A to ZYMV GUIDE TO ERWINIA TRACHEIPHILA INFECTION: AN ECOLOGICAL AND MOLECULAR STUDY

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Lori Shapiro

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The thesis of Lori Shapiro was reviewed and approved* by the following:

Mark Mescher
Assistant Professor of Entomology
Thesis Advisor
Chair of Committee

Consuelo De Moraes
Professor of Entomology

Andy Stephenson
Distinguished Professor of Biology and Associate Dean of Graduate Studies

Shelby Fleischer
Professor of Entomology

Eric Harvill
Professor of Microbiology and Infectious Disease

Gary Felton
Head of the Department of Department or Graduate Program

*Signatures are on file in the Graduate School
ABSTRACT

Many of the most ecologically and economically important diseases of plants are transmitted by insects, and the spread of these diseases ultimately depends on complex interactions among plants, pathogens, and insect vectors. Yet, empirical evidence regarding the mechanisms by which plant pathogens are transmitted by insects is sparse. In this dissertation, I take a systems biology approach to understanding ecological and molecular interactions driving the emergence of the bacterial phytopathogen *Erwinia tracheiphila*, a microbe with exceptional economic importance and a highly restricted host range. Induced changes in the emission of plant volatiles—airborne chemicals released by plants that serve as key foraging cues for insects but are not well characterized in response to pathogen infection. In the field and greenhouse, I found that *E. tracheiphila* induced a unique volatile blend from foliage, and caused a reduction in the emission of floral volatiles. The co-occurring viral pathogen Zucchini Yellow Mosaic Virus suppressed both leaf and floral volatiles. Behavioral assays found these volatile differences had effects on vector attraction and were important for beetle recruitment to *E. tracheiphila* infected plants and dispersal to healthy ones, and that few beetles were recruited to virus infected plants. Controlled inoculations show that healthy and ZYMV infected plants are equally susceptible to *E. tracheiphila* infection, and so vector behavior is largely driving lack of coinfection of the two pathogens in the same host plant that is often documented in the field. Next, I developed a qPCR method to clarify uncertainty in rates of *E. tracheiphila* colonization of beetle vectors exposed by feeding on wilting leaf tissue. I found that *E. tracheiphila* does not colonize beetles very efficiently: While at least 80% of beetles are exposed to *E. tracheiphila* at both short (3 hr) and long (24 hr) exposures, the longer exposure results in significantly higher long-term retention rates. Quantitative changes in bacterial titre in frass and whole beetles also show that transmission is not simply mechanical, and that *E. tracheiphila* exhibits complex colonization dynamics of the
beetle’s digestive tract characterized by attachment, growth, and shedding phases. Finally, I analyze the genome of *E. tracheiphila* and find it has undergone extensive recombination and pseudogenization compared to the closest sequenced relative, and contains many putatively horizontally transferred genes. Phylogenetic and comparative data suggest *E. tracheiphila* is undergoing rapid evolution and may have recently experienced a host jump to squash. Taken together, these studies provide an ecological mechanism for transmission from infected to healthy plants; a quantitative description of how beetles are colonized and consequences for transmission; and a genome analysis hypothesizing factors influencing *E. tracheiphila* emergence as a squash pathogen and persistently transmitted insect symbiont.
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Chapter 1

Introduction

An Historical Perspective of Cucurbit Research

In response to herbivory pressure, plants have developed a chemical arsenal of metabolically costly traits that deter damages and losses from herbivory. Induced chemical traits, which are not known to have any primary metabolic function, are referred to as secondary metabolic compounds, and encompass a diverse array of chemical structures that have shaped animal evolutionary history (Fraenkel 1959; Ehrlich & Raven 1964). In response, many insect herbivores evolved the ability to detoxify, sequester, or otherwise tolerate secondary metabolites from plants. Because of the cost to the insect from interacting with plant secondary metabolites, many herbivores specialize on one or a few closely related host plant species (Becerra 1997).

One plant-insect system of ecological, agricultural, and cultural interest comprises Cucurbitaceae plants and coevolved Luperini leaf beetles (Coleoptera: Chrysomelidae: Galerucinae). While Cucurbitaceae has a cosmopolitan distribution (Schaefer 2009), Cucurbita is native to Mesoamerica and has important historical and cultural significance. The wild gourd Cucurbita pepo ssp. texana is the wild progenitor of cultivated squashes, pumpkins, and gourds, and has its center of evolutionary origin and diversity in Mesoamerica (Whitaker & Bemis 1976; Whitaker & R.J. Knight 1980), with a range that extends northward through the American Southwest and the Mississippi River Valley. The wild gourd C. pepo ssp. texana has undergone an estimated six confirmed domestication events, with the oldest being in the Oaxaca Valley,
Mexico approx. 10,000-8,000 years ago (Smith 1997; Sanjur 2002). Domesticated *Cucurbita pepo* varieties retain important cultural and agricultural uses throughout their native range.

**Cucurbita Defensive Chemistry**

The production of a group of oxygenated tetracyclic triterpenes with variable side chains, called cucurbitacin (cucs), is diagnostic of many wild Cucurbitaceae species (S. Rehm 1957; Rehm 2006), although there is isolated documentation of cucurbitacin production in several other plant families (Curtis & Meade 1971; Pohlmann 1975; Dryer & Trousdale 1978). Cucurbitacins are highly toxic to both mammalian and insect herbivores: Cucs can comprise up to 1% fresh weight of fruits but are detectable by humans concentration as low as 1ppb (Enslin 1954; David & Vallance 1955), and cucs have caused poisoning when ingested by foraging cattle (Watts & Breyer-Brandwiyk 1962). In *C. pepo ssp. texana*, the predominant form of cucurbitacin is cuc-E-glycoside (Metcalf 1982). Because of their intense bitterness and cytotoxicity, cucs are selected against in crop breeding, although the complexity of the genes involved in cucurbitacin biosynthesis has resulted in occasional production of bitter fruits from non-bitter parents (Rehm & Wessels 1958).

While cucurbitacins deter almost all mammalian and insect herbivores (Tallamy et al. 1997), they act as potent arrestants and feeding stimulants for Old and New World Luperini leaf beetles that can detoxify and sequester cucurbitacins (Chambliss 1966; Metcalf et al. 1980; Ferguson & Metcalf 1985; Tallamy 2000; Gillespie et al. 2004). Cucurbitacin feeding is common in geographically distant Luperini (Nishida et al. 1992; Lewis & Metcalf 1996) and phylogenetic analysis show that an affinity for Cucurbitacins among luperine chrysomelids is not ancestral (Gillespie et al. 2003). *Acalymma* and *Diabrotica* spp. beetles can sequester a small proportion of ingested cucurbitacins (Ferguson et al. 1985), which can then function as protection against predators (Ferguson & Metcalf 1985) and to decrease the probability that eggs succumb to fungal pathogens (Tallamy 1998). If fed continuously on bitter, cuc-containing fruit, *Diabrotica* and
Acalymma spp. both experience a decrease in longevity (Ferguson et al. 1985), and a limit in the amount of bitter leaves consumed has been observed for A. vittatum that have already sequestered a large amount of cucs (Smyth 2002). In addition to being chemically defended by cucurbitacins that are highly effective against non-coevolved herbivores, cucurbits produce sticky phloem sap that can interfere with specialist herbivore feeding and even cause beetle death if mouthparts become too gummed (Cronshaw et al. 1973; Cronshaw 1975; McCloud et al. 1995; Eben 2007).

**Insect Attraction to Cucurbita**

Most plants release low molecular weight volatile organic compounds as part of their normal metabolic processes. These volatile cues can convey important information about host plant identity, status, and nutritional content to mammalian and insect herbivores, including humans (Goff & Klee 2006). The Cucurbita floral volatile blend has been a subject of intensive study for many decades as host location cues for coevolved beetles (Metcalf et al. 1980) and the basis for an attractant bait to control diabroticite pests in many agricultural systems (Lampman & Metcalf 1987, 1988). While most work has concentrated on volatile attraction of Diabrotica spp. to Cucurbita floral volatiles for the purpose of pest control in maize plantings (Metcalf & Lampman 1991), isolated studies have also found Acalymma vittatum to be strongly attracted to volatiles of cucurbit flowers and seedlings (Lewis et al. 1990).

Cucurbita spp. are pollinated by solitary Peponapis and Xenoglossa spp. (Hymenoptera: Anthophoridae) (Hurd 1971). However, this is an evolutionarily ‘recent’ interaction, and diabroticites are hypothesized to be the ancestral pollinators of Cucurbita. The fossil record dates the establishment of holometabolous development and the proliferation of Coleoptera, Neuroptera, Diptera, and Mecoptera to the Permian period (299-250 MYA) (Martynova 1961), while hymenoptera are not confirmed in the fossil record until the Tertiary period (65-2.6 MYA). Cucurbita floral traits, including large, yellow, open bowl shaped flowers are congruent with originally being beetle pollinated (Baker & Hurd 1968; Kevan & Baker 1983). While cucumber
beetles are highly destructive foliar cucurbit pests (Balduf 1925; Ferguson et al. 1983), Acalymma and Diabrotica mandibles and mola are structurally suggestive for florivory and to prepare pollen for digestion (Cabrera & Durante 2001; Cabrera & Durante 2003), and florivory is believed to be the ancestral state of all chrysomelids (Crowson 1960). Cucurbit pollen is found in the gut of Diabrotica and Acalymma and thought to compose an important part of their diet (Samuelson 1994). A. vittatum are also often found foraging on the flowers of rosaceous and composite plants (Balduf 1925; Houser 1925) although larval development is restricted to cucurbits.

**Diversity of Cucurbita herbivores**

Approximately 900 species of diabroticites have been described, with 338 valid Diabrotica species (Wilcox 1972) and 72 Acalymma species (Cabrera & Durante 2001)(Munroe & Smith 1980). It is estimated at least that many diabroticites remain undescribed (Smith 1966), mostly in the neotropics (Krysan & Smith 1987). Diabrotica has been informally divided (Eben et al. 2004) into the signifera, fucata (305 species), and virgifera (21 species) species groups with the pest species confined to the multivoltine fucata and univoltine virgifera groups (Clark 2001). While fucata species are somewhat polyphagous and can threaten the production of many crops (Barbercheck 1993), virgifera species threaten maize cultivation. Acalymma spp. are specialists on cucurbits in both larval and adult stages (Eben 1997b, a).

**Diabroticites as agricultural pests and disease vectors**

While the vast majority of Diabroticite species only occur in low population densities in wild habitats, several species have become some of the world’s most destructive agricultural pests (Branson & Krysan 1981). The most economically damaging among them is Diabrotica virgifera virgifera, a recently diverged subspecies of Diabrotica virgifera zea that has emerged as a specialized pest of cultivated corn varieties, and has undergone recent range expansion as corn cultivation has expanded through the American Midwest over the past several decades (Krysan et al. 1980; Branson et al. 1982). Because diabroticite pests are part of a coevolved, native system
and herbivores and pollinators utilize the same host location cues, insecticide-laced volatile baits developed to control diabroticite beetles also attract and kill pollinators, demonstrating the continuing need for more information on ecological interactions to develop sustainable pest and pathogen control strategies (Metcalf et al. 1987).

Several pests and diseases of cucurbits that threaten agricultural production, and possibly undomesticated populations, have emerged in the last several decades. The selection for agricultural traits over functional ecological ones, relative genetic homogeneity of agricultural crops, and landscape changes associated with modern monoculture agricultural methods, is likely associated with the emergence and dispersal of new pests and pathogens (Bach 1979; Hopkins & Purcell 2002; Stukenbrock & McDonald 2008; Pagán et al. 2012). *Erwinia tracheiphila* (Enterobacteriaceae) (Smith) is an emerging pathogen of wild and domesticated cucurbits. The first published description of *E. tracheiphila* was at the PSU experimental station (Fulton et al. 1911), but cucumber beetles were not implicated in bacterial spread until later work (Smith 1911, 1914, 1915; Rand & Enlows 1916, 1920; Rand 1920). The first demonstration that striped and spotted beetles

Most plant pathogenic bacteria are dispersed by rain, dew, or passive association with insects, but *E. tracheiphila* is one of the few known plant pathogenic bacteria that is obligately transmitted by an insect vector. *E. tracheiphila* has a highly restricted range on some cucurbit host plants. Susceptibility to infection varies, with *Cucumis* spp. (melons and cucumbers) being the most susceptible, *Cucurbita* spp. (squashes, pumpkins, and gourds) susceptible but showing some resistance, and watermelon (*Citrullus* spp.) thought to be completely resistance to *E. tracheiphila* infection.

*E. tracheiphila* is only confirmed to be transmitted by two species of diabroticite leaf beetles, the spotted cucumber beetle *Diabrotica undecimpunctata howardii* and the striped cucumber beetle *Acalymma vittatum* (Rand & Cash 1920). The geographical range of and
economic losses from *E. tracheiphila* have historically been restricted to the northeast and midwest, but the first case of *E. tracheiphila* infection was recently recorded in the Southwest (Sanogo et al. 2011), suggesting that *E. tracheiphila* could be expanding its range towards the evolutionary origin of *Cucurbita* in Mesoamerica where there are scores more possible diabroticite species that could function as competent disease vectors.

Because *E. tracheiphila* is obligately transmitted by insects and not through movement of infected plant material (which was responsible for the dispersal of the closely related fire blight pathogen *E. amylovora* from New York State to threatened pome fruit production worldwide), it is likely that factors related to vector competence or insect population levels affect geographical distribution of *E. tracheiphila*. Northeastern agricultural fields are largely dominated by a single species, the striped cucumber beetle *A. vittatum*. Striped cucumber beetles diapause as adults in Northeastern climates, and emerge in the spring and thus are a threat to cucurbit production from spring, when adults converge on fields of seedlings, until the end of the growing season. In contrast, the spotted cucumber beetles (*D. undecimpunctata*) do not overwinter well in the Northeast, and do not achieve large population levels until midsummer after they have migrated from warmer Southern areas. Vector competence of *D. undecimpunctata* compared to *A. vittatum* is not currently known, and the contribution of spotted cucumber beetles, which are more polyphagous and arrive later in the season, toward maintaining *E. tracheiphila* epidemics in the Northeast is uninvestigated. The current distribution of *E. tracheiphila* mostly conforms to the current distribution of the vector *A. vittatum*, suggesting *A. vittatum* are likely the dominant species driving *E. tracheiphila* disease dynamics in the Northeast.

Because *E. tracheiphila* currently occurs in the Midwest and Northeast where *A. vittatum* is the single dominant species, the potential for other diabroticites to transmit *E. tracheiphila* is largely unaddressed. All beetles from the *virgifera* group, which are univoltine and polyphagous as adults (including the pest species *Diabrotica virgifera virgifera*, *Diabrotica barberi*,
Diabrotica longicornis), and therefore do not emerge as adults until late in the growing season and feed on a wider variety of host plants are unlikely to be competent vectors or ecologically important in driving disease dynamics (which can be confirmed with simple transmission tests). Additionally, since virgifera species overwinter as eggs they cannot act as an overwintering (or over-dry season reservoir) of E. tracheiphila, and are likely to have a less co-evolved relationship with the bacterium. D. virgifera virgifera was tested for ability to transmit E. tracheiphila decades ago, and was found not to be a competent vector. However, multivoltine fucata beetles (such as the pest species Diabrotica undecimpunctata and Diabrotica balteada), and any species of Acalymma found in high abundance in agricultural fields, have a higher potential of being competent disease vectors and possibly important for the broader disease ecology of this system since they overwinter as adults and could be potential reservoirs. Determining which beetles are important pests of cucurbits in the southern and western regions, if there are unrecognized losses from E. tracheiphila, comparing area devoted to cucurbit production in different regions, whether production is increasing and decreasing, and whether seasonal production corresponds to beetle phenology, are likely all important components affecting E. tracheiphila distribution and emergence.

Both the striped and spotted cucumber beetles have geographically isolated Western subpopulations/species. The western striped cucumber beetle is a named species, A. trivittatum, while spotted cucumber beetles subpopulations consist of the Southern cucumber beetle (D. undecimpunctata howardi), the Central American spotted cucumber beetle (D. undecimpunctata duodecimnotata) and an Arizona subspecies(D. undecimpunctata tenella). The presence of spotted and striped cucumber beetles in the West that are assumed to be competent vectors, yet where there is a lack of loss from E. tracheiphila suggests that local landscape or differences in local population levels may also affect geography of E. tracheiphila disease range. While not a
comprehensive analysis, distribution maps provided by Ray F. Smith of some pest species (Krysan & Miller 1986) suggest Western populations could be lower. Population measures of diabroticites in different locales are often not well documented, so a more arid, sparse west might support lower overall populations of beetles than population levels required to sustain *E. tracheiphila* epidemics. Additionally, it is possible that among the greater diversity of Western and Southwestern diabroticite species, few species are competent disease vectors, and the populations of competent species may be proportionally low compared to total diabroticites. How the interaction of local climate and environment, agricultural landscapes, diabroticite diversity and abundance, and vector competence of different species interacts to explain disease dynamics is an interesting yet uninvestigated aspect of disease ecology in this system. It is possible that *E. tracheiphila* range is expanding, or that it is constantly present in the West and Southwest, but the losses are low enough that incidence of the disease are not often reported. If the aridity and climate of the Southwest contribute to low vector populations and disease pressure, then this region may be acting as a geographic buffer preventing expansion of *E. tracheiphila* towards wild *Cucurbita* populations in Mesoamerica.

**Cucurbita viral diseases**

Cucurbiti are also susceptible to a number of aphid-transmitted viral pathogens (Choi *et al.* 2002; Klas *et al.* 2006). Several viral pathogens are emerging as agricultural threats; among them is *Zucchini Yellow Mosaic Virus* (*ZYMV*: Potyviridae) (Desbiez *et al.* 2002). While ZYMV is rarely fatal, infection causes a reduction in plant fitness, leaf mottling, and spotting on fruits that render them unmarketable. Viral infections are also common in wild *Cucurbita* populations (Prendeville *et al.* 2012). In response to these viral diseases, transgenic cultivated varieties resistant to CMV, WMV, and ZYMV have been developed and released (Fuchs 1995; Tricoli *et al.* 1995), and transgenic squash varieties have been deregulated throughout the range of native
Cucurbita in the US and Mexico. Gene flow between wild and cultivated Cucurbita pepo varieties occurs often, (Spencer 2001), and hybrids are fully fertile (Laughlin et al. 2009), raising the possibilities of frequent gene flow between domesticated (perhaps transgenic) and wild varieties. Additionally, while transgenic varieties are highly resistant to viral infections, these varieties are unavailable for use by organic growers, and environmental concerns about transgenic gene flow into native populations exist.

**Disease interactions in complex pathosystems**

How plants respond to simultaneous pressure from herbivores and multiple circulating diseases in complex pathosystems, and how pathogen induced effects on plant phenotype may alter those interactions is largely unknown (Sasu et al. 2009b; Sasu et al. 2010b). The epidemiology of E. tracheiphila interactions in agroecology are largely uninvestigated and molecular interactions are completely unknown. E. tracheiphila was recently shown to be capable of ephiphytic colonization of Cucumis leaves under favorable environmental conditions (Rojas, Gleason, & Pathology, 2012), but infection has not been shown to be caused by leaf application (Smith) and inoculation success decreases as time post wounding increases (Brust 1997), and so the contribution of ephiphytic populations to disease dynamics remains to be resolved. Control strategies for bacterial wilt traditionally aim to keep vector numbers low (Necibi et al. 1992; Brust 1999; Ellers-Kirk et al. 2000), although trap cropping shows some effectiveness in this system (Cavanagh et al. 2009). High rates of parasitism by specialist tachinid (Balduf 1925) and braconid parasitoids has been documented (Toepfer et al. 2009; Smyth & Hoffmann 2010), but natural enemies in naturally occurring populations dynamics do not appear to be effective in controlling cucumber beetle populations in agricultural settings.

Sustainable and biologically based pest and pathogen control strategies are dependent on a more complete understanding of plant/pathogen/vector ecological and molecular interactions.

To that end: **The scope of the current dissertation includes the following**
Chapter 2 assays changes on wild gourd leaf and floral volatile phenotype as the result of *E. tracheiphila* and ZYMV infection. Volatile collections are repeated in both the field and greenhouse. Results of these experiments show that infection with either pathogen suppresses the release of floral volatiles. Symptomatic, wilting vines on *E. tracheiphila* infected plants were found to emit significantly more volatiles of a blend largely composed of low molecular weight green leaf volatiles. A series of beetle behavioral assays in the field and greenhouse showed that beetles prefer the stronger volatile blend emitted by flowers on healthy plants compared to the other treatments, and also prefer the volatile blend released by wilting leaves on *E. tracheiphila* – infected plants compared to the volatiles (or lack of volatiles) released by leaves on healthy, non-symptomatic leaves on *E. tracheiphila* – infected plants, or ZYMV – infected plants. Results of feeding preference assays show that beetles prefer the foliage of both ZYMV and *E. tracheiphila* infected foliage relative to leaves on healthy plants. Taken together, these results posit that preferential vector attraction to both volatiles from healthy flowers and wilting leaves, and non-attraction to leaves or flowers of ZYMV – infected plants, is largely responsible for the lack of co-infection repeatedly documented.

Chapter 3 tests whether induced systemic resistance is responsible for the suppression of bacterial wilt symptom development in field populations of ZYMV infected plants. Levels of the defense-signaling hormones jasmonic acid and salicylic acid were measured in healthy and ZYMV – infected plants before and after inoculation with *E. tracheiphila*. Virus – infected plants contain significantly higher constitutive and induced levels of salicylic acid compared to healthy plants, suggesting that induced systemic resistance may be partly responsible for disease suppression. However, a controlled inoculation trial was performed by collecting frass from beetles exposed to wilting plants, and results show equivalent rates of symptom development in both healthy and ZYMV-infected plants at the end of the experimental period. Comparisons if the results in this chapter with documented cases of induced resistance (specifically in cucumber, but
in other model hosts as well) in the literature are discussed, and hypothesis for other factors that could suppress *E. tracheiphila* infection in virus infected plants are proposed.

**Chapter 4** develops a qPCR methodology to describe colonization dynamics of cucumber beetles by *E. tracheiphila*. Previous work in the system has established that the beetles transmit *E. tracheiphila* in a persistent manner, that beetles are the only overwintering reservoir, and that *E. tracheiphila* may be transmitted when frass from infective beetles contacts floral nectaries or leaf wounds created by herbivory. However, quantitative and temporal dynamics of beetle colonization and consequences for transmission were still largely undescribed. In this study, I found that the relative amount of bacteria in whole beetles and shed in frass immediately after feeding on symptomatic, wilting plants displays a large amount of variance, likely as a result of the heterogeneous distribution of *E. tracheiphila* in the xylem of infected plants. At 5 days post AAP, there is significantly less bacteria in frass but the amount in the beetle is not significantly different, and by 28 days post exposure, the relative amount of bacteria in frass is significantly higher than 5 days and the amount in the beetle is again unchanged. Transmission experiments with frass collected 5 days after an acquisition access period showed less wilt symptom development in the group of plants inoculated with frass collected from beetles 0 days or 28 days after an acquisition access period. Thus, the ability of bacteria that is ‘plant derived’ and mechanically passes through the beetle does not differ in infection probability compared to bacteria that are ‘beetle derived’, and shed directly from colonies on the insect’s digestive tract. A series of fitness experiments show that this symbiosis appears to be neutral for the beetle: It appears to be neither substantially harmed nor helped by being colonized by *E. tracheiphila*. A better understanding of colonization dynamics will identify possible points in the transmission cycle for optimal interventions.

**Chapter 5** utilized computational methods and a comparative genomics approach to understand the interactions of *Erwinia tracheiphila* with host plants and insect vectors, and the
relationship of *E. tracheiphila* to other sequenced *Erwinia* spp. and closely related enterobacterial plant pathogens (that were recently reclassified to new genera from *Erwinia*). Overwhelming evidence supports the closest sequenced relative as the non-pathogenic floral commensal *E. billingiae*, and not the devastating fire blight pathogen *E. amylovora*. The presence of many putative horizontally transferred genes, DNA integrated phage genes and what appear to be active infection by two phages replicating in culture, and the proliferation of insertion elements have led to large chromosomal rearrangements and likely are contributing to the emergence of *E. tracheiphila* in an ecological niche dramatically different than other characterized *Erwinia* spp.

