinides *et al.*, 2006, 2008, 2010; Korotkova *et al.*, 2007). Alternatively, it was proposed that the C terminus of Ucp1 does not bind to eukaryotic proteins, but instead to other exposed Ucp C termini of nearby cells, thereby promoting the linking of the structures to produce a bacterial matrix. Although its precise function is unclear, the *ucp1* locus appears to be essential for the pathogenicity of *P. stewartii* in pea aphids.

One insect for all occasions

Many insects are efficient vectors of phytopathogenic bacteria, transporting them to candidate host plants in the environment. In these associations, the bacterium is not expected to harm its vector, although there may be a reduction in the fitness of the insect as a result of carriage (Stavrinides *et al.*, 2010). These interactions can be characterized as commensalistic or slightly parasitic depending on the specific insect–-bacterium association. In contrast, some insects have been shown to function as a primary host for bacteria, and are exploited by these pathogens as equivalents to their plant hosts (Table 1). In cases where the insect is exploited as an alternative primary host, the association is effectively parasitic, with the fitness of the bacteria increasing at a cost to the insect. These bacteria exhibit entomopathogenic characteristics, utilizing specific virulence factors to overcome insect host defences, propagate, and disperse. However, what if an insect can function both as a primary host and as a vector for a given phytopathogen?

Although there is a tendency for us to categorize the interactions among organisms into discrete groups, the general biology and ecology of many phytopathogens and their interactions with other organisms in the environment are still rather nebulous. More recent studies of

Table 1. Properties of bacterial pathogens

Pathogen	Family	Genome size (Mb)	Plant hosts	Insect hosts	Nature of insect association	Plant pathogenicity factors	Insect pathogenicity factors
Candidatus Liberibacter	*Rhizobiaceae*	1.2	Citrus	Psyllid	Vector	–	–
Dickeya dadantii	*Enterobacteriaceae*	4.8	Potato, maize	Pea aphid	Host	*hrp/hrc*	*cyt*
Erwinia amylovora	*Enterobacteriaceae*	3.8	Apple, pear	Pollinating insects	Vector	amylovoran, levan, *hrp/hrc*	–
Erwinia aphidicola	*Enterobacteriaceae*	~4	Bean, pea	Pea aphid	Host	–	–
Erwinia tracheiphila	*Enterobacteriaceae*	~4	Cucumber, melon	Cucumber beetle	Vector	–	–
Pantoea stewartii	*Enterobacteriaceae*	5.0	Maize	Flea beetle Aphid	Vector Host	*hrp/wts*	*ysa* (*ysaN*) *ucp1*
Pectobacterium carotovorum	*Enterobacteriaceae*	5.0	Potato	Fruit fly	Vector	*expI/hor*	*evf/hor*
Pseudomonas syringae	*Pseudomonadacae*	5.6	Bean	Pea aphid	Vector/host	*hrp/hrc*	*fliL*
Serratia marcescens	*Enterobacteriaceae*	4.6	Pumpkin, squash	Squash bug	Vector	*oxyR*	–
Xylella fastidiosa	*Xanthomonadaceae*	2.1	Citrus, grape	Sharpshooter, spittlebug	Vector	*rpfC*	*rpfF*

–, unknown/not determined.

phytopathogens have uncovered unique interactions with insects, including one case where the insect appears to serve as a suitable primary host, as well as a vector (Table 1). This particular association raises several interesting issues, including the difficulty of reconciling the commensalistic and pathogenic life stages of the pathogen. Pathogens are known to reach a virulence optimum, which maximizes their aggressiveness and transmission potential (Woolhouse *et al.*, 2002). High levels of aggressiveness may result in the death of the host insect before the bacterium is able to disperse to other hosts; thus, natural selection will favour a reduction in the aggressiveness of the pathogen to allow dispersal of the bacterium and thereby increase bacterial fitness (Thrall & Burdon, 2010). To overcome this trade-off, the bacterium can exploit its insect host maximally by replicating rapidly, and yet this would come into direct conflict with the dynamics of association between a typical phytopathogen and vector. For the following example, the application of the fundamental concepts of host–microorganism associations is complicated by the fact that the insect can function both as a host and as a vector. It is an especially exciting interaction for the simple idea that it may represent a transitional stage in the evolution of phytopathogen–vector and phytopathogen–host associations.