The genomic data generated as part of this chapter provide a wealth of opportunity to study basic questions about pathogen evolution and emergence, as well as to design successful control strategies and guide crop breeding and development.

**Chapter 6** synthesizes the importance for the findings for the system of study, the applicability of the research in this dissertation to applied questions in agriculture, and to the intersection of plant/insect/microbe interactions. The utility of next generation sequencing technology to these problems is discussed, specifically as it applies to providing a way to quickly and cheaply generate data that can be used to advance our understanding of native pathosystems and nonmodel systems.

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Chapter 2

Pathogen effects on vegetative and floral odors mediated vector attraction and host exposure in a complex pathosystem

Abstract

Pathogens can alter host phenotypes in ways that influence interactions between hosts and other organisms, including insect disease vectors. Such effects have implications for pathogen transmission, as well as host exposure to secondary pathogens, but are not well studied in natural systems, particularly for plant pathogens. Here, we report that the beetle-transmitted bacterial pathogen *Erwinia tracheiphila*—which causes a fatal wilt disease—alters the foliar and floral volatile emissions of its host (wild gourd, *Cucurbita pepo* ssp. *texana*) in ways that enhance both vector recruitment to infected plants and subsequent dispersal to healthy plants. Moreover, infection by *Zucchini yellow mosaic virus* (ZYMV), which also occurs at our study sites, reduces floral volatile emissions in a manner that discourages beetle recruitment and therefore likely reduces the exposure of virus-infected plants to the lethal bacterial pathogen—a finding consistent with our previous observation of dramatically reduced wilt disease incidence in ZYMV-infected plants.
Introduction

The transmission of obligately vector-borne pathogens requires that competent vectors interact with both infected and (subsequently) uninfected hosts in ways that are compatible with pathogen acquisition and transmission (O’Shea et al. 2002; Vyas et al. 2007). These interactions take place within complex pathosystems and are shaped by myriad biotic and abiotic factors (Medel et al. 2004). Our current understanding of these processes and their implications for the establishment and spread of insect-borne diseases remains limited, despite their relevance for global health, agricultural production, and ecology of natural systems (Burdon & Thrall 2008). However, increasing attention has focused on the role of pathogens themselves in manipulating—or otherwise altering—interactions between hosts and vectors in ways that enhance transmission (Lefèvre et al. 2006). This increased focus on pathogen-induced effects complements a growing appreciation of the prevalence of manipulative parasites in natural systems and their far-reaching influences on community structure and ecosystem dynamics (Lefèvre et al. 2009)—though, compared to work in animal systems, relatively little attention has to date been paid to the broader ecological significance of pathogen effects on plant hosts (Mescher 2012).

Insect-borne pathogens can potentially influence host-vector interactions through direct effects on vector behavior (Koella 1999; Ngumbi et al. 2007; Mann et al. 2012), but also via effects on the quality of the primary host as a resource for the vector (Mauck et al. 2010), or on the production of host-derived cues that mediate vector attraction (Lefèvre et al. 2006). Host-derived olfactory cues are likely targets for manipulation by pathogens, as olfaction is a key sensory modality for insects and plays a central role in host location by both plant and animal disease vectors (Eigenbrode et al. 2002; O’Shea et al. 2002; Lacroix et al. 2005; McLeod et al. 2005; Mauck et al. 2010). Moreover, volatile chemistry is relatively labile in comparison to many other traits impacted by pathogens and is known to be altered by disease in many systems (e.g.,
(Kavaliers et al. 2005; Mauck et al. 2010).

In plant systems, recent work on the role of pathogen-induced changes of host odors in mediating vector attraction to infected hosts (e.g., (Eigenbrode et al. 2002; McLeod et al. 2005; Mauck et al. 2010) builds on an extensive literature showing that volatiles play a key role in host-plant location for insect herbivores (Bolter et al. 1997; Halitschke et al. 2008) and that induced volatile emissions following insect damage are crucial host location cues utilized by parasitoids and predaceous insects (De Moraes et al. 1998). Several recent studies have reported vector attraction to the elevated and/or altered volatile emissions of plants infected by plant pathogens (e.g., (Eigenbrode et al. 2002; Jiménez-Martínez et al. 2004; McLeod et al. 2005; Jiménez-Martínez et al. 2009; Mauck et al. 2010). Many of these studies have posited positive effects of vector attraction to infected hosts on pathogen acquisition and transmission, although the epidemiological implications of such attraction are likely to be influenced by the frequency of infection within populations, vector abundance, the mechanism of pathogen transmission by vectors, and the details of the trophic (or other) interactions between hosts and vectors (e.g., (McElhany et al. 1995; Sisterson 2008; Mauck et al. 2012).

Currently, we have detailed information about the effects of plant pathogens on host-vector interactions in only a few—primarily agricultural—systems, and know relatively little how such effects function within the broader context of complex ecological communities. Some fungal plant pathogens elicit dramatic alterations of host phenotypes (e.g., the production of pseudo-flowers that attract insect pollinators) that constitute obvious examples of host manipulation (Roy & Raguso 1997), but the effects of most pathogens on their hosts are more subtle. Initial efforts to characterize broader patterns in the effects of viral pathogens on host-vector interactions (including those mediated by host-plant odors) suggest that variation in transmission mechanism may be an important factor shaping the evolution of virus effects on host phenotypes (Mauck et al. 2012)—though the certainty of this conclusion is constrained by the limited representation of
many virus groups in the existing literature, a near-total lack of research addressing non-crop systems, and the paucity of studies providing reliable data about vector attraction to virus-induced visual and olfactory cues. For other classes of insect-transmitted plant pathogens, still less is known about the ecology of pathogen effects on host-vector interactions (but see Mann et al. 2012).

In the current study we examine how pathogen-induced changes in the vegetative and floral volatiles of a wild gourd *Cucurbita pepo* ssp. *texana* (Cucurbitaceae) influence the behavioral responses (e.g., attraction to and aggregation) of striped cucumber beetles *Acalymma vittatum* (Coleoptera: Chrysomelidae: Luperini), which transmit *Erwinia tracheiphila*, the causative agent of a usually lethal wilt disease. Beetles acquire *E. tracheiphila* by feeding on the leaves of infected plants (Yao 1996; Garcia-Salazar et al. 2000a) and may also transmit the pathogen when infective frass falls onto open wounds at sites of foliar feeding damage (Rand 1915; Mitchell & Hanks 2009). However, beetles also aggregate to feed and mate in wild gourd flowers, and recent findings suggest that transmission through floral nectaries may be particularly important in this system. We have previously shown that *E. tracheiphila* is present at very high rates in frass collected from flowers in the field (Sasu et al. 2010a) and that the incidence of wilt disease is strongly influenced by the presence and number of flowers (Ferrari et al. 2007; Sasu et al. 2009). Furthermore, we recently demonstrated that *E. tracheiphila* is efficiently transmitted through floral nectaries on a time frame consistent with the relatively short window (~6 hrs) (Sasu et al. 2010a) available for pathogen transmission prior to floral abscission (each wild-gourd flower opens only for a single morning). Thus, we suspect that *E. tracheiphila* is frequently acquired by beetles feeding on infected leaf tissue and transmitted to healthy plants though frass deposition into the flowers of healthy plants.

Our previous work has also shown that the incidence of wilt disease is greatly reduced among plants exhibiting symptoms of infection by *Zucchini yellow mosaic virus* (ZYMV) (Potyviridae)
(Sasu et al. 2009b; Sasu et al. 2010b), which slows plant growth and reduces reproductive output, but unlike wilt disease, does not kill plants. Furthermore, while a good deal of theoretical and empirical work has addressed the dynamics of competitive interactions among pathogens occurring in mixed infections (e.g., (Choisy & De Roode 2010), relatively little is known about how pathogen-induced changes in host plant volatiles (or other aspects of host phenotypes that influence interactions with insect vectors) may affect subsequent rates of infection by secondary pathogens. Therefore, in addition to exploring how E. tracheiphila affects foliar and floral plant volatiles and influences the recruitment of beetle vectors to the leaves and flowers of healthy and wilting plants, we examine how ZYMV infections affect cucumber beetle attraction to explore the possibility that the reduced incidence of wilt disease in ZYMV-infected plants is mediated, at least in part, by reduced exposure to the beetle vectors of E. tracheiphila.

Materials and Methods

The Cucurbita pathosystem

Cucurbita pepo ssp. texana is an annual monoecious vine, native to the Southern US, with indeterminate growth and reproduction, and is either the progenitor of cultivated squash or an early escape from cultivation (Decker 1988). Single large yellow flowers (male or female) are borne in leaf axils, remain open for a single morning (~6h), and are abscised the following day (Sasu et al. 2010b). They release a volatile blend attractive over long distances to solitary squash bee pollinators (Hurd 1971) and herbivorous diabroticite cucumber beetles (Metcalf & Lampman 1991; Andrews et al. 2007) —hypothesized to be the ancestral pollinators of Cucurbita spp. (Kevan & Baker 1983). Cucumber beetles are the only known vectors of E. tracheiphila bacteria, which proliferate in the xylem of infected plants and eventually block xylem flow. Wilt symptoms typically develop 10-15 days after exposure, and the disease is typically fatal (within
2-3 weeks) once symptoms appear (Yao 1996). Bacterial wilt is the most economically important disease of cultivated cucurbits (cucumbers, melons, pumpkins, squash) in the Eastern US (Fleischer et al. 1998; Brust 1999).

Generalist aphids also feed on Cucurbita spp. and transmit various viral diseases including ZYMV, an important agricultural pathogen (Fuchs 1995) and the most common viral disease at our field sites in Central Pennsylvania (Sasu et al. 2009b). ZYMV causes leaf and fruit deformities and depresses reproductive output but does not typically kill plants. Details of the epidemiology of ZYMV and E. tracheiphila at our field sites can be found in Ferrari et al. (2007) and Sasu et al. (2009).

**Beetle colony propagation**

*Acalymma vittatum* rearing followed Mitchell (2009). Adult beetles were kept in 1 ft x 1 ft Bioquip mesh cages (cat #1466A), with moist potting soil provided as an oviposition substrate. Beetles were fed thrice weekly with leaves and flowers of *C. pepo ‘Dixie’*. Potting soil (with eggs) was transferred weekly to a 6L plastic rearing container where the root-feeding larvae were continuously supplied with sprouted *C. pepo ‘Raven’* seedlings until pupation. Newly eclosed adults were placed with other adults from the same age cohort.

**Foliar and floral volatile collections:**

*Field volatile collections* were made in two 0.4ha plots (each containing 180 plants) at the Russell E. Larson Research Farm at Rock Springs Pennsylvania in 2009. Plants contracted *E. tracheiphila* and ZYMV from herbivory by naturally occurring vector (cucumber beetle and aphid) populations. Our diagnoses of plant disease status (for all field studies) was confirmed for a sample of symptomatic and asymptomatic plants, by using DAS-ELISA tests for ZYMV (Agdia Inc., Elkhart, IN) and by isolating *E. tracheiphila* from wilting plant tissues and using the isolate.
to infect new, greenhouse-grown plants. Foliar volatiles were collected from one branch of 21 healthy plants; one symptomatic branch of 19 E. tracheiphila-infected plants; one non-symptomatic branch of 13 E. tracheiphila-infected plants; and one symptomatic branch of 9 ZYMV infected plants. For foliar volatile collections, 1ft x 2ft rectangular Teflon bags were placed over the first 7 fully expanded leaves on a branch and tied around the vine to create a closed chamber (bags were cleaned with water and hexane between uses). Using a portable volatile-collection system (Volatile Assay Systems, State College, PA), charcoal-filtered air was pushed into the Teflon bags at 1.2L/min, and pulled at 1.0L/min through adsorbent SuperQ filters. Foliar volatiles were collected for 25 minutes between 1030 and 1500. Filters were subsequently eluted with 150µl dichloromethane (with 400ng of n-octane and nonyl acetate added as internal standards) and the eluate analyzed by gas chromatography with a Hewlett-Packard (Paolo Alto, CA) model 6890 gas chromatograph (GC) with a flame ionization detector (FID). Compounds were determined by GC-mass spectrometry (MS) with electron ionization and chemical ionization sources. All resulting data from field leaf volatile quantifications were log +1 transformed for normality and visualized with a PCA using the ade4 package (Dray & Dufour 2007) in R (R 2008) and then analyzed using SAS PROC MIXED (SAS Institute, 2011). All compounds present (excluding a very small number that were observed only sporadically and not consistently released in any treatment group) were analyzed using a repeated measures statement to account for multiple measurements on the same branch. The model statement for both individual compounds and total summed compounds was Volatiles = Date + Disease; Repeated Date / Subject = Branch.

Floral volatiles were collected from field-grown plants in 2009 and 2010 (only male flowers were utilized because wild gourd plants produce far more male than female flowers and because wilt symptomatic branches often selectively abort female flower buds while male flower buds progress to anthesis). In 2009, volatiles were collected from 36 flowers on 26 healthy plants and
from 46 flowers from symptomatic branches on 16 *E. tracheiphila*-infected plants. In 2010 volatiles were collected from 23 flowers on 17 healthy plants and 22 flowers on 17 symptomatic ZYMV-infected plants. The corolla of each flower was prevented from opening at sunrise by applying twist-ties the evening before anthesis to ensure that pollinators did not remove nectar or pollen that would affect volatile profiles (Mena Granero 2005). When the twist ties were removed the next morning, flowers opened immediately and were then placed in 1ft x 1ft square Teflon collection bags that were tied around the stem or pedicel with string to create a closed chamber. Twenty-five minute volatile collections (conducted as above) occurred between sunrise and 1030 and samples were analyzed as above. Floral volatiles were log + 1 transformed for normality and analyzed separately, with repeated measures to account for sampling different flowers on the same plant, in SAS PROC MIXED (SAS 2011) using the model statement Volatiles = Date + Disease; Repeated Date / Subject = Plant.

**Greenhouse Volatile Collections** were made to confirm the patterns made in the field and to characterize the volatile blend induced by pathogen infection under controlled conditions. All plants were grown in Pro-Mix (Premier Horticulture Reviere Du Loup, Quebec, Canada) and maintained in growth chambers at 25C day 22C nights with 14L 10D light cycle. For analyses of foliar volatiles induced by *E. tracheiphila*, plants were either mock inoculated at 4 weeks (apprx. 4-5 true leaves) (n = 19), or inoculated—using the colony-stab protocol (Mitchell & Hanks 2009)—with a virulent field isolate of *E. tracheiphila* maintained on nutrient peptone agar at 26C (n = 19). Once symptoms appeared (several days after inoculation), individual plants were placed under 4L glass domes, 15cm tall x 15 cm wide (Analytical Research Systems, Gainesville, Florida, USA) with Teflon bases that closed around plant the stem to prevent air flow. Volatiles were collected between 0800 and 1400 using a closed push/pull system, in which filtered air is pumped into the chambers at 1.3L/min and pulled at 1L/min through traps containing 40mg of adsorbent SuperQ (Alltech, Deerfield, Illinois, USA). In a separate experiment, volatiles were...
similarly collected from virus-infected and mock-inoculated plants. A locally collected ZYMV isolate was ground in 0.1M phosphate buffer (K$_2$HPO$_4$ and KH$_2$PO$_4$). Carborundum was used to break cells on the surface of the cotyledons, and the virus-buffer slurry was applied with cotton swabs. Mock inoculations employed the carborundum abrasive and phosphate buffer.

Floral volatiles were collected from flowers on greenhouse-grown, two-month-old plants that had been either mock inoculated (n=15 plants) or inoculated with the same virulent field *E. tracheiphila* isolate used in the leaf volatile inoculations (n=12 plants). In a separate experiment, plants at the 5-leaf stage were inoculated with ZYMV (as above) on the newest fully expanded leaf (n=12 plants) or mock inoculated (n=13 plants). Once infected plants began showing symptoms, individual flowers were placed in 4L glass domes while still attached to the vine. Flower volatiles were collected between 0600 and 1000, when flowers were open. Volatiles from greenhouse experiments were analyzed separately in SAS using a Mixed Effects Model ANOVA to determine the effects of Disease Status (Healthy, Infected) and Date on Total Volatile production and, separately, on individual volatile compounds.

**Recruitment of beetles to plants in the field:**

Field trapping of vector insects employed rectangular cages (3 ft long x 1 ft tall x 1 ft wide) made of galvanized 19-gauge hardware cloth designed to fit over individual non-flowering branches. The sides of the cage were coated with Tree Tanglefoot™ (a sticky non-water soluble substance commonly used for insect sampling). Cages were placed over single non-flowering branches of 20 healthy plants, non-symptomatic branches of 11 *E. tracheiphila*-infected plants, and symptomatic branches of 18 *E. tracheiphila*-infected plants. Beetles were counted and removed from traps twice weekly, and Tanglefoot was re-applied weekly. Data were analyzed with a repeated measures model using SAS PROC MIXED Total Beetles = Disease + Date; Repeated Date / Subject = Branch.
We also counted beetles in one male flower (haphazardly selected, from a distance of ~5m, prior to approaching the plant) on all plants from one of our field plots that possessed at least one male flower on August 12, 2010. The plot contained healthy plants (no visible symptoms of disease), ZYMV infected plants (visible symptoms of ZYMV) and *E. tracheiphila*-infected plants (visible symptoms of wilt disease). The resulting data were analyzed using a 1-Way ANOVA with Disease as the independent variable.

**Laboratory behavioral assays:**

**Volatile choice tests:** We employed a Y-tube olfactometer (ARS, Inc., Gainesville, FL; cat #YTO-A10) to test beetle responses to foliar and floral odors. The Y-tube was impressed vertically into a fitted cardboard box, the top of which was covered with two layers of red plastic. For foliar-volatile assays, wild gourd plants at the 4–5 leaf stage were either mock-inoculated or stab inoculated with *E. tracheiphila* (as above). After symptoms developed on infected plants, plants were enclosed in Teflon bags and placed at the Y-tube terminals. Incoming air (hydrated by passing through a bubbler) passed into the Teflon bags at 1.5 L/min, and air was pulled through the system at 3 L/min. Three- to 4-week-old, lab-reared striped cucumber beetles were starved for 24 hours prior to choice tests. Beetles were individually placed into the base of the Y-tube and allowed to make a choice. Six beetles were recorded per unique plant pair and then a new pair of plants was used. The Y-tube terminal to which a given plant was attached to was alternated after three beetles had made choices. The resulting data were analyzed using Chi-square tests for independence. Floral-volatile assays were conducted similarly, with flowers on healthy or pathogen-infected plants enclosed in 1ft x 1ft Teflon bags positioned at the Y-tube terminals. For these assays, 1-month-old wild gourd plants were inoculated with *E. tracheiphila* or ZYMV, or appropriately mock inoculated (as above). In separate trials, we compared beetle
choice for flowers from (a) healthy plants vs. clean air (empty Teflon bags); (b) mock-inoculated vs. *E. tracheiphila*-infected plants; and (c) mock-inoculated vs. ZYMV-infected plants.

We also assessed beetle feeding preferences for healthy and pathogen-infected plants. Pairs of *E. tracheiphila*-inoculated (as above) or mock-inoculated 4-leaf stage greenhouse-grown wild gourd plants were placed in 1ft x 1ft mesh Bioquip cages (cat # 1466A). Six striped cucumber beetles from a lab colony (three males and three females) that had been starved for 24 hours were placed in each of six replicate cages and allowed to feed for 48 hours. Leaf area consumed from each plant was quantified using SigmaScan Pro v. 5.0 (SPSS Inc., Chicago, IL, USA). A similar assay tested feeding preferences for ZYMV-infected (as above) or mock-inoculated plants. Leaf area data were analyzed with a 1-way ANOVA with disease as the independent variable against total leaf area, and separately against total area consumed as dependent variables, with “cage” entered as a random variable.

**Results**

**Foliar volatiles:**

In the field, the wilting leaves of *E. tracheiphila*-infected plants exhibited significant elevation of volatile emissions compared to those of healthy plants, which exhibited low levels of constitutive volatile emissions (Figure 2.1, Figure 2.2, Table 2.1). Overall, 10 of 12 compounds were released in higher amounts from the symptomatic leaves of *E. tracheiphila* infected plants. Non-symptomatic leaves on *E. tracheiphila*-infected plants displayed volatile profiles indistinguishable from those of healthy plants. ZYMV-infected leaves released the same volatile blend as healthy leaves and non-symptomatic leaves on *E. tracheiphila*-infected plants, except for elevated release of nonatriene, and their total volatiles were not significantly higher than healthy or non-wilt branches in pairwise tests.
Figure 2-1. Field foliar volatiles

The 12 most abundant volatile organic compounds emitted from the foliage of healthy plants, nonsymptomatic branches on *E. tracheiphila*-infected plants, symptomatic wilting branches on *E.tracheiphila*-infected plants, and branches on zucchini yellow mosaic virus-infected plants in the field. LS means ± SE bars. Sample sizes n=20 healthy branches; n=8 non-wilt branches; n=18 wilting branches; n=9 virus branches.
Figure 2.2. PCA of field foliar volatiles.

A principle component bi-plot of foliar volatiles emitted Cucurbita pepo ssp. texana foliage in the field. Principle component 1 explains 33.7% of the variation, and principle component 2 explains 16.6% of the variation. All volatile compounds except nonatriene and 1-pentanol are positively associated with wilting foliage (blue). The volatiles emitted by ZYMV (green), nonwilt (red), and healthy (black) treatments are indistinguishable from each other.

Under greenhouse conditions, we observed a similar pattern: symptomatic E. tracheiphila-infected plants exhibited elevated volatile emissions compared to healthy (mock-inoculated) plants. Overall, the seven consistently E. tracheiphila-induced compounds were all released in higher amounts by symptomatic E. tracheiphila-infected foliage in the greenhouse, while no compound was released in a higher concentration from healthy plants. Most E. tracheiphila-induced compounds were green-leaf volatiles or monoterpenes (Figure 2.3, Table 2). As in the
field, the volatile blend from ZYMV-infected foliage was indistinguishable from that of healthy (mock-inoculated) plants (Table 3). Foliage on healthy and ZYMV-infected plants produced the same volatile organic compounds, and no compound differed significantly in concentration based upon pairwise comparisons (Figure 2.3, Table 3).

**Figure 2-3.** Greenhouse foliar volatiles

Two separate pairwise greenhouse volatile collection experiments measured volatiles emitted by healthy mock-bacterial-infected plants vs. wilting *E. tracheiphila*-infected plants, and healthy mock-infected plants vs. ZYMV-infected plants. Sample sizes are mock-virus n = 11, ZYMV n = 12; mock-*E. tracheiphila* n = 19, *E. tracheiphila*-infected n = 19.

**Floral volatiles:**

In the field, flowers from symptomatic *E. tracheiphila*-infected branches and virus-infected plants exhibited significantly lower total volatile emissions than flowers on healthy plants (Figure 2.4, Table 4, Table 5). Neither *E. tracheiphila* nor ZYMV altered the overall composition of the floral volatile blend, but *E. tracheiphila* infection attenuated the release of linalool, 1,4-methoxybenzene, and nonatriene, while ZYMV infection decreased the amount of 1,4-methoxybenzene emitted (Figure 2.4).
Figure 2-4. Field floral volatiles.

Floral volatiles from healthy and wilting *E. tracheiphila*-infected plants in the field in 2009, and healthy and virus-infected plants in the field in 2010. Mean ± SE of the three main components of the field floral volatile blend. Sample size: n = 36 healthy flowers and n = 46 wilt flowers in 2009 and n = 23 healthy flowers and n = 22 virus flowers in 2010.

In the greenhouse, we observed a pattern similar to our field results. In pairwise experiments, infection with either ZYMV or *E. tracheiphila* reduced the total floral volatiles released compared to healthy, mock-inoculated plants (Figure 2.5, Table 6, Table 7).

Figure 2-5. Greenhouse floral volatiles

Floral volatiles emitted by healthy mock-infected vs. ZYMV-infected flowers, and flowers on healthy mock bacterial infected plants vs. flowers on *E. tracheiphila*-infected wilting plants in
two greenhouse experiments. Sample sizes: mock-ZYMV n = 13; ZYMV n = 12; mock-\textit{E. tracheiphila} n = 12; \textit{E. tracheiphila}-infected n = 15.

**Recruitment of beetles to plants in the field:**

More than twice as many cucumber beetles were captured on symptomatic (i.e., wilting) branches compared to non-symptomatic branches of \textit{E. tracheiphila}-infected plants or branches on healthy plants (Figure 2.6A). In contrast, more beetles aggregated in the flowers of healthy plants (4.6 ± 0.9; N = 34) than in the flowers of \textit{E. tracheiphila}- (2.0 ± 0.7; N = 15) or ZYMV-infected (2.0 ± 0.3; N = 82) plants (1-Way ANOVA, $F_{2,128} = 6.22$, $P = 0.003$) (Figure 2.6B).

![Figure 2-6. Beetle attraction in the field](image)

A. Cucumber beetle attraction to Tanglefoot cages covering branches on healthy plants, non-symptomatic branches on infected plants, or wilting branches in the field. Means ± SE bars. n = Sample size. B. Number of beetles per flower on healthy, \textit{E. tracheiphila}-infected, and ZYMV-infected plants under field conditions. Sample size: N = 85 healthy plants; N = 23 wilt diseased plants; and N = 127 ZYMV-infected plants.

**Laboratory behavioral assays:**
In Y-tube choice assays (Figure 2.7), beetles exhibited a significant preference for the odor of *E. tracheiphila*-infected foliage compared to that of healthy foliage ($\chi^2 = 5.88, P = 0.015, 1\text{ df}$). But, the odors of healthy flowers were significantly more attractive than those of flowers on *E. tracheiphila*-infected plants ($\chi^2 = 12.51, P < 0.001, 1\text{ df}$) or ZYMV-infected plants ($\chi^2 = 5.12, P = 0.023, 1\text{ df}$), or clean-air controls ($\chi^2 = 8.53, P < 0.005, 1\text{ df}$).

**Figure 2-7.** Greenhouse behavioral assays

Results of Y-tube beetle pairwise choice tests for volatiles from healthy or infected leaves or flowers. Beetles did not have a significant preference for the foliage of either healthy or virus-infected plants ($\chi^2 = 0.72, P = 0.386, 1\text{ df}$); but beetles were more attracted to flowers on healthy plants compared to a clean air blank ($\chi^2 = 8.53, P = 0.003, 1\text{ df}$); to flowers on healthy plants compared to flowers on ZYMV-infected plants ($\chi^2 = 5.12, P = 0.023, 1\text{ df}$); to flowers on healthy plants compared to flowers on symptomatic *E. tracheiphila*-infected plants ($\chi^2 = 12.51, P \leq 0.005, 1\text{ df}$); and to foliage on *E. tracheiphila*-infected plants compared to foliage on mock-inoculated plants ($\chi^2 = 5.88, P = 0.015, 1\text{ df}$).

In laboratory feeding assays, cucumber beetles consumed more leaf area from wilting *E. tracheiphila*-infected plants relative to healthy plants (ANOVA $F_{1,10} = 6.59, P = 0.028$), even though *E. tracheiphila* infection reduced the total leaf area available (ANOVA $F: 1, 10 = 5.18 P = 0.046$). ZYMV infection had no similar impact on available leaf area (ANOVA $F_{1,10} = 2.82, P =$
35

0.124), but cucumber beetles consumed more of the leaf area of ZYMV-infected leaves relative to those of healthy plants (ANOVA $F_{1,10} = 31.42, P < 0.001$) (Figure 2.8).

![Graph showing leaf area comparison between wilt and mock, virus and mock conditions.]

**Figure 2.8.** Dual-choice feeding preference test of healthy vs. pathogen-infected plants. Sample sizes are six cages with mock vs. virus and six cages with mock vs. *E. tracheiphila*

**Discussion**

The findings presented here demonstrate that infection with the bacterial wilt disease pathogen *Erwinia tracheiphila* changes the volatile phenotype of leaves and flowers of *Cucurbita pepo* ssp. *texana* relative to healthy plants, and that these changes affect the foraging and aggregation behavior of the beetle vectors in a manner that not only increases the contact rate between wilting foliage and insect vectors, but also increases the exposure rate of healthy host plants to the pathogen. Significantly more cucumber beetles were recruited to the wilting foliage
of *E. tracheiphila*-infected plants in the field (Fig. 6A), and this attraction is likely explained by
the elevated, and altered, volatile emissions of wilting leaves (Fig. 1), which we found to elicit
positive behavioral responses from the beetles (Figs. 6A, 7). The characteristic volatile response
to infection by *E. tracheiphila* includes the release of green-leaf volatiles, which are frequently
associated with plant stress (Farag & Paré 2002) and have been shown to be important as host
location cues for many herbivorous insects (Bolter *et al.* 1997). In the field, this preference for the
odors of wilting leaves increases vector attraction to symptomatic foliage on plants infected with
*E. tracheiphila*, which is acquired during feeding on wilting leaves (Garcia-Salazar *et al.* 2000a;
Mitchell & Hanks 2009).

Furthermore, in dual-choice feeding assays, we observed a significant beetle
preference for wilting (diseased) leaves, consistent with previous reports that cucumber beetles
aggregate to feed on the leaves of wilt-diseased plants (Yao 1996). This preference may in part
reflect enhanced accessibility of wilting tissue owing to diminished defensive secretion by wilting
leaves of phloem sap high in P-proteins that can dramatically impede herbivore feeding by
gumming mouth parts (McCloud *et al.* 1995). Thus, *E. tracheiphila* infection induces symptoms
that likely make it physically easier for cucumber beetle vectors to consume symptomatic leaf
tissue relative to healthy foliage.

While pathogen-induced changes in the volatiles produced by symptomatic *E. tracheiphila*
infected plants can increase rates of vector attraction to wilting plants and induce symptoms
making wilting foliage easier to consume, infective vectors must also have an incentive to visit
healthy hosts in order for pathogen dissemination to occur (Sisterson 2008). As noted above,
there is reason to believe that transmission through floral nectaries is important for disease spread
in this system: In field studies conducted over several years, we have found that the probability of
a plant contracting wilt disease is positively correlated with the number of flowers produced by
the plant over the 15 days preceding the first appearance of wilt symptoms (Ferrari *et al.* 2007;
Sasu et al. 2009b). Furthermore, using a PCR assay, we found that > 95% of the flowers on healthy plants contained *E. tracheiphila*-infected frass on or in the nectaries by 11:00 AM, the approximate time when flowers close (Sasu et al. 2010a). And we recently demonstrated that *E. tracheiphila* is efficiently transmitted through floral nectaries—through controlled greenhouse studies using green-fluorescent-protein transformed bacteria—and documented the progress of the pathogen through the nectaries and into the pedicel within 24 hrs (i.e., prior to floral abscission) (Sasu et al. 2010a).

Given the likely importance of transmission via flowers, our findings regarding *E. tracheiphila*-induced changes in floral volatiles may hold significant implications. It is well-established that cucumber beetles are attracted over long distances to the floral volatiles of *Cucurbita* (e.g., Andersen and Metcalf 1987; Andrews et al. 2007) and that cucumber beetles aggregate in the flowers of *Cucurbita* to mate and feed (Andersen 1987). Our results indicate that the floral volatile emissions of *E. tracheiphila*-infected plants are significantly attenuated relative to those of healthy plants (Figs. 2.4, 2.5). Moreover, we observe reduced beetle attraction to the floral odors of *E. tracheiphila*-infected plants relative to those of healthy plants (Figs. 2.6B, 2.7), and we found more beetles aggregating in the flowers of healthy plants than in the flowers of wilt diseased plants in the field (Fig. 2.6B). These results suggest that healthy plants are likely to experience significant exposure to *E. tracheiphila* through flowers as a consequence of the relative attractiveness of their flowers to foraging beetle vectors compared to those of already-infected plants.

Taken together, the observed effects of *E. tracheiphila* infection on floral and vegetative volatile emissions suggest a pattern conducive to the effective transmission of this pathogen: the elevated volatile emissions induced in symptomatic leaves appear to recruit beetles to wilting tissues on which they preferentially feed, increasing the likelihood of *E. tracheiphila* acquisition by vectors. However, beetles also exhibit a preference for the floral volatiles of healthy plants,
and preferentially aggregate in the flowers of healthy plants where *E. tracheiphila* shed in the frass of infected beetles can access the plant vasculature via the floral nectaries. This pattern is consistent with previous observations that vector borne—and other parasites—often alter the phenotypes of their hosts in ways that facilitate their own transmission (Lefèvre *et al.* 2006).

Our results also suggest a potential explanation for our previous observations that the incidence of wilt disease is greatly reduced among plants exhibiting symptoms of infection by ZYMV—in a three-year field study, Sasu *et al.* (2009) found that 0.4% of virus-infected plants on 15 July contracted wilt disease before 1 September, while between 5-27% of healthy plants contract *E. tracheiphila* during the same period. Our data indicate that ZYMV infection decreases floral volatile production (Fig. 2.4, 2.5); that fewer beetles aggregate in the flowers of virus-infected plants under field conditions (Fig. 2.6b); and that fewer beetles are attracted to floral volatiles of ZYMV infected plants than to the floral volatiles of healthy plants in laboratory choice tests (Fig. 2.7). Together with our previous observation that ZYMV-infected plants produce fewer flowers than healthy plants (Sasu *et al.* 2009b; Sasu *et al.* 2010c), these findings strongly suggest that ZYMV-infected plants may experience reduced rates of exposure—through flowers—to the vectors of *E. tracheiphila* compared to uninfected plants in the same populations and consequently reduced opportunity for *E. tracheiphila* transmission through floral nectaries. This reduced exposure may, at least in part, explain the reduced incidence of wilt disease on ZYMV-infected plants, and given the widespread cultivation of virus-resistant transgenic squash in the United States and Mexico, may mitigate some of the fitness advantage that the escaped transgene may have in wild populations in which both diseases are present (Sasu *et al.* 2009; 2010c).

In conclusion, while a growing number of studies document the effects of arthropod-transmitted pathogens on the nature of host-vector interactions—with potentially important implications for community and ecosystem ecology (McLeod *et al.* 2005; Lefévre *et al.* 2009),...
agriculture (Eigenbrode et al. 2002; Mauck et al. 2010; Mann et al. 2012), and human health (Hurd 2003; Lefévre et al. 2006)—we are only now beginning to explore the implications of such pathogen-induced effects within the context of complex ecological systems in which plant hosts simultaneously interact with multiple herbivores and pathogens. The current findings document the role of pathogen-induced olfactory cues in mediating both the recruitment of cucumber beetles to the foliage of plants infected by *E. tracheiphila* and the relative attractiveness of healthy flowers to these vectors. Furthermore, they reveal effects of ZYMV infection on volatile cues that influence beetle attraction and thereby likely reduce the exposure of ZYMV-infected plants to bacterial wilt. Though we are only beginning to document the occurrence of such effects, it seems likely that similar processes occur frequently in complex pathosystems, with potentially far-reaching implications both for basic ecology and for the management of disease processes in natural and agricultural settings.

**Table 2-1.** Foliar volatiles from healthy, non-symptomatic, wilting, and ZYMV-infected branches under field conditions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Healthy</th>
<th>Non-wilting branch</th>
<th>Wilting branch</th>
<th>ZYMV-infected</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown</td>
<td>2.6 ± 1.6</td>
<td>1.9 ± 1.8</td>
<td>10.3 ± 1.4</td>
<td>0.8 ± 2.6</td>
<td>≤0.0001</td>
</tr>
<tr>
<td>1-pentanol</td>
<td>13.5 ± 1.0</td>
<td>14.3 ± 1.1</td>
<td>16.0 ± 0.9</td>
<td>19.2 ± 1.6</td>
<td>0.0041</td>
</tr>
<tr>
<td>Toluene</td>
<td>4.3 ± 4.2</td>
<td>3.7 ± 4.6</td>
<td>20.8 ± 3.5</td>
<td>0 ± 6.6</td>
<td>≤0.0001</td>
</tr>
<tr>
<td>Hexenal</td>
<td>23.1 ± 10.2</td>
<td>25.8 ± 11.7</td>
<td>74.7 ± 8.9</td>
<td>42.8 ± 16.5</td>
<td>≤0.0001</td>
</tr>
<tr>
<td>e-2-hexenal</td>
<td>10.2 ± 29.7</td>
<td>13.8 ± 34.3</td>
<td>125.8 ± 26.0</td>
<td>34.3 ± 48.3</td>
<td>≤0.0001</td>
</tr>
<tr>
<td>z-3-hexen-1-ol</td>
<td>2.1 ± 3.7</td>
<td>2.4 ± 4.1</td>
<td>17.6 ± 3.4</td>
<td>1.7 ± 6.0</td>
<td>≤0.0001</td>
</tr>
<tr>
<td>Heptanal</td>
<td>2.5 ± 9.4</td>
<td>6.2 ± 10.5</td>
<td>51.5 ± 8.9</td>
<td>16.3 ± 15.2</td>
<td>≤0.0001</td>
</tr>
<tr>
<td>Xylenes</td>
<td>1.2 ± 0.9</td>
<td>0.7 ± 1.0</td>
<td>9.5 ± 0.8</td>
<td>3.8 ± 1.4</td>
<td>≤0.0001</td>
</tr>
<tr>
<td>Ocimene</td>
<td>4.5 ± 2.8</td>
<td>6.5 ± 3.2</td>
<td>19.1 ± 2.5</td>
<td>7.8 ± 4.6</td>
<td>≤0.0001</td>
</tr>
<tr>
<td>Nonanol</td>
<td>12.6 ± 2.5</td>
<td>14.3 ± 2.9</td>
<td>29.0 ± 2.2</td>
<td>16.9 ± 4.0</td>
<td>≤0.0001</td>
</tr>
<tr>
<td>&quot;Nonatriene&quot;</td>
<td>20.8 ± 5.3</td>
<td>9.3 ± 6.1</td>
<td>14.6 ± 4.6</td>
<td>35.6 ± 8.6</td>
<td>0.517</td>
</tr>
<tr>
<td>Unknown</td>
<td>6.3 ± 11.9</td>
<td>4.2 ± 13.7</td>
<td>40.0 ± 10.5</td>
<td>3.2 ± 19.3</td>
<td>0.0496</td>
</tr>
<tr>
<td>Sum</td>
<td>99.2 ± 49.5</td>
<td>101.3 ± 53.6</td>
<td>411.7 ± 40.5</td>
<td>171.6 ± 77.4</td>
<td>≤0.0001</td>
</tr>
</tbody>
</table>
Field foliar volatiles: Mean ± SE (ng) of foliar leaf volatiles collected from healthy plants, non-symptomatic (non-wilting) branches on *E. tracheiphila*-infected plants, symptomatic (wilting) branches on *E. tracheiphila*-infected plants and ZYMV-infected plants growing in experimental fields. *P*-values are from log +1 transformed values analyzed with repeated measures model SAS PROC MIXED = Disease + Date; Repeated = Date/ Subject = Vine.

**Table 2-2.** Foliar volatiles from (mock) healthy vs. *E. tracheiphila*-infected plants under greenhouse conditions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mock</th>
<th><em>E. tracheiphila</em>-infected</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1-pentanol</td>
<td>1.1 ± 4.2</td>
<td>32.9 ± 4.2</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Toluene</td>
<td>2.7 ± 3.2</td>
<td>11.3 ± 3.2</td>
<td>0.0043</td>
</tr>
<tr>
<td>Hexenal</td>
<td>0.01665 ± 2.19</td>
<td>13.8 ± 2.2</td>
<td>≤0.001</td>
</tr>
<tr>
<td>e-2-hexenal</td>
<td>0 ± 490</td>
<td>2297.4 ± 490</td>
<td>≤0.001</td>
</tr>
<tr>
<td>z-3-hexen-1-ol</td>
<td>2.3 ± 12.9</td>
<td>36.0 ± 12.8</td>
<td>0.0092</td>
</tr>
<tr>
<td>Xylenes</td>
<td>10.2 ± 24.8</td>
<td>69.7 ± 24.8</td>
<td>0.0131</td>
</tr>
<tr>
<td>Ocimene</td>
<td>0 ± 75.0</td>
<td>161.9 ± 75.0</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Sum</td>
<td>0 ± 627.0</td>
<td>2382.1 ± 627.0</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>

Results of a 1-Way Anova for the effects of *E. tracheiphila* infection on foliar volatile production under greenhouse conditions. Mean ± SE (ng) for foliar volatiles collected from *E. tracheiphila* infected and mock inoculated plants.

**Table 2-3.** Greenhouse ZYMV vs. mock (healthy) foliar volatiles

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mock</th>
<th>ZYMV-infected</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>E-2-hexenal</td>
<td>87.11 ± 14.5</td>
<td>54.4 ± 13.9</td>
<td>0.1819</td>
</tr>
<tr>
<td>Unknown1</td>
<td>9.3 ± 5.5</td>
<td>7.6 ± 5.2</td>
<td>0.823</td>
</tr>
<tr>
<td>Unknown2</td>
<td>39.3 ± 12.5</td>
<td>46.5 ± 12.0</td>
<td>0.862</td>
</tr>
<tr>
<td>Unknown3</td>
<td>51.5 ± 15.8</td>
<td>33.0 ± 15.1</td>
<td>0.408</td>
</tr>
<tr>
<td>Unknown4</td>
<td>23.5 ± 6.2</td>
<td>19.9 ± 5.9</td>
<td>0.673</td>
</tr>
<tr>
<td>Total</td>
<td>257.8 ± 53.1</td>
<td>177.7 ± 50.85</td>
<td>0.118</td>
</tr>
</tbody>
</table>

Results of a 1-Way Anova for the effects of ZYMV infection on foliar volatile production under greenhouse conditions. Mean ± SE (ng) for foliar volatiles collected from ZYMV infected and mock inoculated plants.

**Table 2-4.** Floral volatiles from healthy and *Erwinia* tracheiphila-infected plants under field conditions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Healthy</th>
<th><em>E. tracheiphila</em>-infected</th>
<th><em>P</em></th>
</tr>
</thead>
</table>
Field floral volatile collections show that *E. tracheiphila* infection suppresses the mean sum of volatiles and the individual compounds nonatriene and 1,4-methoxybenzene emitted from flowers on infected plants relative to healthy plants. The table shows mean ± SE (ng) for floral volatiles and significance testing. Model statement is SAS PROC MIXED volatiles = Date + Disease; Repeated Date / Subject = plant. Absolute mean ± SE are reported in the table, but quantities were log transformed for normality to produce *P*-values.

**Table 2-5.** Floral volatiles from healthy and ZYMV-infected plants under field conditions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Healthy</th>
<th>ZYMV-infected</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonatriene</td>
<td>22.6 ± 4.3</td>
<td>5.6 ± 4.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Linalool</td>
<td>12.9 ± 1.1</td>
<td>11.8 ± 1.2</td>
<td>0.8301</td>
</tr>
<tr>
<td>1,4-methoxybenzene</td>
<td>62.2 ± 11.3</td>
<td>41.9 ± 11.6</td>
<td>0.0312</td>
</tr>
<tr>
<td>Total</td>
<td>97.7 ± 13.6</td>
<td>59.4 ± 13.9</td>
<td>0.0247</td>
</tr>
</tbody>
</table>

The following year (2010), field floral volatile collections show that virus infection also suppresses total volatiles, driven by a suppression 1,4-methoxybenzene, corresponding with greenhouse volatile collections. Model statement is SAS PROC MIXED volatiles = Date + Disease; Repeated Date / Subject = plant. Absolute mean ± SE are reported in the table, but quantities were log transformed for normality to produce *P*-values.

**Table 2-6.** Floral volatiles from *E. tracheiphila* and healthy plants under greenhouse conditions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mock</th>
<th><em>E. tracheiphila</em>-infected</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Linalool</td>
<td>97.2 ± 15.4</td>
<td>41.8 ± 13.4</td>
<td>0.0146</td>
</tr>
<tr>
<td>Nonatriene</td>
<td>90.4 ± 8.5</td>
<td>41.4 ± 8.5</td>
<td>0.0088</td>
</tr>
<tr>
<td>1,4-methoxybenzene</td>
<td>4090.30 ± 628.5</td>
<td>1177.9 ± 546.6</td>
<td>0.0001</td>
</tr>
<tr>
<td>Unknown</td>
<td>48.9 ± 10.4</td>
<td>24.6215 ± 9.0844</td>
<td>0.0200</td>
</tr>
<tr>
<td>Unknown</td>
<td>203.5 ± 58.1</td>
<td>20.1 ± 50.5</td>
<td>0.0011</td>
</tr>
<tr>
<td>Total</td>
<td>4545.0 ± 667.8</td>
<td>1353.9 ± 580.7</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>
Results of a 1-Way Anova for the effects of E. tracheiphila infection on floral volatile production under greenhouse conditions. Mean ± SE (ng) for floral volatiles collected from E. tracheiphila infected and mock inoculated plants.

Table 2-7. Floral volatiles from ZYMV-infected and healthy plants under greenhouse conditions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mock</th>
<th>ZYMV-infected</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linalool</td>
<td>45.2 ± 5.8</td>
<td>40.8 ± 6.0</td>
<td>0.5795</td>
</tr>
<tr>
<td>Nonatriene</td>
<td>12.8 ± 5.0</td>
<td>3.4 ± 5.2</td>
<td>0.204</td>
</tr>
<tr>
<td>1,4-methoxybenzene</td>
<td>687.9 ± 116.6</td>
<td>266.6 ± 121.4</td>
<td>0.0199</td>
</tr>
<tr>
<td>Total</td>
<td>959.3 ± 134.6</td>
<td>515.4 ± 140.1</td>
<td>0.0367</td>
</tr>
</tbody>
</table>

Results of a 1-Way Anova for the effects of ZYMV infection on floral volatile production under greenhouse conditions. Mean ± SE (ng) for floral volatiles collected from ZYMV infected and mock inoculated plants.

References


Chapter 3

Direct interactions between a viral pathogen, a bacterial pathogen, and a specialist herbivore in a shared host plant

Abstract

Exposure to biotic and abiotic challenges can result in the induction of broad-spectrum resistance against subsequent pathogen challenge. However, there are limited data on induced resistance in host plants outside of model systems and the ecological significance of induced resistance in natural settings. To address this, we tested the contribution of induced systemic resistance as a factor explaining several years of field studies showing healthy wild gourds (Cucurbita pepo ssp texana) contract a fatal bacterial wilt infection caused by Erwinia tracheiphila at a significantly higher rate compared to Zucchini yellow mosaic virus infected plants. Our results show that inoculation with Erwinia tracheiphila does not induce the defense signaling hormone SA, while ZYMV-infected wild gourds have higher constitutive and induced SA levels. In controlled inoculation trials, ZYMV – infected plants contract bacterial wilt disease (caused by Erwinia tracheiphila) at the same rate as healthy plants but experience delays in wilt development at high bacterial doses. In addition, healthy and pathogen – infected plants have divergent responses to herbivory by specialist cucumber beetles.
Introduction

Plants deploy a vast array of general, inducible chemical and physical defensive responses to biotic and abiotic challenges. For several decades, the signaling networks and molecular mechanisms of plant resistance to herbivores, pathogens, and abiotic stress have been revealed to be highly complex and tightly regulated (Heil & Baldwin 2002). However, plant defensive signaling in non-model systems, and how induced resistance affects ecological interactions between co-occurring herbivores and pathogens is often uninvestigated. Here, we examine the relationship of induced plant defenses of the wild gourd *Cucurbita pepo* ssp. *texana* to co-infection dynamics between a co-circulating bacterial wilt pathogen *Erwinia tracheiphila* and the viral pathogen *Zucchini Yellow Mosaic Virus*, and how infection with either a bacterial or viral pathogen affects wild gourd induced responses to herbivory by a specialist beetle herbivore.