Pseudomonas syringae and the pea aphid

Pseudomonas syringae is a phytopathogenic bacterium noted for its diverse interactions with different plant species. Although many strains are known to cause disease on various plants, many epiphytic strains have also been identified (Clarke *et al.*, 2010). *Pseudomonas syringae* propagation onto and between host plants involves rain splash-mediated inoculation from infected to uninfected plants, facilitated by the aggressive epiphytic and aggregation capabilities of *P. syringae. Pseudomonas syringae* has also been shown to disperse via precipitation (Pietrarelli *et al.*, 2006).

Pseudomonas syringae was considered to be a very strict phytopathogen, capable of infecting a variety of different plants (Hirano & Upper, 2000); however, a recent study by Stavrinides *et al.* (2009) demonstrated that some strains of this species also have entomopathogenic potential. The bean strain, *P. syringae* pv. syringae B728a (B728a), which is an aggressive epiphyte and pathogen of bean, was shown to exploit the pea aphid as a suitable alternative primary host. Within 36–48 h of ingesting B728a, aphids succumb to infection, with the growth of up to 3×10^6 CFU per aphid. In contrast, ingestion of the tomato strain *P. syringae* pv. tomato DC3000 (DC3000) by aphids, results in bacterial titres of 1×10^9 CFU per aphid, with no evidence of disease, and aphid survivorship remaining unaffected beyond 72 h. This suggests that the presence of strain-specific virulence factors contributes to the colonization of the aphid by B728a.

Whole-genome comparisons of DC3000 and B728a identified toxin complex (*tc*) genes in both strains whose homologues have been implicated in insect association (Lindeberg *et al.*, 2008). The *tc* genes present in DC3000 appeared degenerate, with mobile genetic elements and deletions disrupting the reading frame, whereas the orthologues in B728a were intact. These genes were strong candidates for explaining the virulence of B728a and the avirulence of DC3000. Mutation of two of the B728a *tc* genes does not attenuate virulence, indicating that these genes were not the primary virulence determinants for B728a in the aphid. To identify those genetic factors that were involved in aphid colonization, Stavrinides *et al.* (2009) performed a mutagenesis screen to identify the mutants that had reduced or abolished virulence. Multiple hypovirulent B728a mutants were recovered, including one that was defective in the *fliL* gene, which is required for flagellar formation. The *fliL* mutant was completely avirulent, growing to titres of 4×10^7 CFU per aphid, which were not lethal to the aphid, much like the avirulent DC3000 wild-type strain. To identify the phenotypic effects of the *fliL* mutation, various motility assays were undertaken. A swarming assay revealed that the *fliL* mutant was incapable of swarming – a type of movement commonly seen in bacteria that allows for coordinated movement over a solid or a semi-solid surface. It is unclear, however, whether it is swarming specifically that is required for virulence in the aphid or whether there are pleiotropic effects, where motility regulates other virulence factors.

In exploring the pathogenicity of B728a toward the aphid, Stavrinides *et al.* (2009) noted that infected aphids exhibited some very unusual behaviours. After the onset of disease, aphids would discontinue feeding and commence to wander around, depositing and moving honeydew behind them. Stavrinides *et al.* (2009) hypothesized that the honeydew that was passing through the aphids contained high titres of B728a. Using a simple culturing method, they found that viable B728a was present in the deposited honeydew, with up to 10^7 phytopathogenic bacteria cm^{-2}, suggesting that the bacteria propagate in the aphid, and are then redeposited back onto plant surfaces; however, because many of these feeding experiments were performed under artificial conditions, Stavrinides *et al.* (2009) attempted to demonstrate that healthy aphids could indeed become infected by feeding on plants that were colonized epiphytically by B728a. Aphids were introduced onto plants that had been surface-inoculated with B728a, and after a feeding period, aphids were harvested and screened for the presence of B728a. Aphids were shown to acquire the bacteria, which likely colonized the digestive tract, multiplied, and were then excreted in the aphid honeydew. The acquisition of the