Several chemical hormones that regulate interconnected genetic and phenotypic responses have been implicated in controlling expression of defensive traits in response to herbivore and pathogen challenge. Jasmonates are multifunctional oxylipins synthesized from fatty acid precursors through the octadecanoid pathway. There is continuing debate about the contribution of jasmonic acid (JA) precursors and isoforms to resistance induction, but cis-JA has been confirmed as an active form mediating expression of many defensive responses (Creelman & Mullet 1997; Engelberth *et al.* 2007). JA signaling is important for many aspects of senescence and plant growth regulation (Wasternack 2007), but most research attention has focused on JA as a key signaling molecule mediating induced defensive responses to physical damage from chewing herbivores. JA mediated defenses include *de novo* synthesis of toxic secondary metabolites, proteinase inhibitors, and insect growth suppressors (Farmer & Ryan 1992; Creelman & Mullet 1997). In addition to induced herbivore defenses, a functional role for JA in defense against some necrotrophic pathogens has also been proposed (Glazebrook 2005).
Salicylic acid is an endogenous phenolic signaling hormone best investigated for its role in disease resistance (Vlot et al. 2009). SA induction and signaling after pathogen challenge can lead to cell wall fortification (Dean & Ku 1987), accumulation of pathogenesis related (PR) proteins and broad spectrum, systemic acquire resistance (SAR) against subsequent pathogen challenge (An & Mou 2011). In some systems, ‘cross – talk’ occurs, where the expression of one pathway can be antagonistic to expression of the other (Bostock 2005). However, this signaling interference is often not well understood and is not thought to be universal.

The common cucumber Cucumis sativa (Cucurbitaceae) has been an important historical model for unraveling the mechanisms of induced plant defense responses. Cucurbits are susceptible to a variety of bacterial, fungal, and viral pathogens, and susceptible to attack from specialist Diabroticite herbivores (Coleoptera: Chrysomelidae: Luperini) that can sequester and detoxify cucurbit secondary metabolites (Zehnder et al. 1997). Early work in the cucumber model led to the identification of salicylic acid as a mobile signal responsible for systemic acquired resistance (Metraux et al. 1990). Salicylic acid and pathogenesis-related (PR) proteins are induced in cucumber after inoculation with a variety of bacterial, viral, and fungal pathogens. In cucumber, SA associated induced resistance has been shown to be nonspecific: abiotic stresses like phosphates (Mucharromah & Kuc 1991) in addition to biotic challenges from virus can induce PR proteins and systemic, long-term, broad scale resistance to subsequent pathogen challenge (Kuć 1982).

While common cucumbers have been an essential laboratory model for understanding plant defense responses to a variety of biotic and abiotic challenges, little is known about how pathogen-induced changes in hormone levels and induced defenses might affect broader epidemiological patterns when several diseases are circulating in natural populations. The wild gourd Cucurbita pepo ssp. texana is susceptible to many insect-transmitted plant pathogens that also threaten cultivated cucurbits, including the bacterial wilt pathogen Erwinia tracheiphila is
only transmitted by specialist cucumber beetle vectors (Coleoptera: Chrysomelidae: Luperini) (Garcia-Salazar et al. 2000b). Zucchini Yellow Mosaic Virus (ZYMV) (Potyviridae) is an emerging viral pathogen of cucurbits worldwide, and is transmitted in a non-persistent manner by several generalist aphid species (Desbiez & Lecoq 1997).

Several years of field studies show that both ZYMV and *E. tracheiphila* are endemic in field populations of wild gourd at the PSU Experimental Farm in Rock Springs, PA (Sasu et al. 2009b; Sasu et al. 2010b), yet co-infect the same individual host plant at a significantly lower rate than would be expected by chance. Viral infection has been shown to suppress floral volatiles, which reduces recruitment of beetle vectors that transmit *E. tracheiphila* (Shapiro in press), but the contribution of direct *in planta* interactions between the two pathogens to this phenomena is not yet known. In light of this, the present study was undertaken to explore the direct interactions between the viral pathogen *Zucchini yellow mosaic virus* (ZYMV), the bacterial wilt pathogen *Erwinia tracheiphila*, and herbivory by specialist cucumber beetles that transmit *E. tracheiphila* in a shared plant host, and specifically to test if there is an induced systemic acquired resistance in ZYMV-infected wild gourd plants that makes virus-infected plants resistant to subsequent exposure to the bacterial wilt pathogen *E. tracheiphila*.

**Methods**

**Plant and pathogen materials**

The wild gourd *Cucurbita pepo* spp. *texana* is thought to be the wild progenitor of cultivated squash varieties. For all the following experiments, we used *C. pepo* spp. *texana* seeds from wild maternal families originally collected in Texas and grown yearly in experimental plots in Rockspring, PA. After germination, seedlings were transplanted in pots with compost soil supplemented with 3 g of osmocote slow-release fertilizer (NPK:14-14-14) and micronutrients.
All experiments were performed in climate-controlled chambers under incandescent and fluorescent lights (25 C, 16h:8h photoperiod).

The same strain of ZYMV, originally sampled in 2009 in a squash field, was used for all virus-inoculation experiments. We mechanically inoculated the experimental plants by dusting leaves with carborundum and applying an inoculum of 0.1 Potassium-phosphate buffer grinded with infected frozen C. pepo leaf tissue. Mock-inoculated controls consisted of plants inoculated by the same protocol with clean buffer only. A local field isolate of E. tracheiphila was maintained on nutrient peptone agar at 27C for 3 days before plant inoculations.

**Hormone Extractions**

To test differences in constitutive and induced defense-related hormones in wild gourd, 83 C. pepo spp. texana seedlings at the two true leaves stage were randomly either mock inoculated, inoculated with a ZYMV isolate, or used as untouched controls. Virus symptoms uniformly appeared on inoculated plants within 5 days of inoculation. Seven days after virus or mock inoculation, when the seedlings uniformly showed virus symptoms, half of the plants in each treatment group (control, mock, ZYMV) were randomly chosen and inoculated with a virulent field isolate of E. tracheiphila grown for 72 hours on nutrient peptone agar at 28C. Bacterial inoculations were performed by scraping all bacteria from a single plate into 10ml tap water in a beaker, mixing gently until homogeneous dispersal of cells, and applying 10μl of the bacterial suspension to a small break on the petiole of the newest fully expanded leaf.

48 hours after bacterial inoculation, 0.1-0.2 g of the same leaf that received the bacterial inoculation or the mock (clean water only) inoculation was quickly weighed and flash frozen in liquid nitrogen. Hormone extraction through vapor phase extraction was performed following (Schmelz et al. 2003; Schmelz et al. 2004) with modifications. Briefly, frozen tissue was ground with stainless steel beads in a Genogrinder under liquid nitrogen. Dichloromethane and a weak acid solution and 100 ng internal standards of synthetic phytohormones were added to partition
the phytohormones into an organic layer, which was then dried under house air. Trimethylsilyldizomethane (Sigma-Aldrich, St. Louis, MO) was used to derivatize phytohormones from carboxylic acids to methyl esters. The remaining solvent was then allowed to evaporate, and the vial with dried hormones was heated for 2 min at 200°C for volatilization under 1L/min vacuum attached to volatile traps containing 30mg SuperQ. SuperQ traps were eluted with 150μl dichloromethane and the eluate analyzed by GC-MS with isobutane chemical ionization and select monitoring of phytohormone ions described in (Schmelz et al. 2004). Final concentrations of free phytohormones (cis- and trans-JA, SA) were calculated against the initial internal standards (2H6-SA, dhJA) corrected by original weight of the sample. Data were analyzed with treatment as the independent variable and either cisJA or SA as the dependent variable in SAS PROC MIXED and SAS GLM with the model statement Hormone = Disease + Herbivory + Disease*Herbivory.

**Herbivory interactions with ZYMV and E. tracheiphila on defense signaling**

To test whether pathogen infection (either bacterial or viral) affects wild gourd induced responses to herbivory by specialist cucumber beetles, 68 *C. pepo* ssp. *texana* seedlings were assigned for inoculation either as mock-virus, ZYMV, or inoculated with *E. tracheiphila* that had been grown on nutrient peptone agar at 28°C for 48 hours. Mock inoculations and ZYMV inoculations occurred when the seedlings had two true leaves, and *E. tracheiphila* infections were performed when when plants had 4 true leaves so that ZYMV infected plants and *E. tracheiphila* infected plants would show symptoms concurrently. Five days after *E. tracheiphila* inoculations when all inoculated plants showed either ZYMV or *E. tracheiphila* symptoms, half of each group (mock, ZYMV, *E. tracheiphila*) were randomly subjected to herbivory from one striped cucumber beetle confined to the newest fully expanded leaf for 15 hours (overnight). After the insects were removed, 0.1-0.2 g of leaf tissue adjacent to the herbivory damage was weighed and frozen, and processed for vapor-phase acid hormone extraction as described above. Raw values
(ng/g fresh weight) were ln transformed to meet assumptions of normality and analyzed with both SAS PROC MIXED and SAS PROC GLM (SAS 2011) with the model statement ln_hormone = Disease + Herbivory + Disease*Herbivory.

**Infection success**

*Manual leaf inoculations of E. tracheiphila*

To test differences in wilt susceptibility between healthy and ZYMV infected plants at realistic levels of *E. tracheiphila* exposure, 125 wild gourd seedlings were randomly mock-inoculated or inoculated with ZYMV. Seven days after viral or mock-inoculations, with viral symptoms prominent, all plants were inoculated with beetle frass containing *E. tracheiphila*. *E. tracheiphila* inoculations were accomplished by allowing 125 lab-reared *Acalymma vittatum* a 24hr acquisition access period on symptomatic, *E. tracheiphila* infected wild gourd plants. Groups of 10-12 beetles were then immediately placed in petri dishes with a clean cucumber leaf. After 2 hours of feeding, all the frass from all petri dishes was collected and pooled in 1ml of tap water within a 1.7ml Eppendorf tube. The tube was inverted several times to disperse the frass evenly. A small break was made at the base of the petiole of a fully expanded leaf was made, and 10μl of the frass homogenate was applied to the leaf breakage. Symptom appearance was recorded over time and proportions of wilting, *E. tracheiphila* infected plants in each treatment tested with a χ² test of two proportions in R (R 2008).

To test differences in wilt symptom development between healthy and ZYMV infected plants at high *E. tracheiphila* levels, 150 plants were randomly mock-inoculated, inoculated with ZYMV as above or left untouched. They were then inoculated seven days later with *E. tracheiphila* following the same protocol as above, using a mixture of infected beetle frass and *in vitro*-grown bacterial colonies in the same water solution that ensured high doses of bacterial exposure. All but one plant developed wilt symptoms within 17 days and the dates of first wilt symptoms on the inoculated leaf, second wilting leaf and whole plant wilting (when wilt reaches
the primary stem and the whole plant collapses) were recorded over time. The data, excluding one dead untouched control and three ZYMV inoculated plants without viral symptoms, was ln-transformed and analyzed with a one-way ANOVA for differences among the three treatments. Two plants apparently recovered from the disease and were excluded from the whole plant wilting analysis.

**Results**

**Hormone responses to *E. tracheiphila* inoculation**

Leaves on ZYMV-infected wild gourd seedlings contained significantly more constitutive and induced salicylic acid compared to mock-virus infected (healthy) and true control plants (Figure 3.1, Table 3.1). The SA levels in mock and untouched control plants are statistically indistinguishable, and no induction of SA 24 hr after *E. tracheiphila* inoculation was observed. cis-JA and trans-JA were present in negligible amounts in all treatment (cis-JA shown in Figure 3.1) and were not affected by *E. tracheiphila* inoculations.
3-1a.

**Constitutive and Induced Salicylic Acid Levels**

![Graph showing constitutive and induced salicylic acid levels](image)

3-1b

**Jasmonic Acid Induction after *E. tracheiphila* inoculation**

![Graph showing jasmonic acid induction](image)

**Figure 3-1.** Induced hormone responses of healthy and ZYMV-infected wild gourd to inoculation with *E. tracheiphila*

Salicylic acid is present in significantly higher amounts in ZYMV-infected plants compared to healthy or *E. tracheiphila* – infected plants. Inoculation with *E. tracheiphila* had no effect on SA levels in healthy plants, but resulted in significant induction of SA in ZYMV-infected plants.
Consitutive and induced mock cisJA levels in healthy and pathogen-infected plants is negligible. Sample sizes Control n = 14; Control + Et n = 14; Mock n = 14; Mock + Et n = 13; ZYMV n = 15; ZYMV + Et n = 13.

Table 3-1. Mean + SE of ng/gMeSA present in foliage of healthy and ZYMV infected plants exposed to *E. tracheiphila*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean</th>
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<tr>
<td>Control</td>
<td>29.5</td>
<td>110.9</td>
</tr>
<tr>
<td>Control + <em>Etrach</em></td>
<td>39.5</td>
<td>110.9</td>
</tr>
<tr>
<td>Mock</td>
<td>32.1</td>
<td>110.9</td>
</tr>
<tr>
<td>Mock + <em>Etrach</em></td>
<td>43.1</td>
<td>115.1</td>
</tr>
<tr>
<td>ZYMV</td>
<td>416.5</td>
<td>107.2</td>
</tr>
<tr>
<td>ZYMV + <em>Etrach</em></td>
<td>1299.5</td>
<td>115.1</td>
</tr>
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</table>

Table 3-2. ANOVA table of ZYMV * E. tracheiphila* interactions in *C. pepo texana*

<table>
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<tr>
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<th>Denom DF</th>
<th>F value</th>
<th>Pr &gt; F</th>
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<td>Disease</td>
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<td>77</td>
<td>10.91</td>
<td>&lt; 0.0015</td>
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<td><em>E. tracheiphila</em> exposure</td>
<td>2</td>
<td>77</td>
<td>36.22</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Disease<em>Etracheiphila</em> exposure</td>
<td>2</td>
<td>77</td>
<td>10.21</td>
<td>&lt; 0.0001</td>
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</table>

Disease by Exposure ANOVA table to assess differences in SA levels among control, mock-virus infected, and ZYMV - infected plants that were either untouched or inoculated with a virulent field isolate of *E. tracheiphila*. Model run in SAS PROC Mixed with model statement SA = Disease + Etrach + Disease*Etrach.

**Inoculations of healthy and ZYMV-infected plants**

The first controlled leaf inoculation experiment with infectious beetle frass showed that there was no significant difference in the overall rate of *E. tracheiphila* infection success on ZYMV relative to mock-infected (healthy) plants (Figures 3.2 and 3.3). There was a one-day delay in the appearance of the first plant with wilt symptoms on ZYMV-infected compared to mock-infected plants (7 days vs. 6 days post inoculation), but no difference was observed in the rates of wilt disease, 35% and 40% for mock-infected and ZYMV-infected respectively, at the end of the experiment ($\chi^2 = 0.1167; P = 0.733$).
In the second controlled leaf inoculation with infectious frass supplemented with *in-vitro* bacteria, 99.32% (all plants except one) of inoculated plants developed wilt symptoms (Figure 3.3). There was no difference in the timing of symptom appearance on the first leaf (One-way ANOVA, $F_{2,143}=2.59$, $P > 0.05$) but wilt symptoms appeared on a second leaf on average one day later in ZYMV infected plants than mock-inoculated or untouched controls (One way ANOVA, $F_{2,143}=5.23$, $P = 0.0065$, Figure 3.4), demonstrating that bacterial colonization past the inoculated leaf was slower at high exposure levels. The total wilting of the whole plant was also slower in virus-infected plants compared to controls (One way ANOVA, $F_{2,141}=6.11$, $P = 0.0029$, Figure 3.3)

![Wilting rates after low E. tracheiphila exposure](image)

**Figure 3-2.** Inoculation success rates with *E. tracheiphila* on mock-infected (healthy) and ZYMV-infected plants after exposure to infective frass

Inoculation success rates after 26 days for *E. tracheiphila* infection on mock-infected (healthy, n=62) and ZYMV-infected plants (n=65) using frass pooled from 125 beetles allowed a 24hr
acquisition period on wilting, *E. tracheiphila* infected plants that results in less than 50% overall wilt disease transmission rates

**Figure 3-3.** Inoculation success rates for *E. tracheiphila* infection on mock-infected (healthy) and ZYMV-infected plants with frass and *in vitro* *E. tracheiphila* added

Overall rate of *E. tracheiphila* wilt symptom development and disease progression from the inoculated to systemic leaves on untouched control (n=49), mock (n=50), and ZYMV – infected (n=47) plants after inoculation with frass supplemented with bacteria cultivated on peptone agar plates that achieves close to 100% wilt symptom development. Closed symbols: appearance of first wilt symptoms; open symbols: Complete collapse of the diseased plant.
Figure 3-4. Timing of *E. tracheiphila* colonization of *C. pepo texana* measured by time the second leaf showed wilting symptoms

Mean ± SE number of days post-inoculation until the appearance of wilt symptom on the second (non-inoculated) leaf in untouched control (n=49), mock (n=50), and ZYMV – infected (n=47) plants. Letters indicate groups that are significantly different (p-value < 0.05) by a Tukey-Kramer test.

Figure 3-4. Visual comparison of *E. tracheiphila* symptom development on a healthy (left) and a ZYMV-infected (right) wild gourd plants
**Hormone responses of healthy and pathogen-infected plants to herbivory**

Cucumber beetle feeding preferences for pathogen-infected plants (either ZYMV or *E. tracheiphila*–infected) suggests that there may be nutritional, physical, or chemical differences that make pathogen-infected plants more palatable, and defense signaling may play a role in inducing these changes. In this study, cisJA levels in all undamaged plants were measured at very low levels, but in response to herbivory, there was a significant induction of cisJA within all disease treatments (Figure 3.5a, Table 3.3). Between disease treatments, herbivory induced significantly more cisJA on *E. tracheiphila* infected plants compared to ZYMV or mock (Table 3.4). No difference was observed in the induced levels of JA between healthy and ZYMV-infected plants (Figure 3.5a, Table 3.3). As in Figure 3.1, SA levels are significantly induced in ZYMV compared to *E. tracheiphila* – infected and healthy plants, and herbivory did not affect or interact with SA levels (Figure 3.5b, Table 3.5).

3.5a
cisJA Levels in response to Disease and Herbivory

Salicylic Acid Induction after Herbivory

3.5b
**Figure 3-5.** Herbivory effects on cis-JA levels for healthy, ZYMV, and *E. tracheiphila* infected plants

Hormonal response of healthy mock inoculated, *E. tracheiphila* inoculated, and ZYMV-infected plants 15h after exposure to herbivory relative to non-herbivorized plants. (a) Mean SA production ± SE ; (b) Mean cis-JA (grey bars) and trans-JA (black bars) ± SE.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Denom DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
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<tr>
<td>Disease</td>
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<td>67</td>
<td>12.53</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Herbivory</td>
<td>1</td>
<td>67</td>
<td>102.32</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Disease*Herbivory</td>
<td>2</td>
<td>67</td>
<td>8.7</td>
<td>0.0004</td>
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</tbody>
</table>

**Table 3-3.** ANOVA of Disease, Herbivory, and Disease*Herbivory effects on cisJA production in wild gourd leaves

Disease, herbivory, and the interaction of disease*herbivory are all significant factors affecting cisJA. cisJA levels were ln transformed for normality and analyzed in SAS PROC GLM = Disease + Herbivory + Disease*Herbivory

<table>
<thead>
<tr>
<th>cisJA</th>
<th>Mean</th>
<th>SE</th>
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<tr>
<td>Etrach</td>
<td>9.7</td>
<td>38.2</td>
</tr>
<tr>
<td>Etrach + H</td>
<td>332.03</td>
<td>41.9</td>
</tr>
<tr>
<td>Mock</td>
<td>17.31</td>
<td>36.7</td>
</tr>
<tr>
<td>Mock + H</td>
<td>47.1</td>
<td>38.3</td>
</tr>
<tr>
<td>ZYMV</td>
<td>2.4</td>
<td>36.7</td>
</tr>
<tr>
<td>ZYMV + H</td>
<td>78.9</td>
<td>9</td>
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</tbody>
</table>

**Table 3-4.** Mean + SE cisJA present in healthy and pathogen infected leaves that are undamaged and damaged by cucumber beetles

Mean and SE ng cisJA/g fresh weight levels of all treatments: Non-transformed (raw) values were used to compare constitutive and induced cisJA levels in *E. tracheiphila* – infected, ZYMV – infected, and healthy (mock) infected plants exposed to herbivory. Data were generated with SAS PROC MIXED = All Treatments

JA is induced in response to herbivory in both healthy and pathogen-infected plants, with significantly more JA present in *E. tracheiphila* herbivorized leaves compared to mock or ZYMV herbivorized leaves. Herbivore damaged *E. tracheiphila* leaves and undamaged ZYMV leaves show elevated levels of the fatty acids linolenic and linoleic acids, and these compounds are not elevated in either mock treatment.
Table 3.5. ANOVA for salicylic acid in pathogen-infected and healthy plants

Disease is a significant factor explaining variation in SA levels, but Herbivory and the interaction between Herbivory*Disease do not significantly affect SA levels

<table>
<thead>
<tr>
<th></th>
<th>Num DF</th>
<th>Denom DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
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<tr>
<td>Herbivory</td>
<td>1</td>
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<td>1.7</td>
<td>0.197</td>
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<td>Disease</td>
<td>2</td>
<td>66</td>
<td>21.5</td>
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<tr>
<td>Herbivory*Disease</td>
<td>2</td>
<td>66</td>
<td>1.14</td>
<td>0.3261</td>
</tr>
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</table>

Discussion

Salicylic acid mediated systemic acquired resistance has been documented in cucumber in response to viral, bacterial, and fungal challenge (Kuć 1982; Hammerschmidt 1999). Here, we find that despite higher constitutive and induced salicylic acid levels in ZYMV infected plants relative to healthy plants (Figure 3.1a), healthy and ZYMV-infected wild gourds are equally susceptible to infection by *E. tracheiphila* in both low dose (infectious frass only, Figure 3.2) and high dose (infectious frass supplemented with *in-vitro* bacteria, Figure 3.3). Although healthy and virus-infected plants had the same proportions of wilt development, we observed a one-day delay in the first appearance of wilt symptoms on a ZYMV-infected plant compared to a healthy plant in the low bacteria infection experiment. When naturally occurring amounts of *E. tracheiphila* in frass are supplemented with *in vitro* bacteria, there is a significantly slower progression of the disease with a one day delay in the appeared of wilt symptoms on a second leaf and a delay in the complete collapse of virus infected plants (Figure 3.4). These results suggest that SAR induced by ZYMV possibly delays disease progression within virus-infected plants at high exposure levels, but does not prevent co-infection with *E. tracheiphila*.

While bacterial interactions with plant defense signaling networks vary widely between specific plant-pathogen interactions, some bacterial pathogens explicitly exploit cross-talk in host
signaling networks. Some strains of the model plant pathogen *P. syringiae* produce coronatine, a JA mimic that suppresses SA signaling (Uppalapati *et al.* 2007). Other gene products have been found to suppress SA signaling directly, such as *P. syringiae avrE* genes (DebRoy *et al.* 2004). The fire blight pathogen *E. amylovora* contains an *avrE* homologue DspA/E (Disease-specific region) that is required for pathogenicity on hosts (Bogdanove *et al.* 1998a; Bogdanove *et al.* 1998b). This gene (among other potential candidates, Shapiro *et al.* in prep), which was also found in the *E. tracheiphila* genome [ETR 3867], might also function as an SA signaling suppressor for both *E. tracheiphila* and *E. amylovora*.

*E. tracheiphila* inoculation does not induce SA on healthy plants shortly after inoculation (Figure 3.1a), or on leaves five days post inoculation when wilt symptoms had developed (Figure 3.5b). SA induction after *E. tracheiphila* inoculation was only observed in virus-infected plants that had constitutively higher SA levels. Since many bacterial pathogens directly suppress host defenses, it is possible that *E. tracheiphila* suppressed the induction of SA signaling in healthy control plants. Because virus-infected plants with high SA induction experienced only a slight delay in wilt symptom progression without any change in the final outcome of the disease, the SA pathway might not have a strong impact on *E. tracheiphila* disease progression—at least under laboratory inoculations—or only slows systemic bacterial movement through xylem vessels, but does not inhibit bacterial establishment in xylem.

Pathogen–induced effects on plant physiology may also affect interactions with insect herbivores. Cucurbitacins (toxic oxygenated triterpenes) are induced in cucurbit plants by feeding damage and are sufficient to deter herbivory from most herbivores, but cucumber beetles have been shown to feed preferentially on induced leaves (Tallamy 1985). In a previous study (Shapiro *et al.*, 2012), striped cucumber beetles preferred feeding on leaves from either ZYMV or *E. tracheiphila* infected plants relative to mock-infected, healthy plants. Constitutive JA levels are extremely low in all treatments (Figure 3.1b, Figure 3.4a), and beetle herbivory induces JA on
both healthy and pathogen infected plants, although significantly more JA is induced by herbivory on *E. tracheiphila* - infected plants compared to either healthy or ZYMV – infected. Healthy, wilting, and ZYMV-infected plants all had higher JA levels after cucumber beetle herbivory. However, *E. tracheiphila*-infected and ZYMV-infected treatments had opposite patterns of phytohormonal responses, with higher SA production in virus treatments compared to *E. tracheiphila* treatments, while herbivory-induced JA was higher in *E. tracheiphila* treatments. This antagonistic pattern suggests that *E. tracheiphila* inhibition of SA production could possibly have favored the induction of JA as a result of cross-talk between these two signaling pathways, and the higher SA levels in virus – infected plants may inhibit the induction of JA in ZYMV – infected plants. Virus infection can also alter plant nutritional content (Belliure *et al.* 2005; Belliure *et al.* 2010), and possible nutritional changes from virus infection may be more important than JA mediated defenses in feeding preference for ZYMV – infected plants by striped cucumber beetles (Tallamy & Krischik 1989; McCloud *et al.* 1995; Huang *et al.* 2003).

Our results show that the viral pathogen ZYMV and the bacterial pathogen *E. tracheiphila* have divergent effects on plant defense hormone induction in both undamaged and herbivore-damaged plants. While ZYMV infection resulted in a large induction of SA, the absence of SA induction in undamaged plants and high JA levels in beetle damaged plants with *E. tracheiphila* infection suggests that *E. tracheiphila* interferes with SA signaling. After inoculation onto healthy plants, *E. tracheiphila* appears to be suppressing SA signaling and presumably some SA mediated defenses, which include PR protein accumulation and lignification of cell walls (Gessler & Kuc 1982; Mucharromah & Kuc 1991). While *E. tracheiphila* does not induce SA after inoculation on healthy plants while ZYMV does, it is notable that inoculation rates with *E. tracheiphila* are not suppressed on ZYMV infected compared to healthy plants. However, wilt disease progression is slower on ZYMV – infected plants at high inoculation levels, suggesting that ZYMV infection has a negative effect on *E.
tracheiphila movement in the plant, but that SA mediated plant defenses may not be critical in the establishment of E. tracheiphila infection in wild gourds. The slight delay in wilt symptom development on ZYMV – infected plants might have consequences on infection probabilities or rates of symptom development when transmission opportunities of E. tracheiphila consist of daily exposure to low doses of bacteria deposited by many beetles daily in the field, conditions that are impossible to replicate in the lab. Overall, antagonistic interactions in plant defensive signaling pathways appear to be conducive to E. tracheiphila infection progression and beetle feeding preferences. Furthermore, attenuation of floral volatile emissions from virus-infected plants is associated with a reduction in recruitment of cucumber beetles that transmit E. tracheiphila to wild gourd (Shapiro et al, in press). A small negative effect of ZYMV on E. tracheiphila movement in planta at high inoculation levels through direct pathogen interactions or antagonistic effects on defense hormones would thus have contributed only in a limited way to differences in wilt disease dynamics observed between healthy and virus infected plants in the field. These limited direct effects support previous findings that, in this system, indirect viral effects through the attenuation of floral volatiles and reduced beetle vector recruitment is the major contributor to the observed field disease dynamics.

References


Chapter 4

Colonization dynamics of an insect vector by a bacterial phytopathogen

Abstract

*Erwinia tracheiphila*, the causal agent of bacterial wilt of cucurbits, is predominately transmitted in the Eastern US by the striped cucumber beetle *Acalymma vittatum* (Luperini: Chrysomelidae: Coleoptera), but the colonization dynamics of *A. vittatum* by *E. tracheiphila* are not well understood. Here, we develop a quantitative Real-Time PCR method using a TaqMan probe specific to an *E. tracheiphila* outer membrane protein (OmpA) to examine the colonization of *A. vittatum* by *E. tracheiphila* and to describe the quantitative dynamics of bacterial colonization of the beetle digestive tract and shedding in frass over time. Our results show that acquisition of *E. tracheiphila* does not differ between short-term (3hr) and long-term (24hr) acquisition access periods (AAP), but that long-term retention increases for the longer AAP. The amount of *E. tracheiphila* shed in frass is significantly higher 0 and 28 days post acquisition compared to 5 days, suggesting successful *E. tracheiphila* colonization of the hindgut determines long term ability to transmit from infected to healthy hosts. Inoculation experiments confirm that the amount of bacteria shed in frass significantly affects the probability of transmission to a wild gourd seedling. Fitness experiments fail to find a cost or benefit to *E. tracheiphila* exposure for cucumber beetle vectors. A better understanding of pathogen-vector interactions will allow for improvement of control systems in agricultural settings, which currently depends on broad-spectrum chemical control of vectors or expensive mechanical barriers to prevent insects from contacting plants.
Introduction

For insect-borne pathogens, efficiency of dispersal from infected to healthy hosts is influenced by interactions with the insect vector. However, detailed knowledge of pathogen-vector interactions, and how these interactions drive disease dynamics in natural systems, are often poorly understood. Arthropod transmitted plant pathogens that are able to persistently colonize herbivorous insects as alternate hosts can continually contact new, healthy plant hosts through the foraging behavior of the herbivorous vectors for long periods after initial acquisition (Eigenbrode et al. 2002), and the vast majority of plant pathogens are viruses obligately transmitted by homopteran insects. While many bacterial strains cause important plant diseases, they are numerically fewer, and most are mechanically spread through the environment (citations). Very few plant pathogenic bacteria are obligately insect vectored, and colonization dynamics of vectors and correlation with pathogen dispersal are often under-investigated (citations). Here, we investigate the colonization dynamics of the causal agent of bacterial wilt of cucurbits, *Erwinia tracheiphila* Smith (Enterobacteriaceae), within its specialist insect vector, and how vector colonization contributes to host plant exposure.

Cucumber beetles (Coleoptera: Chrysomelidae: Luperini) have been recognized as vectors of *E. tracheiphila* for more than a century (Smith 1911). *E. tracheiphila* replicate in xylem and secretes an extracellular polysaccharide (EPS) that prevents the passage of xylem sap, which causes vessel occlusion and characteristic wilting symptoms (Watterson 1971; Goodman & White 1981). Bacterial wilt infection is usually fatal in most wild and cultivated cucurbits once symptoms appear (Sasu et al. 2009a), and can cause major economic losses in cultivated fields (Brust 1999). Quantification of *E. tracheiphila* within beetles was first attempted with fuschin
staining (Leach 1964), which indicated so few bacteria in the beetle’s intestinal tract that the authors proposed the since-disproven (de Mackiewicz et al. 1998) hypothesis that *E. tracheiphila* have another environmental reservoir (Leach 1964). Previous work using bacteria-smeread cotyledon disks as inoculation sources to beetles and DAS-ELISA and immunoperoxidase localization as a detection method has suggested stronger fluorescence in the hindgut 10 and 30 day post acquisition period compared to 3 day post acquisition (Garcia-Salazar et al. 2000b), but another study failed to detect *E. tracheiphila* in beetle frass with standard PCR 6d post acquisition (Mitchell & Hanks 2009).

In this study, we developed TaqMan probes specific to a single copy *E. tracheiphila* outer membrane protein gene (ompA) and the eukaryotic ribosomal 18S gene as a normalizing gene to compare *Erwinia tracheiphila* acquisition and retention rates as a function of acquisition access period (AAP) through natural herbivory on infected plants. Relative *E. tracheiphila* levels in frass and entire beetles were determined at several time points post acquisition access period, as well as time-dependent changes of *E. tracheiphila* present in the frass and in whole beetles at 0, 5, and 28 days after an acquisition period. We then conducted fitness experiments to understand how feeding on wilt diseased plants affects cucumber beetle oviposition and longevity, and a series of inoculation experiments were performed to determine how elapsed time post acquisition affects rates of transmission of *E. tracheiphila* via frass to healthy wild gourd *Cucurbita pepo* ssp. *texana* seedlings.

### Materials and Methods

**Beetle Rearing**

A clean, *E.tracheiphila*-free striped cucumber beetle colony was maintained following Mitchell (2009). Field captured adult beetles were kept in 1 ft x 1 ft Bioquip mesh cages (cat
Pro-Mix potting soil (Premier Horticulture Reviere Du Loup, Quebec, Canada) is provided as a water source and as an oviposition substrate. Beetles were fed 3x per week with leaves and flowers of ‘Raven’ C. pepo. The potting soil with eggs was transferred weekly to a 6L plastic bin rearing container. Root-feeding larvae were continuously supplied with sprouted C. pepo ‘Raven’ seedlings until pupation. Newly eclosed adults were removed from the rearing container and placed with adults from the same weekly age cohort in 1ft x 1ft Bioquip mesh cages.

DNA extraction from beetle frass

Two wild gourd Cucurbita pepo ssp. texana plants were infected with a local field isolate of E. tracheiphila by the colony-stab procedure (Mitchell & Hanks 2009). Both plants were placed together in a 1ft x 1ft Bioquip mesh cage (cat #1466A) when most leaves showed wilt disease development. Seventy-five 5-day old A. vittatum beetles from our E. tracheiphila-free lab colony that had been starved for 48 hours were placed in the same cage with the infected plants. Groups of 23 beetles were removed after 3hr and 24hr acquisition access periods. Frass was collected immediately by placing beetles individually in 0.7ml Eppendorf tubes until frass was visible inside the tube (~2 hours). The beetles were then kept individually in petri dishes, and transferred 4x weekly to new petri dishes with water soaked cotton and a clean cucumber leaf as food to prevent re-inoculation. Frass was collected from each beetle immediately after removal from the cage with the host plant, and then again at 5 days and 28 days post exposure. DNA was then immediately extracted from the frass using prepGem Bacteria extraction kits (ZyGEM Corp Ltd. Hamilton, New Zealand) following label instructions scaled from 100ul to 55ul extractions.

Quantitative Realtime PCR

5μl of DNA was used as template in triplicate 20 μl RealTimePCR reactions with 10mM primers and probes and ABI TaqMan® Gene Expression MasterMix (cat #4369016). Primers and
probe were designed to anneal to conserved regions of a single copy gene encoding an outer membrane protein A (EtOMPA) of *E. tracheiphila*, such that all published strains of *E. tracheiphila* are indiscriminately detected (Et73: GGCGATCACGACACAGTTGT, Et140: CAGTTTTTGTCAGGCATACTC) P94: TCCCCTCTGGCAGCCATAGGTGC, Probe: TCCCCTCTGGCAGCCATAGGTGC). 18S was used as a normalizing gene for the total amount of DNA in each sample (18S F: CGGCTACCACATCCAAGGAA; 18S R: TGCTGGCACCAGGACTTGC; 18S Probe: CGCAAATTACCCACTCCCGGCAC) (Broackes-Carter et al. 2002). Standard curves were generated for both EtOMP and 18S using 5 serial dilutions of DNA extracted from pure bacterial culture for the EtOMP curve and DNA extracted from clean colony beetles for 18S. The cycling program consisted of an initial denaturation step at 95°C for 3 minutes, and 40 cycles of denaturation at 95°C for 10 seconds, and annealing/extension at 55°C for 30 seconds and was carried out on an ABI Prism 7900 HT sequence detector (Applied Biosystems, Foster City, CA). Fluorescence reads were taken at the end of each annealing/extension step. We compared the sensitivity and accuracy of this Taqman assay with PCR reactions using the same primers but detecting the amount of product via the fluorescence of SYBRgreen. CT values did not differ by more than 1 cycle between the two methods. Both detection methods identified beetles having fed on *E. tracheiphila*-infected plants correctly. The TaqMan probe assay did not show fluorescence signal for beetles that had not been exposed to *E. tracheiphila* (negative control beetles). On the other hand there were sporadic fluorescence signals for the negative control beetles when analyzed by the SYBRgreen assay and only after close inspection of the melting curves could those samples be pronounced negative. In short, the PCR assay using Taqman probes is as sensitive as the previously used SYBRgreen assay, but it eliminates the ambiguity of inspecting melting curves and it is faster (because of the omission of the melting curve in the actual PCR run).
DNA extraction from whole beetles

In a separate experiment 75 beetles from a clean lab colony that had been starved for 48hr were allowed to feed for 24hr on symptomatic *E. tracheiphila*-infected plants and then maintained as described above. At three time points post-acquisition (0, 5 days, 28 days) 25 whole beetles were ground individually under liquid N₂ in a Genogrinder (OPS Diagnostics, LLC). DNA was extracted using 100μl volume prepGem Bacteria following label instructions. 5μl of this DNA was used in triplicate qPCR reactions for 18S and EtOMP as described above.

*Erwinia tracheiphila* Transmission Potential

To determine whether *E. tracheiphila* levels in frass affect transmission potential, 150 beetles were fed on two infected plants (as described above). Frass from 100 randomly selected beetles was collected, pooled, and homogenized in tap water from the beetles at 0, 5, and 28 days post acquisition. 10μl of homogenate was applied to a small break in the leaf petiole of *C. pepo* ssp. *texana* seedlings at 14 days (2 fully expanded leaves). Symptom development was recorded 3x weekly.

*Erwinia tracheiphila* Exposure Effects on *Acalymma vittatum* Fitness

Oviposition Rate and Longevity To test how exposure to *E. tracheiphila* through feeding on symptomatic plants affects *A. vittatum* fitness, a series of experiments was performed to test oviposition rates, egg development time and hatch rate, and longevity. Seventy-five adult beetles that had eclosed within 2-3 days were separated by sex. Two mesh 1ft x 1ft Bioquip cages (cat #1466A) each contained a symptomatic, *E. tracheiphila* infected plant, and two more cages each contained a healthy, mock-inoculated plant. Half of the newly eclosed females and half of the males were separately placed in the two cages with the infected plants, and the other half were separately placed in the cages with healthy (mock-inoculated) plants. Beetles were allowed to an 96 hr AAP on infected or healthy plants. One male and one female of the same plant treatment
were then placed in petri dishes and allowed to mate for 48 hours. Females were then kept individually in petri dishes with moistened cotton as an oviposition substrate and 3x3cm cucumber leaf as a food source. Females were re-mated 14 days after the first mating because stored sperm quality and subsequently egg quality declines after this period. Females were transferred to new, clean petri dishes with new food and oviposition substrates 3x weekly. Rates of female survivorship and oviposition were recorded. The experimental insects were kept in an incubator set at 25°C with 14:10 L/D cycle.

**Egg Hatch Rate and Developmental Time** In a separate experiment, newly-eclosed beetles were divided by sex and randomly assigned to feed on a healthy (mock inoculated) *C. pepo* ssp. *texana* seedling or a wilting *E. tracheiphila* infected seedling for 96 hours (as above). One female and two male beetles were then placed in petri dishes and allowed to mate for 48 hours. Females were then placed individually in petri dishes with moist potting soil as an oviposition substrate (Quebec supplier) and 3x3cm cucumber leaf provided as food. The first 17-27 eggs oviposited by each female were collected from the soil in the petri dish and then placed in 1 gallon black pot with four *C. pepo 'Raven'* seedlings provided as food for the rootworms. 'Raven' seedlings were replenished as needed. The rate of adult emergence from each pot was recorded. Pots with seedlings and eggs were kept in a growth chamber set at 25°C/22°C and 14:10 day:night cycle.

**Results**

**Frass Acquisition and Retention Rates**
Of the 138 frass samples collected from beetles at 0, 5, and 28 days after feeding for either 3 or 24 hrs on *E. tracheiphila* infected plants, only 124 samples yielded usable DNA. The remaining samples that gave no product for either gene were not included in the analysis. A chi-square test reveals that the probability that the frass will contain *E. tracheiphila* immediately after feeding on infected plants is independent of the acquisition access period (3 hr vs. 24 hr) ($\chi^2 = 0.0341$, df = 1, $P = 0.8535$) (Fig 2.1). At five days post acquisition, there are significantly more beetles in the 24hr acquisition group that are *E. tracheiphila*-positive compared to the 3hr acquisition group ($\chi^2 = 6.4736$, df = 1, $P = 0.01095$), and at 28 days post acquisition there is again a significantly higher proportion of *E. tracheiphila*-positive beetles in the 24hr acquisition group compared to the 3hr exposure group ($\chi^2 = 7.621$, df = 1, $P = 0.0058$) (Figure 4.1). Therefore, the time period of acquisition does not significantly affect initial acquisition rates but does significantly affect the probability of a beetle becoming a persistent carrier.

![Figure 4-1](image_url) Frass acquisition and retention rates

**1A:** Chi-square tests of the percentages of beetles with frass positive for *Erwinia tracheiphila* detected immediately, 5 days, and 28 days post AAP for a 3hr or 24hr acquisition access periods.
Whole Beetle Acquisition and Retention Rates

In the second experiment, 72 of the 75 whole beetles were unambiguously scored as positive or negative for *E. tracheiphila* after feeding for 24 hrs on *E. tracheiphila* infected plants. Three beetles were not scored due to a failed DNA extraction determined by lack of product for both 18S and EtOMP. There is a moderately higher percentage of *E. tracheiphila*-positive beetles immediately (82%) and at 5 days (81%) than at 28 days (64%), but the proportion of *E. tracheiphila*-positive beetles is not significantly different between any time points (0:5days $\chi^2 = 0$, df = 1, $P = 1$; Immediate:28 days $\chi^2 = 1.4749$, df = 1, $P = 0.2246$; 5 days: 28days $\chi^2 = 1.9056$, df = 1, $P = 0.1675$) (Figure 4.2). The frequency of *E. tracheiphila* positive beetles from the whole beetle experiment is statistically indistinguishable from the proportion of *E. tracheiphila* positive beetles in the 24 hr acquisition group in the frass experiment at the 0 and 28 day time points, but a significantly higher proportion from the whole beetle experiment are positive compared to frass at 5 days (0 days frass: 0 days beetle $\chi^2 = 0.4312$, df = 1, $P = 0.5114$; 5 days frass:5 days beetle $\chi^2 = 3.9305$, df = 1, $P = 0.0472$; 28 days frass: 28 days beetle $\chi^2 = 0$, df = 1, $P = 1$). In both the whole beetle and 24hr feeding experiments, more than 80% of beetles acquired *E. tracheiphila* through feeding on infected plants, and more than 60% retained it for the duration of the experiment (Figures 4.1, 4.2).
Figure 4-2. Whole beetle acquisition and retention rates
Chi-square tests of the percentages of whole beetles positive for Erwinia tracheiphila detected immediately, 5 days, and 28 days post AAP for a 24hr acquisition access periods.

Whole Beetle Quantitative Dynamics

To achieve a quantitative understanding of changes in bacterial titre during long-term colonization of A. vittatum by E. tracheiphila, we used the amount of the single copy gene ETOMP as a measure for the amount of Erwinia present and the amount of 18S as a normalizing gene for the amount of total DNA present. The underlying assumption is that bacterial DNA contributes only negligibly to the total amount of DNA present in whole beetles that fed on symptomatic, infected plants and that dead bacteria contribute negligible amounts of EtOMP to total bacterial DNA. Bartlett’s test for equal variances of Et/18S ratios rejects the null hypothesis of equal variances (Bartlett’s $K^2 = 175.93; \, df=2, \, P \geq 0.005$), so a non-parametric test was used to compare relative E. tracheiphila levels in whole beetles 0 days, 5 days, and 28 days post acquisition. Kruskal-Wallis test of relative bacteria levels at the three time points showed they are not statistically different ($\chi^2 = 2.63, \, df = 2, \, P = 0.2676$). Re-testing for difference in median
bacterial levels after the removal of two outliers representing high relative bacterial levels immediately after herbivory did not change the test result ($\chi^2 = 3.8194, \text{df} = 2, P = 0.1481$). We note that the two outliers with the largest Et/18S ratio and the four highest absolute relative bacterial levels occur in the first time group of beetles analyzed immediately after exposure, further supporting the hypothesis that beetles are potentially exposed to highly varying amounts of *E. tracheiphila* through natural herbivory on infected plants (Figure 4.3). While the median relative levels of *E. tracheiphila* in whole beetles are not significantly different at the three time points measured, the variance is significantly different. Immediately after acquisition, there is a wide variance in relative levels of *E. tracheiphila* present. By 28 days post acquisition, the median amount of bacteria has not changed but the variance is significantly narrower, indicating that bacterial population levels in the gut lumen converge toward an equilibrium population range in beetles that have been successfully colonized (Figure 4.4).

**Frass Quantitative Dynamics**

The amount of bacteria shed in frass is a factor determining the level of exposure of healthy host plants to the wilt pathogen, so we sought to understand the quantitative and temporal dynamics of bacterial levels in beetle frass. In plant hosts, *E. tracheiphila* form localized blockages in xylem vessels and are thought to be heterogeneously distributed throughout the vasculature. To assess whether this could affect the amount of bacteria that beetle vectors are exposed to through feeding on symptomatic plants, Levene’s test for equal variances was applied to compare the ranges of bacterial levels present in frass 0, 5, and 28 days post acquisition. There is a significantly wider range of relative bacterial levels present in frass at 0 days and 28 days compared to the amount of bacteria present 5 days post acquisition (Test statistic = 3.74, $P = .031$) (Figure 4.4). We applied a repeated measures GLM in SAS Proc Mixed (SAS 2011) to measure the effects of AAP (3 vs. 24 hr) and time since acquisition (0, 5, 28 days) on the amount
of *E. tracheiphila* relative to total plant DNA in the frass. AAP was not significant \((F = 0.61, P = 0.4427)\) so the 3 hr and 24 hr AAP groups were pooled for further analysis of the relative amount of bacteria in frass at three time points post acquisition (0, 5, or 28 days). Frass was repeatedly collected from the same insects, so a repeated measures model was used to compare the bacterial levels in frass from. We found that while the amount of *E. tracheiphila* shed in frass was not significantly different between acquisition groups, time post acquisition is a significant factor \((F = 4.92, P = 0.0241)\). Individual comparisons show there is significantly more *E. tracheiphila* in frass immediately compared to 5 days (Kruskal-Wallis test: \(H = 6.99, \text{df} = 1, P = 0.008\)) and 28 days compared to 5 days \((H = 14.12, \text{df} = 1, P \geq 0.005)\), and that there is marginally more relative *E. tracheiphila* in frass at 28 days compared to immediately \((H = 3.14, \text{df} = 1, P = 0.076)\) (Figure 4.3). The model statement is \(\text{EtOMP/18S} = \text{AAP} + \text{Time post acquisition} + (\text{AAP} \times \text{Time post acquisition})\); Repeated Time/Beetle using the spatial power covariance structure (SAS 2011).

**Figure 4-3.** Quantitative dynamics of frass in whole beetles vs. frass
Comparison of the relative amounts of bacteria detected in frass and whole beetles immediately, 5 days, and 28 days post acquisition. For whole beetles, Kruskal-Wallis test was applied to the model \(\text{Et} = \text{Time}\). For frass, \(\text{Et} = \text{AAP} + \text{time} + \text{AAP*time};\) repeated time.
Figure 4-4. Distribution of *E. tracheiphila* in frass over time

Distribution (variance) of relative *E. tracheiphila*/totalDNA ratios in frass immediately, 5 days, and 28 days post acquisition. Levene’s test for equal variances of bacterial levels in frass shows the variance is lower at 5 days compared to the other two time points (Test statistic = 3.74, \( P = .031 \)).

Figure 4-5. Distribution of *E. tracheiphila* in whole beetles

Distribution (variance) of relative *E. tracheiphila*/totalDNA ratios in whole beetles immediately, 5 days, and 28 days post acquisition. Bartlett’s test for equal variances of Et/18S ratios shows the variance is significantly larger immediately compared to both 5 days and 28 days after an acquisition access period (Bartlett’s \( K^2 = 175.93; \ df=2, \ P \geq 0.005 \)).
### Inoculation Trials

We performed a series of transmission experiments to examine how the amount of bacteria in frass affects transmission potential and inoculation success, and found that inoculation success rates correlate with the amount of bacteria detected in frass. Chi-square tests for difference in two proportions in R was applied to analyze the different inoculation success rates of *C. pepo ssp. texana* seedlings using frass collections from a group of 100 *A. vittatum* 0, 5, and 28 days post acquisition. There is a significantly higher inoculation success rate with frass collected 0 days and 28 days post acquisition compared to frass collected 5 days post acquisition (0:5 days $\chi^2 = 42.812$, df = 1, $P < 0.005$; 5 days:28 days $\chi^2 = 25.967$, df = 1, $P < 0.005$). The difference in inoculation success rates with frass collected 0 days and 28 days post acquisition is not significant ($\chi^2 = 1.348$, df = 1, $P = 0.245$) (Figure 4.6).