bacterium by the aphids most probably occurs via stylet-mediated plant host probing that takes place when aphids land on a new plant and attempt to determine whether it is a suitable host (Kennedy & Stroyan, 1959; Auclair, 1963; Stavrinides *et al.*, 2009). Infection by epiphytic bacteria may occur during this process, where aphids repeatedly push their stylet through the host tissue, pushing down any surface bacteria, and then ingesting those bacteria while sampling plant fluids. Under this model, aphids acquire the pathogen during probing of an epiphytically colonized plant host, with the ingested bacteria subsequently colonizing and propagating within the aphid. The bacteria escape from the aphid via the honeydew and are deposited back onto the plant surface, where they are given an opportunity to reassociate with their plant host. At this stage, the aphid functions as a vector for the pathogen.

To determine the amount of inoculum deposited on the plant surface by infected aphids, Stavrinides *et al.* (2009) introduced infected aphids onto host plants, and bacteria densities were quantified following a feeding period. The phyllosphere was shown to be inoculated with up to 2×10^7 phytopathogenic bacteria cm^{-2} per aphid, suggesting that the aphid is an excellent culturing vessel for this phytopathogen. Because honeydew is carbohydrate rich, the deposition of bacteria in a suspension of nutrients may enable *P. syringae* to enhance its survival and subsistence on the surface of the leaf. Certainly, because B728a is pathogenic to the aphid, successful deposition onto the leaf would have to occur quickly, and before the death of the aphid. In the case of DC3000 and the aphid, however, the bacteria do not kill the aphid, making this particular association more consistent with a true vectoring relationship.

Pseudomonas syringae shows a very high level of aggressiveness in the pea aphid, which results in the death of the aphid in only a few days, but because the bacteria have a direct and continual route of escape from their host, they have the opportunity to replicate maximally without the tradeoff of prematurely killing the host due to high aggressiveness. Such an interaction provides the opportunity to study the dynamics of a unique relationship between a phytopathogen and an insect that can be used not only as an alternative primary host but also as a vector, which can provide an active dispersal mechanism to other plant hosts (Fig. 1).

Aphids and insect defences

It is particularly interesting that many of the interactions between insects and bacteria described above have involved the aphid – one of the most destructive agricultural insect pests (Harada & Ishikawa, 1997) – which causes significant damage to plants as sap feeders, pollutive excreters, toxifiers, and as vectors of viral diseases (Harada & Ishikawa, 1997). Because of their close association with a variety of plants, they are also predisposed to encountering a diversity of epiphytic and phytopathogenic bacteria (Stavrinides *et al.*, 2010). Several studies have highlighted the general affinity of the members of the *Enterobacteriaceae* for aphids, many of which colonize the aphid gut (Grenier *et al.*, 1994; Harada &