<table>
<thead>
<tr>
<th>Wilt Symptoms</th>
<th>0 days</th>
<th>5 days</th>
<th>28 days</th>
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<tr>
<td>Total (n)</td>
<td>84</td>
<td>76</td>
<td>54</td>
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**Figure 4-6.** Inoculation success rates with frass collected 0, 5, and 28 days after AAP

Inoculation experiments were used to show that infection success correlates with the number of *E. tracheiphila* cells shed in frass at different time points post exposure.

**Erwinia tracheiphila exposure effects on Acalymma vittatum fitness**

Prolonged acquisition access periods on wilting *E. tracheiphila* infected plants did not significantly affect striped cucumber beetle longevity, oviposition rates, or adult emergence rates. In the oviposition experiment, 10/13 females that fed on the mock inoculated plants and 11/14 females that fed on the *E. tracheiphila* infected plants survived for the 32 day duration of the experiment. Two females from the *E. tracheiphila* exposure group died during the experiment (the first female died 2 days after feeding, the second died 23 days after feeding) and three females from the mock group died (one died 9 days post feeding and two died 20 days post feeding). Females that oviposited fewer than 20 eggs total (two from the mock group and two from the *E. tracheiphila* group) were excluded from the statistical analysis of oviposition rate but not longevity. The total number of eggs oviposited by females in each group was not different (1-way ANOVA; \( F_{1,25} = 0.32; P = 0.577 \)). In the egg development experiment, there was no difference in the percentage of adult emergence from eggs produced by females fed either on healthy or wilting seedlings (1-way ANOVA, \( F_{1,22} = 0.07, P = 0.791 \)).

**Discussion**

Microbes that persistently colonize vectors can be carried through heterogeneous landscapes and over long time frames. This ability to persistently colonize multiple environments (insect vector and primary host) can be an important factor affecting transmission dynamics and disease persistence in host populations. Here, we show *Erwinia tracheiphila* can be both mechanically transmitted with no latent period after an acquisition period, and persistently
transmitted for up to 28 days, which is approximately the life of the adult beetle (Ellers-Kirk & Fleischer 2006). While many enteric phytopathogens can colonize insects (Hinnebusch 1996; Basset et al. 2000; Ffrench-Constant et al. 2003; Grenier et al. 2006), *E. tracheiphila* is unique among this group in that it is the only pathogen known to be completely dependent on an insect vector for dissemination from infected to healthy hosts. Closely related plant pathogenic *Erwinia* ssp. have only transient, external, and non-specific associations with insect vectors (Hildebrand et al. 2000; Kube et al. 2010b).

Despite the importance of *E. tracheiphila* colonization of *A. vittatum* for transmission over extended time periods (de Mackiewicz et al. 1998), little was known about quantitative and temporal dynamics of *E. tracheiphila*-*A. vittatum* interactions, or how these interactions affect host plant exposure to infective frass. Previous investigations have produced conflicting data about the dynamics of long-term colonization of cucumber beetles by *E. tracheiphila* (Garcia-Salazar et al. 2000b), and the effectiveness of frass as a long-term pathway of transmission (Mitchell & Hanks 2009). To address this shortcoming, we developed a qPCR protocol using TaqMan probes to determine overall acquisition and retention rates when beetles are exposed to *E. tracheiphila* through natural herbivory on wilting plants, and to quantify the amount of *E. tracheiphila* relative to the 18S normalizing gene in whole beetles and frass to better understand the process of bacterial colonization of beetle vectors.

*E. tracheiphila* form occlusions that block water passage in host plants, indicating a heterogeneous distribution of bacteria within xylem vessels that is reflected in the large variance of bacterial levels observed in whole beetles and in frass immediately after natural herbivory on infected plants (Fig 3, 4). The length of AAP significantly affects the probability of becoming an *E. tracheiphila*-positive vector but not the amount of bacteria shed in frass once the beetle is colonized. The high initial acquisition but low long term retention of *E. tracheiphila* by beetles in the 3hr AAP suggests that adhesion of *E. tracheiphila* to the beetle’s digestive tract is inefficient,
but *E. tracheiphila* that do attach to the digestive tract and begin replicating follow the same population trajectory regardless of the length of the acquisition period. Persistently transmitted bacterial pathogens must be able to attach to, replicate within, and detach (shed) from insect vectors in order to be transmitted to new hosts. Comparisons of the relative quantities of bacteria in whole beetles and in frass immediately and then at several time points post acquisition measured in these experiments support a model of complex colonization dynamics of *E. tracheiphila* in beetle vectors (Berk et al. 2012).

To assess how the changing bacterial levels in frass might affect broader disease dynamics, a series of inoculation experiments was performed. Transmission results show that infection success rates correlate to bacterial levels in frass (Fig 5), with significantly higher inoculation success with frass collected from beetles 0 days and 28 days post acquisition compared to frass collected 5 days post acquisition. In these experiments, direct inoculations of infective frass into leaf wounds on small seedlings resulted in a maximum of 50% infection rates. *Cucurbita* ssp. are more resistant to wilt infection as they mature (Brust 1997a), therefore sustaining epidemics in field settings is likely dependent on having high proportions of infective vectors often contacting healthy plants. In a field setting, many factors are likely to affect *E. tracheiphila* disease dynamics. In addition to persistence within vectors, insect preference and orientation towards healthy and infected plants influence host exposure and pathogen transmission to healthy plants (Sisterson 2008). Because plants with bacterial wilt disease usually die within 7-14d after the onset of symptoms, there is limited time for acquisition of the pathogen by the vector. Our recent work (Shapiro 2012) has shown, however, that cucumber beetles are preferentially attracted to the volatiles produced by *E. tracheiphila* infected plants and feed more on infected plants than healthy plants. Consequently the beetles are more likely to acquire the pathogen than would be predicted by the number of wilt diseased plants at any given time in the population. The data presented here show that once the pathogen is acquired, the vector can
transmit *E. tracheiphila* throughout much of its life (and well after the diseased plant from which it acquired the pathogen is dead).

Infective frass must directly contact herbivory wounds or floral nectaries in order for *E. tracheiphila* to access the vasculature and for symptoms to develop (Smith 1915). *C. pepo ssp. texana* can become infected through leaf (Smith 1915) (Mitchell & Hanks 2009) or floral nectary exposure (Sasu *et al.* 2010a). However, it is possible that both the amount of exposure and infection efficiency through leaves and flowers are different. Wild gourd may be more susceptible to *E. tracheiphila* infection through leaves, however phloem exudates from leaf wounds can gum beetle herbivore mouthparts and discourage feeding (McCloud *et al.* 1995; Eben 2007), so it is likely that *Cucurbita* plants receive more exposure through floral nectaries. Symptom development on cucurbit seedlings leaf-inoculated with *E. tracheiphila* varies by growth stage and cultivar (Brust 1995, 1997) and inoculum concentration (Brust 1997b). Previous single-beetle inoculation studies (Rand 1920, Brust 1997b, Fleischer 1999) consistently result in low rates of wilt symptom development. More than 95% of healthy wild gourd plants in a field experiment were found to have *E. tracheiphila* near the nectaries, but fewer than 20% of plants in the field presented with wilt symptoms. Based on these previous investigations and the frass inoculation experiments in this study, we hypothesize that *E. tracheiphila* in frass from a single insect is often not enough to produce infection on a host, especially for large plants. Repeated contacts with bacteria from frass from infective vectors may be an important determinant of disease dynamics and repeated contact with infective vectors is likely necessary, especially for large, mature plants.

The outcome of interactions between phytopathogenic bacteria with insects can be diverse and difficult to predict. *Pectobacterium carotovora* elicits an immune response in *Drosophila* (Basset *et al.* 2000), and both *Pseudomonas syringae* (Stavrinides *et al.* 2009a) and *Dickeya dadantii* (Grenier *et al.* 2006) (Costechareyre *et al.* 2010) are pathogenic to the pea aphid, *Acyrthosiphon pisum*. However, phytopathogens that are obligately insect-transmitted are
predicted to not severely impact the fitness of the vector to allow infective vectors time to contact new hosts (Elliot et al. 2003; Mauck et al. 2012). To assess how prolonged exposure to *E. tracheiphila* infected plants impacts beetle fitness and longevity, experiments were performed to determine how extended acquisition access periods on symptomatic, *E. tracheiphila* infected plants affect longevity, oviposition rate, or hatch rate compared to beetles fed on mock-inoculated (healthy) plants. Herbivore gut microbiota is a complex and poorly described environment (Schloss et al. 2006; Engel et al. 2012) that can have important effects on insect digestion and nutrient absorption (Shi et al. 2010). While being colonized by *E. tracheiphila* may induce other important physiological changes based on changes in gut microbial community composition and nutrient uptake (Ben-Yosef et al. 2010), (de Vries et al. 2004) (Douglas 2009), or changes in beetle preferences for infected or healthy plants (Stafford et al. 2011), these experiments do not suggest there is an overall fitness benefit or cost to ingesting *E. tracheiphila* through feeding on infected plants.

Striped cucumber beetles are the most important vector of *E. tracheiphila* in the northeast, and here we describe quantitative dynamics of *A. vittatum* colonization by *E. tracheiphila* and how colonization dynamics interact with beetle biology to affect disease transmission in a field setting. *E. tracheiphila* was recently confirmed in the southwest (Sanogo 2011), where *Diabrotica* and *Acalymma* are much more speciose (Munroe 1980, Krysan and Smith 1987) providing many potentially competent vector that may be possible transmitters to drive disease emergence in an area that does not currently face losses from wilt infections. Many environmental and ecological factors interact to affect pathogen dispersal and the maintenance of disease epidemics in host populations. For insect-borne pathogens, persistent colonization of insect vectors are a determining factor for sustaining epidemics by providing a mobile environmental reservoir of an infective agent, but quantitative investigations of vector acquisition and retention dynamics are often lacking. A clearer understanding of the factors affecting insect
colonization by *E. tracheiphila* and transmission dynamics will aid in the development of biologically informed pest and pathogen control strategies by identifying when insects are shedding the highest amounts of infective bacteria, and suggesting the most appropriate stage for intervention. Elucidating the molecular basis of beetle-pathogen interactions, such as what cues mediate bacterial attachment to and proliferation within the insect’s digestive tract and plant vasculature tissue, could lead to the development of inhibitors to interfere with colonization and transmission (Verschuere *et al.* 2000) (Shahabuddin & Kaslow 1993).

Bacteria often cause disease in a concentration dependent manner (Dong *et al.* 2000). For example, a cheap and effective way of controlling cholera is to filter the copepods that the bacterium attach to as a way of reducing the amount of bacteria ingested with drinking water (Huo *et al.* 1996) (Heithoff & Mahan 2004), connecting an understanding of bacterial population dynamics and colonization of alternative host to important insight for disease control. Understanding the acquisition and retention dynamics of plant pathogens within insect vectors are important parameters for understanding broader disease dynamics in agricultural and natural systems (Blanc *et al.* 2011). While many obligately insect-transmitted plant pathogens cross from the gut lumen into the haemoceol and display a latent period until they reach the salivary gland and can be transmitted by the probing or feeding behaviors of herbivorous insects (Labroussaa *et al.* 2010) (Hogenhout *et al.* 2008), relatively few but economically important bacteria (Garcia-Salazar *et al.* 2000a) (Esker & Nutter Jr 2003) (Almeida & Purcell 2006), colonize the digestive tract of insect vectors as extracellular symbionts and lack latent periods. A better understanding of bacterial colonization of environmental niches and alternate hosts are increasingly recognized as important to understanding the biology of and devising effective control strategies for pathogens important to human, animal, and plant health (Huq *et al.* 1983; Brandl 2006; Schikora *et al.* 2008).
References


Chapter 5

Comparative genomic insights into emergence and host associations of the bacterial wilt pathogen *Erwinia tracheiphila*

Abstract

*Erwinia tracheiphila* is the causal agent of a bacterial wilt disease with a host range restricted to susceptible wild and cultivated *Cucurbita* and *Cucumis* ssp. Unlike other sequenced *Erwinia* ssp. that have transient, facultative, and external associations with insect vectors, *E. tracheiphila* is obligately transmitted in a persistent manner. Analysis of the draft genome of an *E. tracheiphila* clone isolated from the wild gourd *Cucurbita pepo* ssp. *texana* at the Rock Springs Research Farm shows evidence of active genome decay and chromosomal rearrangements likely associated with phage and transposable element proliferation, and the presence of important genes for host interactions likely acquired laterally. Overall, this suggests that *E. tracheiphila* is undergoing rapid evolution, possibly associated with host adaptation and niche specialization. The genome *E. tracheiphila* associated bacterial wilt of cucurbits results in millions of dollars in crop loss annually, yet mechanisms of virulence to plants hosts and factors mediating growth and attachment to the insect vector have not been discussed in the literature. In the present study, comparative genomic analysis of *E. tracheiphila* with two other sequenced *Erwinia* spp. was conducted to describe conserved and novel traits, and how simultaneous genome decay through pseudogenization and expansion through HGT might be contributing to niche specialization and pathogen emergence.
**Introduction**

*Erwinia tracheiphila* (Enterobacteriaceae), commonly known as bacterial wilt of cucurbits, is the most economically important pathogen of cultivated cucurbits in the eastern US and causes millions of dollars in economic losses annually (Rojas *et al.* 2011). *E. tracheiphila* occupies a distinct ecological niche with divergent host associations and transmission characteristics compared to other characterized *Erwinia* spp., and yet the underlying genetic basis of *E. tracheiphila* host interactions and plant pathogenesis are completely undescribed. To address this shortcoming, we sequenced and described the draft genome of a local field isolate of *E. tracheiphila* and compare it to other characterized strains to identify traits likely contributing to these differences.

The biology of *Erwinia* are historically defined as plant associated (Hauben *et al.* 1998) and many are rosaceous floral symbionts or pathogens. Many *Erwinia* spp. colonize floral stigmas and can be spread by rain. Epiphytic colonization of floral stigmas defines the life histories of the commensal *E. billingiae* and the infection dynamics of the fire blight pathogen *E. amylovora*. Unlike other pathogenic *Erwinia* spp., *E. tracheiphila* leaf transmission through insect herbivory is thought to be a major driver of epidemics (Smith 1914) (Rand 1915; Rand & Enlows 1916), at least until cucurbits flower in early summer (Shapiro *et al.*, in press). *Cucurbita* spp. flowers are only open for a single morning, so *E. tracheiphila* must quickly pass through the nectary without an epiphytic colonization stage (Sasu *et al.* 2010a; Sasu *et al.* 2010c). Similar to other characterized *Erwinia* spp., *E. tracheiphila* maintains epiphytic capabilities on *Cucumis* leaf surfaces under favorable environmental conditions (Rojas & Gleason 2011). However, unlike other pathogenic *Erwinia* spp., direct contact with a recent leaf wound appears to be required for infection to develop (Smith 1914; Brust 1997b).
While many *Erwinia* spp. have transient and non-specific associations with a variety of pollinators, which can transmit bacteria to many new flowers as they forage for pollen (Johnson & Stockwell 1998; Hildebrand *et al.* 2000), *E. tracheiphila* is obligately transmitted through infective frass by two species of cucumber beetle vectors (Coleoptera: Chrysomelidae: Luperini), with which it can maintain persistent, non-pathogenic associations in the beetle’s digestive tract (Shapiro *et al.*, in prep) (Garcia-Salazar *et al.* 2000b) and *E. tracheiphila* only overwinters in the digestive tract of diapausing adult cucumber beetle vectors and not in host plants (de Mackiewicz *et al.* 1998).

Control options of *E. tracheiphila* in agricultural settings is limited, and is currently achieved through environmentally and economically expensive chemical control of insect vectors (Fleischer *et al.* 1998) or the deployment of labor intensive row cover to prevent beetles from contacting plants (Rojas *et al.* 2011). Despite the importance of *E. tracheiphila* control to agricultural production of squashes, pumpkins, melons, cucumbers, and gourds, no investigations into the underlying genetic basis of pathogenesis to plants and persistence within insects have been conducted. To address this, we used 454 Titanium chemistry to sequence a strain of *E. tracheiphila* isolated from a wilt-infected wild gourd (*Cucurbita pepo* ssp. *texana*) grown at the Larson Agricultural Research Station at Rock Springs, PA. The draft genome was compared with genomes of the highly destructive fire blight pathogen *E. amylovora* and the commensal symbiont *E. billingiae*, and screened for laterally transferred genes.

Our analyses reveal that *E. tracheiphila* is the most distant relative to other sequenced *Erwinia* spp., and is more divergent from host-adapted pathogenic strains such as *E. amylovora* compared to epiphytic strains such as *E. billingiae*. *E. tracheiphila* also shows evidence of extensive chromosomal rearrangements and gene inactivations compared to other sequenced *Erwinia* spp., likely associated with an influx of transposable and extrachromosomal genetic material that constitutes a substantial percentage of the *E. tracheiphila* genome. Taken together,
this suggests *E. tracheiphila* is undergoing rapid evolution and may have experienced a recent host jump to cucurbit host plants and is likely in the process of niche specialization. The inclusion of the *E. tracheiphila* genome sequence substantially decreases the size of the *Erwinia* core genome and challenges the hypothesis of relative homogeneity within the genus (Kube et al. 2010a; Kamber et al. 2012). While important traits for metabolism are conserved with other *Erwinia* spp., preliminary lateral gene transfer analysis suggests the acquisition of many important genes that may contribute to differences in host plant and insect associations for *E. tracheiphila*.

**Results/Discussion**

**Phylogenetic Relationships**

A functional division of *Erwinia* spp. into either a commensal ‘epiphytic’ or a pathogenic ‘host-adapted’ group has been proposed. The ‘host-adapted’ group has been described to include *E. tasmaniensis*, *E. amylovora* and *E. pyrifoliae*, although *E. tasmaniensis* is an epiphyte with important loss of some virulence genes. *E. amylovora* and *E. pyrifoliae* are plant pathogens with smaller genomes (Kamber et al. 2012). Like *E. tracheiphila*, *E. amylovora* and *E. pyrifoliae* are necrogenic vascular pathogens that can replicate in xylem and induce characteristic wilting symptoms in their respective host plants through the production of an exopolysaccharide that occludes water movement (Huang & Goodman 1976) (Suhayda & Goodman 1980). The ‘epiphytic’ group, which includes *E. billingiae*, is currently defined by a relatively broad host range as a non-pathogenic floral symbiont of Rosaceous crop and ornamental trees with largely undescribed ecology (Mergaert et al. 1999).

To understand the phylogenetic relationship of *E. tracheiphila* to other *Erwinia* strains and enterobacterial plant pathogens, concatenated ribosomal proteins from sequenced *Erwinia*
spp., sequenced strains from the plant pathogenic genera *Pantoea*, *Dickeya*, and *Pectobacterium* (which were reclassified from *Erwinia* (Hauben et al. 1998)), and important animal associated enteric pathogens were used to create a phylogenetic tree. The tree shows support for the derivation of *E. tracheiphila* from a basal *Erwinia* strain, which does not cluster with other host-adapted *Erwinia* strains. There is strong bootstrap support for *Pantoea* and *Erwinia* as a monophyletic clade. Plant pathogenicity has arisen independently at least twice within Enterobacteriaceae (for the *Pantoea/Erwinia* clade and the ‘soft-rot’ *Dickeya/Pectobacterium* clade). The ‘soft-rot’ clade is defined by the profuse production of pectinolytic enzymes for extensive plant cell wall degradation abilities not described in characterized *Erwinia* spp., which are mostly epiphytes or vascular pathogens (Figure 5.1).
Figure 5-1 Phylogenetic relationships inferred from the concatenated ribosomal sequences of the sequenced *Erwinia* ssp. and other important enterobacterial plant and animal pathogens

Sequenced *Erwinia* strains form a monophyletic clade, with *E. tracheiphila* related to the last common *Erwinia* ancestor and not the host – adapted pathogenic strains. Recently reclassified soft – rot phytopathogens *Pectobacterium/Dickeya* also form a monophyletic clade, suggesting plant pathogenicity arose independently at least twice within enterobacteriaceae. Concatenated sequences were aligned in MEGA with ClustalW, and bootstrap analysis was performed in RaxML on the CIPRES server with the JTT substitution model and 100 bootstrap replicates.
Overall Features and comparisons

The *E. tracheiphila* draft genome size is 4.7 MB, approximately the same size as the commensal symbiont *E. billingiae* (5.1MB) (Table 5.1). BLASTP-based sequence similarity comparisons generated with EDGAR (Blom et al., 2009) show *E. tracheiphila* shares more gene families with the floral symbiont *E. billingiae* than *E. amylovora* (Figure 5.2). The core *Erwinia* genome of these three species is much smaller (1,739) than previously hypothesized when only rosaceous floral genomes *E. billingiae*, *E. tasmaneinsis*, and *E. pyrifoliae* were compared (2,241) (Kube et al. 2010a). *E. tracheiphila* shares fewer homologous genes with *E. billingiae* (1,970) or *E. amylovora* (1,832) than *E. billingiae* and *E. amylovora* share with each other (2,291).

Of the 4,207 total *E. tracheiphila* ORFs (Table 5.1, Figure 5.3), only 63% are predicted to encode functional proteins, a much lower percentage than *E. amylovora* (85%) or *E. billingiae* (87%). *E. tracheiphila* has 805 CDS annotated as putative pseudogenes, defined here as either containing at least one internal stop codon, or a truncated CDS with 40% or more AA length deletions compared to the closest Blastp homolog. The published genome sequences of *E. billingiae* calls only 13 pseudogenes and 2 fragments (Kube et al. 2010b), but more pseudogenes were identified by searching the intergenic regions of both genomes (Table 5.1) (Pati et al. 2010). The average nucleotide identity (ANI) (Konstantinidis & Tiedje 2005) of the shared functional genes is approximately the same (76-78%; Table 5.2a) in the pairwise genome comparisons of sequenced *Erwinia* spp., while *E. tracheiphila* pseudogenes show lower ANI, suggesting that the latter genes are actively decaying (Table 5.2b).
Table 5-1. General characteristics in the genomes of *E. tracheiphila*, *E. billingiae*, and *E. amylovora*

<table>
<thead>
<tr>
<th></th>
<th><em>E. tracheiphila</em></th>
<th><em>E. amylovora</em> CFBP 1430</th>
<th><em>E. billingiae</em> Eb661</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (bp)</td>
<td>4,717,279</td>
<td>3,805,573</td>
<td>5,100,167</td>
</tr>
<tr>
<td>Sequencing coverage</td>
<td>60-80X</td>
<td>40X</td>
<td>19X</td>
</tr>
<tr>
<td>G+C content (%)</td>
<td>51.5</td>
<td>54.69</td>
<td>56.43</td>
</tr>
<tr>
<td>Coding Sequences</td>
<td>4207</td>
<td>3706</td>
<td>4596</td>
</tr>
<tr>
<td>Coding density (%)</td>
<td>63</td>
<td>85.4</td>
<td>87.7</td>
</tr>
<tr>
<td>Average size (bp)</td>
<td>830</td>
<td>866</td>
<td>966</td>
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<tr>
<td>G+C content (%)</td>
<td>51.63</td>
<td>53.6</td>
<td>56.4</td>
</tr>
<tr>
<td>Assigned function</td>
<td>3339</td>
<td>2822</td>
<td>3768</td>
</tr>
<tr>
<td>Conserved uncharacterized</td>
<td>868</td>
<td>884</td>
<td>515</td>
</tr>
<tr>
<td>Phage related</td>
<td>393</td>
<td>194</td>
<td>321</td>
</tr>
<tr>
<td>Pseudogenes</td>
<td>785</td>
<td>115</td>
<td>82</td>
</tr>
<tr>
<td>Frame shift</td>
<td>533</td>
<td>89</td>
<td>54</td>
</tr>
<tr>
<td>Fragments</td>
<td>252</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>rRNA genes</td>
<td>30</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>tRNAs</td>
<td>68</td>
<td>77</td>
<td>77</td>
</tr>
<tr>
<td>Transposases/Integrases</td>
<td>348</td>
<td>25</td>
<td>14</td>
</tr>
</tbody>
</table>
Figure 5-2 VENN diagram generated using EDGAR to compare the number of shared and unique genes between *E. tracheiphila*, *E. amylovora*, and *E. billingiae*. 
<table>
<thead>
<tr>
<th></th>
<th>ANI All Genes</th>
<th>ANI Pseudogenes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. amylovora</em></td>
<td>76.4</td>
<td>72.7</td>
</tr>
<tr>
<td><em>E. billingiae</em></td>
<td>77.9</td>
<td>73.5</td>
</tr>
<tr>
<td><em>E. pyrifoliae</em></td>
<td>76.6</td>
<td>75.9</td>
</tr>
<tr>
<td><em>E. tasmaniensis</em></td>
<td>76.5</td>
<td>72.4</td>
</tr>
</tbody>
</table>

**Table 5-2.** Average nucleotide identities (ANI) of all ORFs and pseudogenes

ANI compared between all *E. tracheiphila* ORFs and other sequenced *Erwinia* spp. shows that shared gene families *E. tracheiphila* are equally divergent from other sequenced *Erwinia* spp. ANI comparison of just *E. tracheiphila* pseudogenes with the ORFs of other sequenced *Erwinia* spp. shows lower identity, suggesting that pseudogenization was recent and pseudogenes are actively decaying.