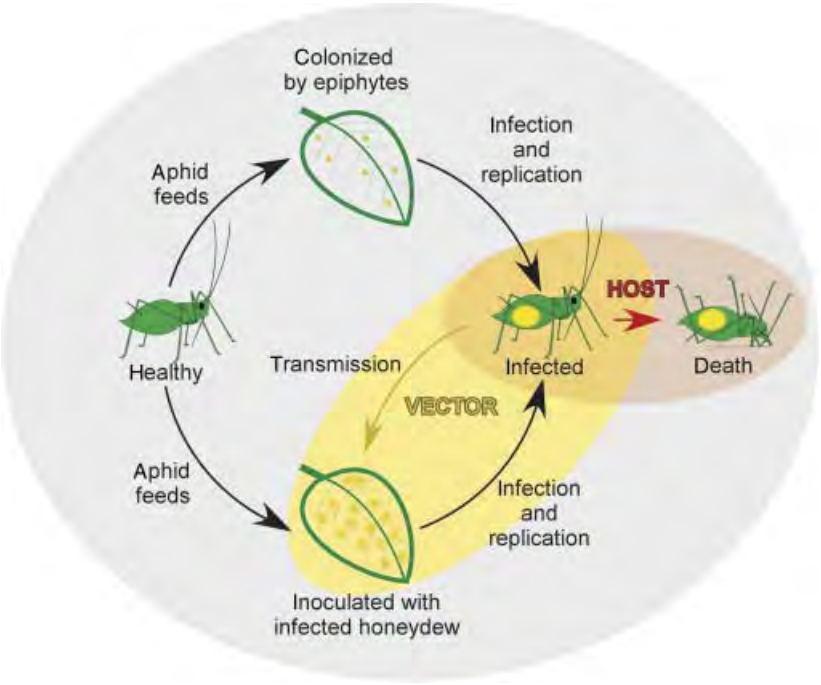

Fig. 1. The role of the aphid as both vector and alternative primary host for the plant pathogen *Pseudomonas syringae* pv. *syringae* B728a (B728a). The acquisition of B728a by aphids occurs through feeding on plants colonized epiphytically by bacteria (yellow dots). The plant pathogenic bacteria replicate within infected aphids, and are excreted in globules of honeydew, which fall onto the plant surface. Infected aphids may wander to other plant hosts, vectoring the bacteria in the process. Shortly after infection with B728a, the host aphid, which has been used as a mass replication vessel by the bacteria, succumbs to sepsis. Adapted from Stavrinides *et al.* (2009).

Ishikawa, 1997). Are aphids, therefore, an ideal insect host for phytopathogen colonization and are they more susceptible to pathogen attack than other insects?

Insects have evolved specific behaviours that allow them to avoid predation, environmental stressors, and pathogens, but when these stressors bypass the defensive behaviours, insects must rely on the physical defences, such as those provided by their protective cuticle or gut pH level for defence (Tarpy, 2003; Ha *et al*., 2005; Francke *et al*., 2008; Hatano *et al*., 2008; Gerardo *et al*., 2010). If these barriers are also breached, immunological defence mechanisms such as clotting, encapsulation, phagocytosis, and the synthesis of antimicrobial substances come into play (Gagneux *et al*., 2006; Govind, 2008; Gerardo *et al*., 2010). Analysis of the recently sequenced genome of the pea aphid has revealed that aphids do have defence mechanisms found universally in other arthropods, including the JAK/STAT and Toll signalling pathways, which are involved in both development and immunity; however, several essential genes involved in the innate immunity of arthropods are absent from the genome, including the IMD signalling pathway, c-type lysozymes, defensins, and peptidoglycan recognition proteins (Gerardo *et al*., 2010). The absence of these genes may be due to an inability to locate homologues, given the large evolutionary distance between aphids and the taxa from where such genes are well studied (Gerardo *et al*., 2010) or due to aphids possessing an alternative, yet equally effective immune response. There is little evidence for the latter. It was also suggested that unlike *Drosophila*, whose source of food is constantly contaminated with a diverse array of microorganisms, aphids would only encounter entomopathogens and bacteria in the phloem sap of plants very rarely, eliminating the need for a more developed defence arsenal (Altincicek *et al*., 2008); however, through probing of plants, aphids have been shown to contact and ingest a diversity of epiphytic bacteria, both pathogenic and nonpathogenic (Stavrinides *et al*., 2009, 2010; Gerardo *et al*., 2010). Another possibility is that aphids invest in terminal reproduction when faced with an immune challenge in contrast to spending extensive amounts of energy attempting to defend themselves (Altincicek *et al*., 2008; Gerardo *et al*., 2010). Indeed, stabbed aphids generated more offspring than those that were untreated (Altincicek *et al*., 2008), although this is also seen in crickets (Adamo, 1999), waterfleas (Chadwick & Little, 2005), and snails (Minchella & Loverde, 1981; Minchella *et al*., 1985), which appear to have more developed immune systems (Gerardo *et al*., 2010). Interestingly, the secondary endosymbionts such as *Hamiltonella defensa*, which provides protection against the parasitoid wasp *Aphidius ervi*, and *Regiella insecticola*, which protects against fungal pathogens, persist within the haemolymph and are detected and managed by the aphid immune system (Gerardo *et al*., 2010).