<table>
<thead>
<tr>
<th></th>
<th><em>E. billingiae</em></th>
<th><em>E. pyrifoliae</em></th>
<th><em>E. tasmaniensis</em></th>
<th><em>E. tracheiphila</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. amylovora</em></td>
<td>78.51</td>
<td>91.37</td>
<td>85.72</td>
<td>76.30</td>
</tr>
<tr>
<td><em>E. billingiae</em></td>
<td>78.67</td>
<td>78.43</td>
<td>77.70</td>
<td></td>
</tr>
<tr>
<td><em>E. pyrifoliae</em></td>
<td></td>
<td>86.58</td>
<td>76.65</td>
<td></td>
</tr>
<tr>
<td><em>E. tasmaniensis</em></td>
<td></td>
<td></td>
<td></td>
<td>76.39</td>
</tr>
</tbody>
</table>

**Table 5-3.** Average nucleotide identities between sequenced *Erwinia* spp.

ANI comparisons of all ORFs between all sequenced *Erwinia* spp. supports the phylogenetic relationships suggested by the ribosomal alignment. *E. amylovora* and *E. pyrifoliae* are most similar. Of the ‘epiphytic’ species, *E. tasmaniensis* has highest identity to the ‘host-adapted’ strains. *E. tracheiphila* has only slightly higher ANI to *E. billingiae* compared to the other strains.
Decay in the genome of *E. tracheiphila* is mostly occurring in the accessory genome comprising transposable elements/phage or conserved uncharacterized or genes assigned general function only, and not within genes for essential metabolic or biosynthetic pathways (Figure 5.4). In *E. tracheiphila*, the functional proportion of each COG category more closely resembles pathogenic *E. amylovora* than commensal *E. billingiae*. *E. tracheiphila* contains more genes for rRNA synthesis, including a number of HGT candidates. There are fewer genes for pyruvate metabolism, glycine/threonine-serine, arginine/proline, metabolism in *E. tracheiphila* compared to *E. amylovora* or *E. billingiae*, but pathways for carbohydrate metabolism, amino acid synthesis, cell envelope biosynthesis and cofactor metabolism (among others), all pathways found in many free-living enterobacterial are present and putatively functional. *E. billingiae* contains notably more genes for transport and metabolism of carbohydrates, amino acids, inorganic ions, and energy production and conversion, and moderately more genes for cell envelope biogenesis, than other sequenced *Erwinia* spp., likely related to an increased need for metabolic flexibility as an ephiphyte in a less predictable environment with unpredictable carbohydrate availability. Taken together, *E. tracheiphila* appears to be decaying towards a niche-adapted plant pathogenic lifestyle resembling the plant pathogen *E. amylovora*. Pseudogenization within some transcriptional regulators [ETR1632] and an AHL synthase [ETR3169] suggests gene function and regulation in *E. tracheiphila* may be significantly altered even for genes with known homologues, and further functional studies are necessary to understand regulatory changes that have occurred (Price et al. 2007).
Figure 5-3. Distribution of pseudogenes on the *E. tracheiphila* chromosome

Distribution of predicted coding sequences in *E. tracheiphila* and visualization of genome decay in all scaffolds of *E. tracheiphila*. Track 1 is leading strand; track 2 is lagging strand; track 3 (brown) is the distribution of pseudogenes and track 4 is the sizes and arrangement of scaffolds. Color assignments for tracks 1 and 2 correspond to COG functional groups: Green is surface associated, pink is phage or IS element, grey is metabolism, dark blue is stable RNA, red is DNA recombination and repair.
Figure 5-4. Comparisons of genome decay across orthologous groups between *E. amylovora*, *E. billingiae*, and *E. tracheiphila*

Most of the genome decay in *E. tracheiphila* is occurring in genes of general or unknown function, or among the insertion elements. Core biosynthetic and metabolic pathways appear to be largely conserved. *E. tracheiphila* is red; *E. billingiae* is blue; *E. amylovora* is orange and pseudogenes are grey.
Phage and Transposable Elements Proliferation

More than 421 million bases reads were generated between paired end and shotgun 454 sequencing (corresponding to 60-80X coverage), yet the chromosome assembled into 25 scaffolds (Table 5.4). The two largest scaffolds account for 1.82 MB and are predominantly composed of bacterial chromosomal DNA. Most scaffolds contain extrachromosomal elements, including regions of phage derived, insertion element, and plasmid conjugative transfer or other plasmid associated genes [ETR0098, or119a, ETR0104-ETR0115, ETR0174, ETR0504, ETR0797, ETR0907, ETR2349, ETR2604, ETR2781-2782, ETR3020, or3212b, ETR3332-ETR3333, ETR3465, ETR3473-ETR3476, ETR3564, ETR3981].

Table 5.4. *E. tracheiphila* scaffold sizes and important features

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Size (nt)</th>
<th>Coverage</th>
<th>Notable Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,160,218</td>
<td>60-80X</td>
<td>Genomic DNA integrated phage proteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Flagellar biosynthesis clusters</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EPS biosynthetic cluster</td>
</tr>
<tr>
<td>2</td>
<td>665,612</td>
<td>60-80X</td>
<td>31 rRNA genes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Putative plasmid replication-associated protein</td>
</tr>
<tr>
<td>3</td>
<td>530,285</td>
<td>60-80X</td>
<td><em>Inv/Spa</em> T3SS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Hrp</em> T3SS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>T6SS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Genomic DNA integrated phage</td>
</tr>
<tr>
<td>4</td>
<td>462,842</td>
<td>60-80X</td>
<td>Genomic DNA integrated phage proteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Putative plasmid proteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Inv/Spa</em> T3SS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rhamnogalacturonase</td>
</tr>
<tr>
<td>5</td>
<td>222,389</td>
<td>60-80X</td>
<td>Genomic DNA integrated phage genes</td>
</tr>
<tr>
<td>6</td>
<td>229,276</td>
<td>60-80X</td>
<td>Genomic DNA; Some integrated phage CDS</td>
</tr>
<tr>
<td>7</td>
<td>244,940</td>
<td>60-80X</td>
<td>Genomic DNA integrated phage</td>
</tr>
<tr>
<td>8</td>
<td>240,771</td>
<td>60-80X</td>
<td>Genomic DNA</td>
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<td></td>
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<td>PsiA/PsiB plasmid genes</td>
</tr>
<tr>
<td>9</td>
<td>217,201</td>
<td>60-80X</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>10</td>
<td>131,409</td>
<td>60-80X</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>11</td>
<td>93,198</td>
<td>60-80X</td>
<td>Genomic DNA integrated phage</td>
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<td></td>
<td></td>
<td>Putative plasmid transfer protein</td>
</tr>
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<td>12</td>
<td>78,767</td>
<td>60-80X</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>13</td>
<td>84,878</td>
<td>60-80X</td>
<td>Many phage genes</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Putative plasmid stabilization protein</td>
</tr>
<tr>
<td></td>
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<tr>
<td>----</td>
<td>-----</td>
<td>-------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>14</td>
<td>84,351</td>
<td>60-80X</td>
<td>Fructose PTS operon adjacent to phage and plasmid related proteins</td>
</tr>
<tr>
<td>15</td>
<td>67,034</td>
<td>60-80X</td>
<td>Expansin, endoglucanase Intact mannitol PTS operon Degraded plasmid replication protein</td>
</tr>
<tr>
<td>16</td>
<td>57,653</td>
<td>60-80X</td>
<td>Genomic DNA</td>
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<td>17</td>
<td>46,611</td>
<td>60-80X</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>18</td>
<td>37,446</td>
<td>60-80X</td>
<td>Plasmid conjugative genes</td>
</tr>
<tr>
<td>19</td>
<td>22,957</td>
<td>200-300X</td>
<td>Phage</td>
</tr>
<tr>
<td>20</td>
<td>7,974</td>
<td>60-80X</td>
<td>Type VI Vgr-family protein</td>
</tr>
<tr>
<td>21</td>
<td>6,473</td>
<td>60-80X</td>
<td>Hypothetical Proteins</td>
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<td>Phage</td>
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<td>23</td>
<td>4,005</td>
<td>60-80X</td>
<td>Mostly Hypothetical Proteins</td>
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<td>24</td>
<td>2,714</td>
<td>60-80X</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>25</td>
<td>2,669</td>
<td>60-80X</td>
<td>Genomic DNA</td>
</tr>
</tbody>
</table>

Scaffolds 19 and 22 are comprised of enterobacterial phage genes, and the contigs that make up these two scaffolds are present in significantly higher coverage (200-300X) compared to the other chromosomal scaffolds (60-80X), indicating that *E. tracheiphila* appears to be co-infected by phages that may be replicating in culture (Table 5.4). Many other scaffolds contain chromosomally integrated phage remnants. In total, 393 CDS (9.7% of the *E. tracheiphila* genome) are also present in the phage pan-genome (Table 5.5). The largest number of phage genes are from general enterobacterial phages, and there are many genes from phages known to infect hosts of putative laterally transferred genes, such as *Pseudomonas* and *Sodalis* phages.

Clustered regularly interspaced short palindromic repeats (CRISPR) associated sequence proteins (*cas* genes) are prokaryotic RNAi-based defenses. *E. amylovora* CFBP 1430 has eight genes homologous to other described *cas* genes (Rezzonico *et al.* 2011), while *E. billingiae* has four putative CRISPR regions (Kube *et al.* 2010b). In contrast, *E. tracheiphila* has no detected CRISPR regions (Grissa *et al.* 2007). Absence of CRISPR repeats and *cas* genes may explain the presence of two putatively replicating phages and the abundance of genes that are likely of phage origin.
Table 5-5. Distribution of phage genes present in *E. tracheiphila* among phages with at least 10 genes

<table>
<thead>
<tr>
<th>Phage Host</th>
<th>Gene Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacteria phage</td>
<td>140</td>
</tr>
<tr>
<td>Burkholderia phage</td>
<td>102</td>
</tr>
<tr>
<td>Pseudomonas phage</td>
<td>99</td>
</tr>
<tr>
<td>Escherichia phage</td>
<td>66</td>
</tr>
<tr>
<td>Salmonella phage</td>
<td>60</td>
</tr>
<tr>
<td>Shigella phage</td>
<td>31</td>
</tr>
<tr>
<td>Bacillus phage</td>
<td>31</td>
</tr>
<tr>
<td>Vibrio phage</td>
<td>27</td>
</tr>
<tr>
<td>Clostridium phage</td>
<td>26</td>
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<tr>
<td>Yersinia phage</td>
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</tr>
<tr>
<td>Phage Gifsy-1</td>
<td>22</td>
</tr>
<tr>
<td>Erwinia phage</td>
<td>21</td>
</tr>
<tr>
<td>Megavirus chilensis</td>
<td>21</td>
</tr>
<tr>
<td>Synechococcus phage</td>
<td>18</td>
</tr>
<tr>
<td>Acanthamoeba polyphaga mimivirus</td>
<td>18</td>
</tr>
<tr>
<td>Ralstonia phage</td>
<td>17</td>
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<tr>
<td>Haemophilus phage</td>
<td>17</td>
</tr>
<tr>
<td>Aeromonas phage</td>
<td>16</td>
</tr>
<tr>
<td>Phage Gifsy-2</td>
<td>16</td>
</tr>
<tr>
<td>Ostreococcus tauri virus</td>
<td>16</td>
</tr>
<tr>
<td>Sodalis phage</td>
<td>15</td>
</tr>
<tr>
<td>Cafeteria roenbergensis virus</td>
<td>15</td>
</tr>
<tr>
<td>Klebsiella phage</td>
<td>15</td>
</tr>
<tr>
<td>Endosymbiont phage</td>
<td>15</td>
</tr>
<tr>
<td>Planktothrix phage</td>
<td>14</td>
</tr>
<tr>
<td>Stx2-converting phage</td>
<td>14</td>
</tr>
<tr>
<td>Prochlorococcus phage</td>
<td>14</td>
</tr>
<tr>
<td>Mannheimia phage</td>
<td>13</td>
</tr>
<tr>
<td>Micromonas sp. RCC1109 virus</td>
<td>13</td>
</tr>
<tr>
<td>Staphylococcus phage</td>
<td>12</td>
</tr>
<tr>
<td>Burkholderia ambifaria phage</td>
<td>12</td>
</tr>
<tr>
<td>Lactococcus phage</td>
<td>12</td>
</tr>
<tr>
<td>Paramecium bursaria Chlorella virus</td>
<td>12</td>
</tr>
<tr>
<td>Mycobacterium phage</td>
<td>12</td>
</tr>
<tr>
<td>Ostreococcus lucimarinus virus</td>
<td>12</td>
</tr>
<tr>
<td>Bacteriophage APSE-2</td>
<td>11</td>
</tr>
</tbody>
</table>
In the *E. tracheiphila* genome, there are 348 non-phage insertion/transposable elements from 28 families. IS200, which is usually reluctant to transpose, has the highest abundance with 37 copies (Table 5.6) (Beuzón *et al.* 2004), and the mutator-type transposase best known as a maize IS element is also present in high abundance (Lisch *et al.* 2001). There are an additional 17 IS element families with 10 or fewer copies in the *E. tracheiphila* genome and 90 putative IS elements without pfam family assignments.

**Table 5-6.** Abundances and rates of decay of insertion sequenes and transposable elements in the *E. tracheiphila* genome

<table>
<thead>
<tr>
<th>Transposable Element Family</th>
<th>Pseudon</th>
<th>Total</th>
<th>% Inactivated</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS200</td>
<td>13</td>
<td>37</td>
<td>35.14%</td>
</tr>
<tr>
<td>Integrase, Catalytic Core</td>
<td>14</td>
<td>30</td>
<td>46.67%</td>
</tr>
<tr>
<td>Mutator Type</td>
<td>14</td>
<td>28</td>
<td>50.00%</td>
</tr>
<tr>
<td>IS240</td>
<td>13</td>
<td>18</td>
<td>72.22%</td>
</tr>
<tr>
<td>IS4/IS5</td>
<td>11</td>
<td>16</td>
<td>68.75%</td>
</tr>
<tr>
<td>IS1_DDE_Tnp_IS1</td>
<td>1</td>
<td>14</td>
<td>7.14%</td>
</tr>
<tr>
<td>Ribonuclease H-like</td>
<td>7</td>
<td>13</td>
<td>53.85%</td>
</tr>
<tr>
<td>Phage Integrase</td>
<td>3</td>
<td>13</td>
<td>23.08%</td>
</tr>
<tr>
<td>Resolvase</td>
<td>2</td>
<td>11</td>
<td>18.18%</td>
</tr>
<tr>
<td>Tn3_DDE_Tnp</td>
<td>7</td>
<td>10</td>
<td>70.00%</td>
</tr>
<tr>
<td>Tnp_IS801/1294</td>
<td>5</td>
<td>10</td>
<td>50.00%</td>
</tr>
<tr>
<td>Tnp_IS3/IS911</td>
<td>1</td>
<td>9</td>
<td>11.11%</td>
</tr>
<tr>
<td>IS1_InsA</td>
<td>1</td>
<td>8</td>
<td>12.50%</td>
</tr>
<tr>
<td>YghA-like Tnp</td>
<td>5</td>
<td>7</td>
<td>71.43%</td>
</tr>
<tr>
<td>IS204_IS1001_IS1096</td>
<td>3</td>
<td>4</td>
<td>75.00%</td>
</tr>
<tr>
<td>IS111A_IS1328_IS1533</td>
<td>1</td>
<td>4</td>
<td>25.00%</td>
</tr>
<tr>
<td>Tnp_IS116/IS110/JS02</td>
<td>2</td>
<td>4</td>
<td>50.00%</td>
</tr>
</tbody>
</table>
Genomic Rearrangements

Whole genome alignments between the plant-associated *Erwinia* spp. (both pathogenic and non-pathogenic) sequenced to date show that there are small-scale genomic rearrangements between species, but all previously sequenced species share large collinear blocks and gene content is highly conserved (Smits et al. 2010a) (Kube et al. 2010b) (Smits et al. 2010c). *E. billingiae* contains only 8 annotated IS elements and *E. amylovora* contains only 10, and these genomes are highly collinear with few chromosomal rearrangements compared to each other (Figure 5.5). In contrast, the *E. tracheiphila* genome has undergone extensive genomic recombination compared to *E. billingiae*, likely associated with bacteriophage infection and the proliferation of insertion sequences.

<table>
<thead>
<tr>
<th>Homeodomain</th>
<th>2</th>
<th>4</th>
<th>50.00%</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS891/IS1136/IS1341</td>
<td>3</td>
<td>3</td>
<td>100.00%</td>
</tr>
<tr>
<td>IS66</td>
<td>1</td>
<td>3</td>
<td>33.33%</td>
</tr>
<tr>
<td>Tnp_IS4</td>
<td>2</td>
<td>2</td>
<td>100.00%</td>
</tr>
<tr>
<td>DDE_3</td>
<td>1</td>
<td>2</td>
<td>50.00%</td>
</tr>
<tr>
<td>HTH_21</td>
<td>2</td>
<td>2</td>
<td>100.00%</td>
</tr>
<tr>
<td>rve_2</td>
<td>1</td>
<td>2</td>
<td>50.00%</td>
</tr>
<tr>
<td>HTH_29</td>
<td>1</td>
<td>1</td>
<td>100.00%</td>
</tr>
<tr>
<td>Tnp_1_2</td>
<td>0</td>
<td>1</td>
<td>0.00%</td>
</tr>
<tr>
<td>HTH_ISL3</td>
<td>1</td>
<td>1</td>
<td>100.00%</td>
</tr>
<tr>
<td>Integrase, SAM-like</td>
<td>1</td>
<td>1</td>
<td>100.00%</td>
</tr>
<tr>
<td>No Family Assignment</td>
<td>66</td>
<td>90</td>
<td>0.7333333333</td>
</tr>
<tr>
<td>Total</td>
<td>118</td>
<td>348</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.5. Chromosomal rearrangements between *E. tracheiphila*, *E. amylovora*, and *E. billingiae*

Comparisons of chromosomal inversions and rearrangements between *E. tracheiphila*, *E. amylovora*, and *E. billingiae* generated with DoubleACT and visualized in ACT. Red lines connect homologous regions in the same orientation while blue lines show homologous regions in inverted orientations. 5.3b Syntenic dot plot comparisons show *E. amylovora* and *E. billingiae* are
highly syntenic with several large chromosomal inversions, while *E. tracheiphila* is not syntenic with either *E. amylovora* or *E. billingiae*.

**Lateral Gene Acquisition**

*E. amylovora* is described as having a ‘closed’ pan-genome due to high sequence similarity among *E. amylovora* strains and presence of few detected gene acquisitions (Smits, Rezzonico, & Duffy, 2010, (Boucher & al 2003). In contrast, initial DarkHorse analysis of the *E. tracheiphila* genome (Podell & Gaasterland 2007) identified 301 protein-coding genes (7.4% of all protein coding genes as candidates for lateral gene transfer. The list of gene transfer candidates contains several genes with purported functions important for both plant pathogenicity and persistence in the beetle digestive tract (Supplemental Table 1), including p450 detoxification and secondary compound synthesis, plant colonization and pathogenicity factors, and a locus with genes for several chitin binding/degradation proteins. Further analysis of these transfer candidates revealed that many are flanked by transposable elements and/or show phylogenetic incongruence with the ribosomal protein tree, strengthening the hypothesis that these genes were acquired by *E. tracheiphila* through lateral transfer. In addition to genomic rearrangements, lateral gene transfers are a source of genetic novelty supporting niche adaptation (Mathee *et al.* 2008) or host jumps (Zhaxybayeva & Doolittle 2011). In the following sections we take an in-depth look into some of these genes and the adjacent genomic regions.
Hypersensitive Response and Pathogenicity (Hrp) T3SS

The Type III secretion system (T3SS) is an inducible, needle-like protein export apparatus found in many plant and animal bacterial pathogens (Hueck 1998) that injects effector proteins directly into host cell cytoplasm. Erwinia tracheiphila has an Hrp T3SS conserved with other pathogenic Erwinia and Pseudomonas spp. (Alfano et al. 2000) and composed of three transcriptional units (hrpC operon [ETR 3853 – ETR 3857], hrpA operon [ETR 3858 – ETR 3862], and hrpJ operon [ETR 3838 – ETR 3848]). Compared to E. amylovora, the core structural genes in the hrp/hrc secretion system, and the sigma factor HrPL that controls expression of all hrp/hrc genes in E. amylovora, are present and conserved with high AA identity in E. tracheiphila (McNally et al. 2011) (Figure 5.6, Table 5.7). The hrp T3SS was acquired laterally from Pseudomonas spp. before proliferation within Erwinia/Pantoea spp. (Naum et al. 2009) and has been eliminated from the epiphytic symbiont E. billingiae, which does not possess an hrp T3SS or any secreted effectors (Kube et al. 2010a).

Effectors

E. tracheiphila shows high divergence in both effector content and AA identity of conserved effectors compared to T3SS structural and regulatory genes (Figure 5.6, Table 5.7). The E. amylovora hrp region is bound by the Hrp effector and elicitor (HEE) and the Hrp associated enzymes (HAE) regions, which are required for full virulence (Oh et al. 2005). Overall, 4/9 secreted proteins from the HEE/HAE region in E. amylovora are absent in E. tracheiphila. In E. tracheiphila, the hrp-associated systemic virulence genes hsvABC were excised from the HAE region, but both hsvA [ETR3340] and hsvC [OR3380b] were found as pseudogenes degrading elsewhere on the chromosome. E. tasmaniensis, which contains the hrp T3SS but lacks the HAE region, is non-pathogenic (Kube et al. 2010b). Harpins are translocated proteins that induce a hypersensitive response on non-host plants and are factors determining disease development on host plants. In the HEE region, E. tracheiphila contains a partial homologue of the harpin HrpN.
[ETR 3863], which is an *E. amylovora* virulence determinant and proteins from fragments of this gene have been shown to induce symptoms (Barny 1995). However, an HrpW homologue is absent in *E. tracheiphila* although a degrading fragment of the hrpW specific chaperone [ETR 3866 ] remains (Figure 5.6).

In addition to HEE/HAE effectors, *E. amylovora* was previously the only other *Erwinia* ssp. known to contain singleton T3SS effector genes (Smits *et al.* 2010b). While the host-adapted pathogen *E. pyrifoliae* contains a functional *hrp* T3SS, the lack of singleton effectors is hypothesized to be a factor in the more restricted host range of this pathogen compared to *E. amylovora*. *E. tracheiphila* also contains several singleton effectors and chaperones without homologues in *E. amylovora*, and that are identified as HGT candidates from *Pseudomonas* spp. (Table 5.8). In *Pseudomonas* spp., effectors are often located near insertion sequence elements (Kim *et al.* 1998), and their importance for determining plant host range promotes both their transfer among phytopathogens and high divergence in AA identity (Ma *et al.* 2006). The functional characterization of individual and synergistic importance of HEE, HAE, and singleton effectors towards plant pathogenicity and disease development will be important for dissecting *E. tracheiphila* molecular pathology.
While the structural genes are the Hrp pilus are largely intact, 4 of the effectors essential for plant pathogenicity found in the *E. amylovora* T3SS regulon are absent in *E. tracheiphila*. The AA identity of the structural components and regulators ranges from 71-92.5%, while the AA identity of effectors ranges from 52.6-68.6%. While the harpin gene HrpW is absent, the specific chaperone remains, albeit with very low AA identity.
**Table 5-7.** Comparison of gene presence and amino acid similarity of different functional genes in the *h*rp/*hrc* regulon

Comparisons of T3SS AA identities for *h*rp pilus structural components, regulators, effectors, and chaperones was compared between *E. tracheiphila* and *E. amylovora* shows that regulators are most conserved, while effectors and their chaperones are more divergent.