Evolution of alternative associations

Bacterial phytopathogens have been, up to now, considered just that – bacteria that are capable of colonizing, reproducing, and disseminating from only plant hosts; however, it is now very evident that these plant pathogenic bacteria have the ability to exploit insects with which they share an overlapping niche as alternative primary hosts (Table 1). Many interesting questions arise from this including those relating to general ecology, pathogenic potential, and host-specific virulence factors. For example, the ability of a microorganism to associate intimately with two hosts across two different kingdoms likely leads to an evolutionary struggle for the microorganism, which must evolve host-specific strategies for associating with each of its hosts. A phytopathogen will undergo adaptation of its overall aggressiveness toward its plant host in order to achieve its fitness optimum, but this optimum may be different in the insect vector or host, requiring the pathogen to achieve an intermediate multihost optimum (Fig. 2). The *X. fastidiosa rpfF* gene, which is required for insect association, causes a reduction in plant virulence (Newman *et al*., 2004; Chatterjee *et al*., 2008), illustrating that there are tradeoffs associated with multihost associations.

Aside from the complexities of multihost associations, the directionality of host association is also an interesting evolutionary question. In the majority of interactions described above, insects serve either as vectors or as alternative hosts for phytopathogenic bacteria, and in cases where the insect presently serves as a vector, the phytopathogen uses the insect cavity as a transport vehicle for moving to its next plant host. But, how did these relationships evolve? The association of phytopathogens and insects may have begun with insects feeding transiently on plant tissues colonized by phytopathogens. Internalized bacteria survived the conditions of the insect cavity as well as immunological defences to be dispersed successfully to a new host (Fig. 3). The reiteration of this process over evolutionary time would have selected for those bacterial variants whose fitness increased as a result of this interaction, namely, those that were capable of surviving in the insect, were less immunogenic, and/or had higher replication and dispersal as a result of associating with the insect. This would have resulted in many of the interactions that exist today between phytopathogens and their vectors; however, did this association begin so pleasantly? Phytopathogens may have first evolved entomopathogenicity and began colonizing insects as alternative primary hosts following recurrent encounters over the course of evolution. These interactions may have then converted to a more benign association, where the entomopathogen became substantially reduced in virulence, but capable of maintaining its association with specific insect hosts long enough to ensure its dispersal. In any host–

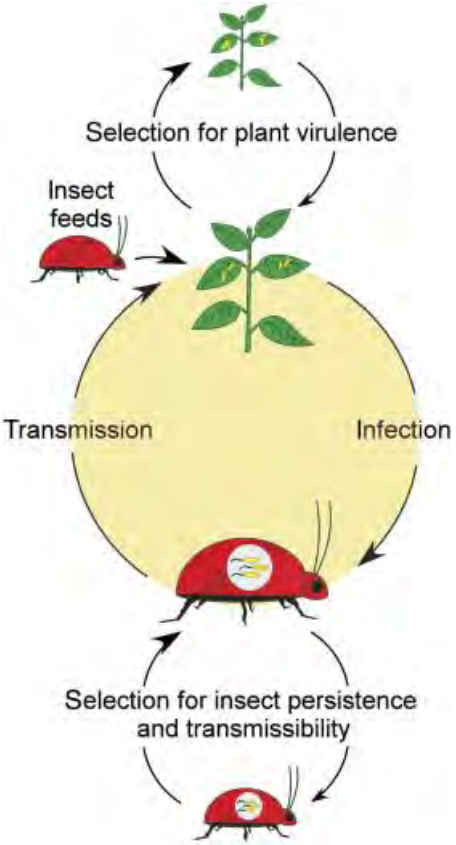

Fig. 2. A bacterium having multiple hosts must achieve a balance with both its hosts to achieve optimal fitness. The lifecycles of some phytopathogens include insect vectors, which function to transmit the bacteria to new plant hosts (middle circle). During their association with the plant (top), phytopathogens utilize plant-specific strategies for colonizing and causing disease in plant tissues, eventually reaching a virulence optimum that maximizes their fitness. This optimum, however, may conflict with the strategies used for colonizing, persisting, and being transmitted from the insect vector, necessitating a balance that maximizes the fitness of the pathogen with both hosts.

microorganism relationship, the specific tradeoffs endured by each partner will dictate the strength and overall success of the association. In the associations where phytopathogens are transmitted by vectors, by definition, there needs to be a very low cost to the insect for carrying the bacterium; however, there may often be a slight cost to carriage that can destabilize the success of the association (Bahri *et al.*, 2009).

The associations between phytopathogens and insects may be promoted and maintained through the direct effects of pathogen infection. Infection of plants by bacterial pathogens has been shown to lead to drastic enhancement of their commonly emitted volatiles, which are known to be attractants for insects (Turlings *et al.*, 1990; Shiojiri *et al.*, 2006). Modifications to the hydroperoxide lyase pathway in

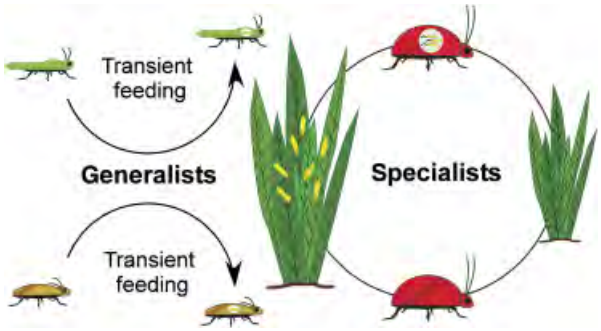

Fig. 3. The plant-first model. Phytopathogenic bacteria (yellow) may have evolved alternative associations with insects following either transient interactions with generalists (left), which may move to an unsuitable plant host for the pathogen, or through interactions with specialized insects (right) that feed on a limited set or subset of plants that overlap with the preferred hosts of the pathogen.

Arabidopsis, for example, which is responsible for the synthesis of the leaf volatiles, resulted in an increase in volatile production during pathogen infection, which in turn made the plant more attractive to the parasitic wasp, *Cotesia glomerata* (Shiojiri *et al.*, 2006). In some cases, the specific volatiles produced have been shown to be dependent on the specific bacterial strain colonizing the plant. Tobacco plants inoculated with virulent strains of *P. syringae* produced qualitatively different volatiles and at higher concentrations than those produced during infection with avirulent strains (Huang *et al.*, 2003); thus, the changes induced by the pathogen may attract insects to the infected plants, increasing the likelihood of the pathogen associating with a particular insect host.

It is interesting to note that many of the phytopathogens shown to have alternative insect associations are in the *Enterobacteriaceae* (Stavrinides, 2009), a group that generally associates with animal and insect hosts. Did these phytopathogens evolve entomopathogenicity, or were they in fact insect-associated microorganisms that evolved phytopathogenicity, but still retain an ancestral insect-association lifestyle? If these bacteria were once insect-associated, either entomopathogens or insect commensals, they may have evolved phytopathogenic capabilities after repeated deposition on plants over evolutionary time (Fig. 4). An increase in bacterial fitness that results from repeated encounters with their own insect hosts or other insects in the environment would have contributed to the maintenance of the determinants necessary for insect association. Many of the enteric plant pathogens described here seem to retain their ancestral gut-associating capabilities. Phytopathogenic *Enterobacteriaceae*, including *Erwinia*, *Dickeya*, *Serratia*, and *Pantoea*, retain relatively tight pathogenic and nonpathogenic associations with herbivorous- and plant-associated insects (Harada & Ishikawa, 1997; de Vries *et al.*, 2001a, b;

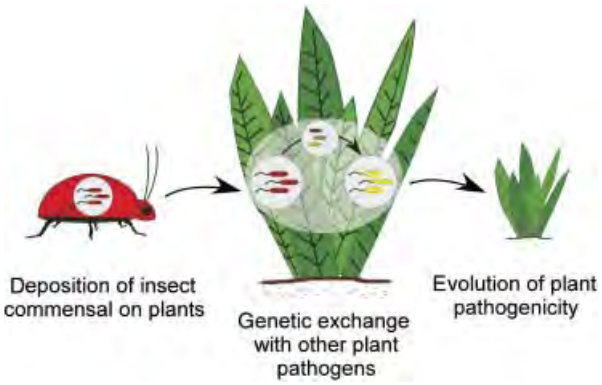

Fig. 4. The insect-first model. Present-day phytopathogens that associate with insects may have had ancestral associations with insects. Following deposition onto plant hosts via their plant-associating insect hosts, genetic exchange with other plant pathogens found within the phyllosphere or rhizosphere may have led to the evolution of phytopathogenicity.

Capuzzo *et al.*, 2005). For example, *E. amylovora* is pathogenic to the olive fly and Western flower thrips (de Vries *et al.*, 2004; Capuzzo *et al.*, 2005), but survives 12 days on aphids, and at least 5 days in association with the green lacewing (Hildebrand *et al.*, 2000), while *D. dadantii, P. stewartii*, and *E. aphidicola* have been shown to be pathogenic to the pea aphid, colonizing the gut and causing death (Harada & Ishikawa, 1997; Grenier *et al.*, 2006; Stavrinides *et al.*, 2010). Similarly, the colonization of *Drosophila* by *P. carotovorum* results in a host defence response, characterized by the production of antimicrobials (Basset *et al.*, 2000); however, bacterial persistence is enabled by the bacterial gene, *evf*, which enhances the survival of the bacteria in the gut by preventing insect excretion (Basset *et al.*, 2003). This gene was suggested to be acquired recently through horizontal gene transfer, possibly suggesting that insect persistence is an acquired and not an ancestral capability. Certainly, there may be issues of host specificity that also come into play, where there may be another true host of *P. carotovorum* in which the bacteria can persist without the *evf* gene. In contrast, genomic comparisons of the enteric phytopathogen *Pectobacterium atrosepticum* and several enteric animal pathogens revealed the acquisition of many different plant-associated pathogenicity islands by *P. atrosepticum*, including a T3SS, and genes for agglutination, adhesion, and phytotoxin biosynthesis (Toth *et al.*, 2003). These islands share homology to genes from other plant-associated bacteria, suggesting acquisition through horizontal gene transfer from phytopathogens. The *P. atrosepticum* genome does not show obvious signatures of having undergone new niche adaptation, suggesting that it has only gained new capabilities through incremental gene loss and gain. The identification of interactions between these phytopathogens and plant-associated insects could indicate that their ancestral gut associations have remained an integral component of their lifecycle, and the evolution of plant pathogenicity may have followed from frequent insect-mediated deposition on plants (Fig. 4).

In the association between *P. syringae* and the pea aphid, strain B728a exhibits pathogenicity toward the insect, with infection resulting in aphid death in < 36 h. The pathogen replicates in the aphid, and is then deposited onto the plant via the aphid honeydew, making the aphid an efficient vector for the phytopathogen. In most microorganism–vector associations, the bacterium is not pathogenic toward the vector, because this would reduce the likelihood of being transmitted to the next plant host; but because the aphid is already plant-associated, the microorganism will be deposited back onto the plant host, allowing it to replicate maximally without having to offset the cost of killing the insect host. In this somewhat atypical interaction, the aphid can be used as both a primary host and vector (Fig. 1), which raises interesting questions about the directionality of the association. Could this interaction represent a transitionary state in the evolution of insect–microorganism interactions, where B728a began as being only vectored by the aphid, but not causing its death, and gradually moved toward entomopathogenicity? Or is it attenuating in virulence as it is becoming more adapted to the aphid, perhaps to a strict vectoring association? The ability of the related tomato pathogen *P. syringae* pv. tomato DC3000 to replicate within, but not cause the death of the pea aphid, would suggest that B728a has moved toward entomopathogenicity. The pea aphid is not known to feed on tomato plants, and would therefore be unlikely to encounter DC3000, supporting the idea that the entomopathogenicity of B728a is an acquired trait. This exciting prospect lends itself to further exploration of this interaction, including the identification and characterization of the specific genetic determinants required for this relationship. The analysis of the genetics of this interaction is presently underway, and will yield an important insight into the evolution and ecological relevance of these alternative associations.

Conclusions and future developments

Our knowledge of the general ecology of phytopathogenic bacteria has begun to expand beyond their immediate interactions with plants to encompass the other ecological players in the environment. There is increasing evidence that plant pathogenic bacteria have evolved specific and nonspecific associations with insects, which they exploit as delivery vehicles or as primary alternative hosts. Specific bacterial genetic determinants have been identified that lend credence to the notion that these associations are not incidental, but have evolved with recurrent encounters, followed by natural selection. While many of these studies

have provided an incredible wealth of information on the genetics and pathology of bacterial association, their ecological relevance remains ambiguous. It is certain, however, that a better understanding of phytopathogen epidemiology will require a better understanding of the nature of specific interactions and associations with other organisms in the environment.

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