<table>
<thead>
<tr>
<th>Function</th>
<th>Average AA Similarity</th>
<th>Number of <em>h</em>rp genes with functional homologues in <em>E. tracheiphila</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulators</td>
<td>83.1</td>
<td>3/3</td>
</tr>
<tr>
<td><em>Hrp</em> pilus structural components</td>
<td>72.4</td>
<td>24/24</td>
</tr>
<tr>
<td>Effectors</td>
<td>60.2</td>
<td>4/9</td>
</tr>
<tr>
<td>Chaperones</td>
<td>48.7</td>
<td>½</td>
</tr>
</tbody>
</table>

**Table 5-8.** Singleton *h*rp/*hrc* Type III Secretion System effectors that are likely horizontal gene transfer candidates from phytopathogenic *Pseudomonas* spp.

T3SS effectors and chaperones from plant pathogenic *Pseudomonas* spp. flagged by Darkhorse as candidates for HGT. In three cases, both a chaperone and effector appear to have been horizontally transferred from the same donor strain. Several singleton effectors without chaperones that appear to be functional are present. All of these effectors and chaperones in the table have highly disjoint phylogenetic distributions (data not shown) and homologues of these genes are not present in other *Erwinia* spp.

<table>
<thead>
<tr>
<th>query_id</th>
<th>Putative Functional Assignment</th>
<th>Annotated Function</th>
<th>Strain of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>or0597</td>
<td>Functional</td>
<td>Type III Effector HopV1</td>
<td><em>Pseudomonas savastanoi</em></td>
</tr>
<tr>
<td>or0598</td>
<td>Fragment</td>
<td>Type III Chaperone ShcV</td>
<td><em>Pseudomonas savastanoi</em></td>
</tr>
<tr>
<td>or4102</td>
<td>Pseudo</td>
<td>Type III Chaperone ShcF</td>
<td><em>Pseudomonas syringae</em></td>
</tr>
<tr>
<td>or4103</td>
<td>Functional</td>
<td>Avr protein</td>
<td><em>Pseudomonas syringae</em></td>
</tr>
<tr>
<td>or2699</td>
<td>Functional</td>
<td>Type III Chaperone ShcF</td>
<td><em>Pseudomonas syringae</em></td>
</tr>
<tr>
<td>or2970</td>
<td>Functional</td>
<td>Type III Effector Hopa1</td>
<td><em>Pseudomonas syringae</em></td>
</tr>
<tr>
<td>or2971</td>
<td>Functional</td>
<td>Type III Chaperone protein shca</td>
<td><em>Pseudomonas viridiflava</em></td>
</tr>
<tr>
<td>or1961</td>
<td>Functional</td>
<td>Type III Effector AvrB4-2</td>
<td><em>Pseudomonas syringae</em></td>
</tr>
</tbody>
</table>
Plant Cell Wall Degradation

No *Erwinia* spp. is described to induce soft rot symptoms on host plants characteristic of those caused by *Pectobacterium/Dickeya* spp. through profuse extracellular pectinase secretion. Characterized *Erwinia* spp. multiply in the apoplast and interact with hosts through T3SS effectors, while ‘soft-rot’ bacterial pathogens also secrete a profuse amount of plant cell wall degrading enzymes, sequentially degrading pectins and then cellulose (Liu *et al.* 2008). *E. tracheiphila* contains a number of plant structural carbohydrate degradative genes not present in other *Erwinia* spp., including several genes to degrade the most abundant pectin in plant cell walls, rhamnogalacturon (Cosgrove 2005), [ETR0494, ETR0742] (Glasner *et al.* 2008). Homologues of these genes in *Dickeya* and *Pectobacterium* spp. are virulence factors functioning in development of characteristic soft rot symptoms (Toth & Birch 2005).

In addition to the two rhamnogalacturon degrading genes identified above, *E. tracheiphila* contains a locus with an expansin [ETR 1276] (Kerff *et al.* 2008) and a cellulase [ETR 1277], and their physical proximity suggests they are likely co-regulated. Expansins are plant proteins involved in important physiological processes, including extension and rearrangements of plant cell walls, and pH dependent cell wall extension (Li *et al.* 2002). Recently, the crystal structure of an expansin homologue EXLX1 from *Bacillus subtilis* was found to be functional as cell-wall loosening factor allowing root colonization (Kerff *et al.* 2008) (Georgelis *et al.* 2011) and essential for movement through xylem and full virulence of *Pantoea stewartii* on sweet corn (Mohammadi *et al.* 2011). Expansins are not thought to exhibit enzymatic activity, but are implicated in loosening of pit membranes and bacterial movement through xylem
vessels through manipulation of cellulose polymers. The presence of a cellulase at the same locus suggests possible rearrangement of cellulose microfibrils so the proper cleavage site can be exposed for subsequent enzymatic degradation.

Phylogenetic analysis of the pectin and cellulose associated genes in *E. tracheiphila* reveals high support for horizontal acquisition of these genes from ‘soft-rot’ strains (Figure 5.7 and 5.8). Expression of genes for plant cell wall degradation enzymes by *E. tracheiphila* would make these carbohydrates available as a glucose source for *E. tracheiphila*, and might also contribute to palatability or digestability of wilting plants by beetle vectors, which prefer to consume leaves of symptomatic, wilting foliage on *E. tracheiphila* – infected plants compared to healthy foliage (Shapiro 2012, in press).

**Figure 5-7.** Schematic of a locus with two genes encoding proteins that manipulate plant cell walls that shows support for horizontal acquisition by *E. tracheiphila*

Two maximum likelihood trees with representative strains showing the phylogenetic distribution of the expansin and cellulose genes found in *E. tracheiphila*. This locus is flanked by IS elements, further supporting horizontal acquisition. Both expansin and cellulase are important for plant cell wall structural carbohydrate interactions and specific to plant-microbe or plant-nematode interactions. Current evidence suggests both genes have very limited phylogenetic distributions among gram-positive and gram-negative bacteria and some plant parasitic nematodes. Expansin tree was run with the WAG substitution model, and the cellulase tree was run with the LG substitution model.
Figure 5-8. Two pectin degradation genes present in *E. tracheiphila* that were likely acquired horizontally

Maximum likelihood phylogenetic trees of two singleton genes that degrade the most abundant pectin in cell walls, rhamnogalacturon, that are found in *E. tracheiphila* and all top Blastp hits. Both genes cluster with homologues from the ‘soft-rot’ pathogen clade and homologues are not present in the *Erwinia/Pantoea* clade. Many plants contain genes for similar proteins utilized in cell wall rearrangement and degradative processes. Both in Figure 5-8 trees are unrooted and use the LG substitution model.
P450

Cytochrome p450s (CYP) enzymes are ubiquitous and diverse in eukaryotic organisms. In contrast, many prokaryotes lack p450s, indicating p450 functions are not a central component of microbial metabolism. Little is known about the functional significance of p450 systems in bacteria, but many appear to detoxify terpenes and other xenobiotic compounds through oxidoreductive reactions (Nelson 2009). While more than 100 families of prokaryotic p450s have been described, the taxonomic distribution of these genes are most abundant in Rhizobia, Streptomyces, and other actinobacteria and α-proteobacteria, with very few identified in γ-proteobacteria (Munro & Lindsay 1996). A p450 region is present in E. tracheiphila [ETR 3367 – ETR3376] that displays a high level of synteny to a p450-containing region found on a symbiosis island of Mesorhizobium loti, a nitrogen-fixing, non-pathogenic symbiont of legumes (Figure 5.9). In both M. loti and E. tracheiphila, the p450 region is adjacent to a transposable element (Uchiumi et al. 2004). In addition to the p450 monoxygenases and hydroxylases, the locus contains two terpene synthases, suggesting the possible production of plant manipulative compounds (Tsavkelova et al. 2006) that may be induced at the same time p450 mediated detoxification occurs. Phylogenetic analysis of a representative p450 monoxygenase from the E. tracheiphila locus shows that the distribution of this p450 is mostly restricted to bacteria with soil reservoirs, where they are likely to encounter a diverse array of toxins (Lamb et al. 2003)(Figure 5.9).

Genes conferring toxin resistance or metabolism (Mathee et al. 2008) (including antibiotic resistance) are among the most commonly horizontally transferred traits because of the strong selective advantage to having these genes when toxins are present (de la Cruz & Davies 2000). Non-domesticated Cucurbita ssp. are chemically defended by a group of highly oxygenated tetracyclic triterpenes known as cucurbitacins (Halaweish 1993) (Tallamy et al. 2005). Cucurbitacins are detectable at 1ppb, bitter, and highly toxic to most mammalian and
insect herbivores (Rehm 2006), but can be detoxified and sequestered by the co-evolved, specialist cucumber beetle herbivores that transmit *E. tracheiphila* (Metcalf 1986; Smyth *et al.* 2002). The presence of toxic cucurbitacins may provide a selective advantage for *E. tracheiphila* to maintain metabolically costly p450 detoxification systems. p450 enzymes are induced in *Bradyrhizobium japonicum* under anaerobic conditions in the bacterioid (plant-associated) stage in legume root nodules (Uchiumi *et al.* 2004) but p450 deletion mutants do not appear to be impaired in symbiotic ability (Keister *et al.* 1999), so the full function of p450 in vivo, in different growth states, and in the environment remain to be described.
Comparison of the p450 regions and AA identity of individual genes between *E. tracheiphila* and *Mesorhizobium loti*. *E. tracheiphila* contains two cytochrome p450 and two p450 hydroxylase

**Figure 5-9.** Comparisons p450 loci in *E. tracheiphila* and *Mesorhizobium loti*.
genes homologous to genes in *M. loti*. In both genomes, the p450 regulon is adjacent to transposable elements 8b. Phylogenetic distribution constructed with a representative p450 monooxygenase strongly shows that p450s are largely absent in enterobacteriaceae, and supports lateral acquisition by *E. tracheiphila*.

**Chitin Association**

Chitin is β(1 -> 4) linked linear homopolymer of N-acetylglucosamine (GlcNAc) that is ubiquitous in aquatic and terrestrial systems (Meibom *et al.* 2004b), and a defining structural polysaccharide of insect exoskeletons, including the digestive tract (Cohen 2001). Several notable arthropod symbionts contain chitinases that promote colonization of the external exoskeleton (Meibom *et al.* 2004a) or digestive tract (Jones *et al.* 1986; Killiny *et al.* 2010), and utilization of chitin as a glucose source (Li & Roseman 2004; Killiny *et al.* 2010).

*E. tracheiphila* contains a locus that encodes three chitin-associated genes [ETR1568, OR1593a, OR1593b]. Phylogenetic analysis of the closest Blastp homologues for each gene and physical proximity to enterobacterial phage genes support the likelihood of lateral acquisition (Figure 5.10). One of the genes is a small fragment of a chitinase A gene with only a chitin-binding domain [ETR 3429] with the closest Blastp homologue found in *S. glossinidius*. However, given the location of this fragment, it is likely functional (Figure 5.10). Additionally, this locus is inserted within a flagellar assembly locus and immediately adjacent to a transcriptional regulator, suggesting chitin detection and degradation is likely co-regulated with expression/suppression of motility associated genes.

*E. amylovora* can adhere to the exoskeleton of insect vectors for up to seven days (Smits *et al.* 2010c), and contains one hexosaminidase with a homologue in *E. tracheiphila* [ETR1196] and one novel chitin associated gene unrelated to genes in the *E. tracheiphila* chitinase locus. However, long-term bacterial growth and persistence either within the digestive tract or on the exoskeleton of insect vectors has not been shown in several studies investigating insect interactions with *E. amylovora* and *E. billingiae* (Johnson & Stockwell 1998; Hildebrand *et al.*
While the genetic basis of insect persistence in *E. tracheiphila* has not been functionally characterized, the presence of this locus containing several novel chitin associated genes is likely a contributing factor.

**Figure 5-10.** Schematic of *E. tracheiphila* chitin associated locus and phylogenetic support for horizontal acquisition

A locus likely conferring enhanced chitin association of the two full length chitin associated genes. The first gene has several binding domains, the second is a fragment compared to the closest homologue but has an intact chitin-binding domain, and the third is a GH18 family chitin degradation gene. This locus is flanked by enterobacterial phage genes and inserted adjacent to a transcriptional regulator and a flagellar synthesis cluster.
General Characteristics

Motility

Flagellar movement allows bacterial dispersal from low quality or nutrient poor environments towards better quality environments, and flagella-mediated swarming motility is important for virulence of many plant and animal pathogenic bacteria, including *E. amylovora* (Bayot & Ries 1986). *E. tracheiphila* contains two complete flagellar genes sets that are highly conserved in other *Erwinia* spp. The first set is scattered in several clusters [ETR 1002-ETR1016; ETR 1040-1043, ETR 2294 – ETR 2297 ], while the second set is located in a single cluster [ETR 1547 – 1560; ETR 1570- ETR 1583]. *E. tracheiphila* contains an additional sigma factor and two flagellar genes [ETR 2967-ETR2968] homologous to *Yersinia* and *Escherichia* spp.

Flagella are the structural ancestor of T3SS, and in *E. amylovora* the T3SS positive regulator sigma factor HrpL is also a negative regulator of motility (Cesbron *et al.* 2006), suggesting a close functional and regulatory link between motility and virulence that is largely unexplored in proteobacteria. This link is unexplored in *E. tracheiphila*, and HrpL is absent in *E. billingiae*, so the expression, regulation, and ecological consequences of flagella-mediated movement is not well understood among these bacteria. Chemotaxis associated genes are located throughout the *E. tracheiphila* genome, and many are located in close proximity to motility clusters, such as flagella. Some are of phage origin or appear to be pseudogenes, suggesting HGT and gene inactivation influence *E. tracheiphila* responses to environmental stimuli and chemical cues [ETR 0611] [ETR 0816] [ETR1005-1010] [ETR 1128][ETR1390][ETR1469-1471][ETR1562][ETR1780-1781][ETR1803][ETR2954] [ETR 3176][ETR3191-3192][ETR 3651][ETR3883]
Additional Secretion Systems

Type II SS

The Type II secretion system is the general secretory pathway and recognizes proteins with specific signal sequences. The *E. tracheiphila* T2SS [ETR 2799 – ETR 2810] is adjacent to transposable elements upstream and downstream, and all proteins show highest sequence identity (80-90%) to *Pantoea stewartii*, a corn wilt pathogen which can also persistently colonize its flea beetle vector (Stavrinides *et al.* 2009b) (Menelas *et al.* 2006). Many of the genes in this locus have low identity (20-30%) with other *Erwinia* spp.

Inv/Spa T3SS

The *inv/spa* T3SS secretion system is a cell invasion and pathogenicity factor towards mammalian hosts characterized mainly from enteroinvasive *Salmonella* spp. (Shea *et al.* 1996), where the *inv/spa* T3SS is located on pathogenicity island 1 (PAI-1) and functions in initial cell attachment and invasion. While less well characterized outside of *Salmonella* spp., an *inv/spa* T3SS was shown to be essential for persistent gut colonization of flea beetle vectors (Coleoptera: Chrysomelidae: Galerucinae) by the corn wilt pathogen *P. stewartii* (Correa *et al.* 2012) and intracellular invasion of the tsetse fly digestive tract by *Sodalis glossinidius* str. *morsitans* (Dale *et al.* 2001) (Dale *et al.* 2002). *E. tracheiphila* [ETR 0461 – ETR 0480] contains one *Inv/Spa* T3SS conserved among *Erwinia* spp. None of the *Erwinia* spp. with an *inv/spa* T3SS are known to have intracellular associations with insects, and current evidence suggests the *E. tracheiphila* association with insects is extracellular (Garcia-Salazar *et al.* 2000a). Additionally, the *inv/spa* region was not shown to affect *E. amylovora* pathogenicity on apple (Zhao *et al.* 2009), so the function of these systems and reasons for continued chromosomal maintenance in plant associated *Erwinia* spp. are unknown.
Type VI SS

*E. tracheiphila* contains two T6SS gene clusters, only one of which is homologous to a T6SS in other *Erwinia* spp. [~ETR 3902 – ETR 3922]. The second shows strong support for lateral acquisition [ETR 0414 - ETR 0426, ETR 0432 – ETR 0434] and contains an internal fimbrial cluster [ETR 0427-ETR0421]. A singleton VgrG effector also shows some support for horizontal acquisition [ETR0003].

Type VI secretion systems show structural similarities to bacteriophage tail proteins (Pell *et al.* 2009) and are receiving increasing research attention as important virulence factor widespread among gram-negative bacteria (Silverman *et al.* 2012). T6SS are almost completely uncharacterized in plant pathogenic bacteria, but in *P. carotovorum*, T6SS hcp effectors appear to be plant virulence determinants (Mattinen *et al.* 2007). In addition to virulence determinants, T6SS effectors can also mediate contact dependent inhibition (CDI) to suppress growth of other co-occurring bacterial strains. For *E. tracheiphila*, this function would be useful for colonization of the beetle gut if many other gut bacteria are present, and possibly within infected plants to prevent other bacteria from using degraded plant cell wall polysaccharides as glucose sources.

Surface Colonization Structures

*E. tracheiphila* contains several divergent surface attachment structures, suggesting that *E. tracheiphila* requires different host adhesion factors than *E. amylovora* and *E. billingiae*, and some of these factors were likely acquired through HGT. Fimbriae are a diverse family of structures in gram-negative bacteria that promote adhesion to surfaces and are important components of multi-cell aggregative behavior. Fimbriae are utilized by *E. amylovora* for attachment to xylem vessels and biofilm formation *in planta* (Koczán *et al.* 2011) and many other enterobacteria to adhere to host digestive tract (Griffin *et al.*, 2012) (Korea, Badouraly, Prevost, Ghigo, & Beloin, 2010). While *E. tracheiphila* contains one conserved fimbrial locus [ETR 3622-ETR 3628], there are multiple fimbrial genes in *E. tracheiphila* that do not have homologues in
other sequenced erwinias [ETR 0427-0431]. A second set of fimbrial genes [ETR 2937-2940] [ETR 1823- ETR 1826, ETR 2849] have homologues in other plant and insect associated pathogenic bacteria. Similarly, Type IV pili are essential for initial attachment to xylem colonization and cell detachment from mature biofilms in E. amylovora (Koczan et al. 2011). In addition to a type IV pili locus conserved among erwinias [ETR 3818- ETR 3820, ETR 2450, ETR 3301-3305, ETR 3553] E. tracheiphila contains a singleton type IV pili gene [ETR 2613] with a homologue in Serratia spp., and an adhesin likely acquired by HGT from a Vibrio strain.

**Exopolysaccharide synthesis**

Capsular polysaccharide protect bacteria against environmental stresses, desiccation, plant resistance factors (Koczan et al. 2009) (Bellemann et al. 1994). Secretion of amylovoran and levan, two Erwinia amylovora exopolysaccharides, are an essential plant pathogenicity factor (Bellemann & Geider 1992), and these exopolysaccharides can induce wilting symptoms when inoculated as cell-free isolate in host plants (Goodman et al. 1974) (Zhao et al. 2005) (Koczan et al. 2009). E. tracheiphila contains an EPS region [ETR 1684 – ETR 1878] showing largely conserved gene synteny and AA identity to the EPS region of E. billingiae (Figure 5.11). While no studies have described the structure or functionally characterized the E. tracheiphila EPS, detection of slime in xylem is considered diagnostic of E. tracheiphila infection in the field, and it is reasonable to assume EPS functions similarly in E. tracheiphila as in E. amylovora to block xylem sap flow and induce wilting symptoms. Capsular polysaccharide EPS is important for other enterobacteriaceae during mammalian gut colonization (Favre-Bonté et al. 1999) (Sonnenburg et al. 2004), but the function of and induction of EPS production in colony formation and persistence in the cucumber beetle digestive tract is not known. Many insect-associated bacteria are contained in specialized structures in an insect’s digestive tract (Shigenobu et al. 2000). However, no such structure has been observed in the striped cucumber beetle digestive tract, and
bacteria assumed to be *E. tracheiphila* have been observed attached to the beetle’s hindgut by a glycocalyx (Garcia-Salazar *et al.* 2000a).

**Capsular Polysaccharide**

<p>| | | | | | | | |</p>
<table>
<thead>
<tr>
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</tbody>
</table>

**Figure 5-11.** Comparisons capsular exopolysaccharide operon in *E. tracheiphila, E. billingiae, and E. amylovora*

**Conclusions**

*Erwinia tracheiphila* is the most important pathogen of cultivated cucurbits in the Northeast and Midwest, yet was completely undescribed at the genetic level. The vast majority of research attention has focused on the economically important *Erwinia* spp. that are associated with rosaceous hosts (apples, pears, quince) as either pathogens or epiphytic biocontrol agents. The agricultural genetic stock of these crop plants is fairly restricted, possibly influencing the
relative homogeneity of the sequenced, rosaceous-associated *Erwinia* strains and leading to the hypotheses that the overall genetic diversity within the genus is relatively low compared to other bacterial genera. The addition of the *E. tracheiphila* genome, which infects different plant hosts and can persistently colonize insect vectors, suggests undiscussed genetic and biological diversity within the genus.

While the draft genome size of *E. tracheiphila* is 4.7MB and within the range of many free-living enterobacteria, it is more similar to ‘epiphytic’ than ‘host-adapted’ *Erwinia* spp. Further, pseudogenization and associated low CDS density suggest *E. tracheiphila* is actively undergoing genome decay, which is a widely recognized part of microbial host adaptation. *E. tracheiphila* is derived from a basal *Erwinia* strain (Figure 5.1), and does not cluster phylogenetically with other host-adapted, plant pathogenic strains in the genus, which comprise a more recently derived clade. Since bacteria are under strong deletion bias to get rid of nonfunctional DNA burden quickly (Andersson & Andersson 2001; Tamas *et al.* 2002; Kuo & Ochman 2010), observed presence of large number of pseudogenes suggests *E. tracheiphila*’s recent colonization of a new niche (McCutcheon & Moran, 2012).

In addition to extensive decay in the accessory genome, *E. tracheiphila* has undergone genome expansion and acquired a large number of genes through HGT, which is not often observed for highly host-adapted pathogens. *E. tracheiphila* occurs in two environments conducive to horizontal gene transfer (insect digestive tract and leaf surface/vasculature), and the genome contains a notably large number of genes from mobile genetic elements, which are often agents of host gene transfer. *E. tracheiphila* contains a number of genes that likely influence plant virulence and pathogenicity, including an expansin, cellulase, pectinases, T3SS effectors, and a p450 detoxification locus, showing strong phylogenetic support for lateral acquisition. In contrast, relatively fewer HGT candidate genes could be classified as likely important in persistent insect interactions, with a locus containing several chitin-associated genes the notable exception. Many
of the HGT candidates have homologues in other enterobacteria. HGT between closely related bacteria is thought to be very common, although more difficult to confirm. The presence of enterobacterial phages and IS elements could provide a mechanism by which these genes were transferred into the *E. tracheiphila* genome.

The proliferation of insertion sequences and replicating phage have likely contributed to extensive chromosomal rearrangements and fragmentation compared to other characterized *Erwinia* strains. Taken together, the extensive chromosomal rearrangements, IS element proliferation, low percentage of protein coding genes, and horizontal acquisition of important host interaction genes suggests *E. tracheiphila* is undergoing rapid evolution and is in the process of niche specialization. Agricultural landscapes are likely promoting the emergence of *E. tracheiphila*, both by providing large fields of highly susceptible and non-native host plants (*Cucumis* spp.) cultivated at high densities, and by supporting high population densities of the striped cucumber beetle *A. vittatum*, which is the predominant vector (Shapiro, Chapter 4).

The addition of genome sequences of poorly known and predominantly insect associated *Erwinia* spp. (de Vries *et al.* 2001) (Estes 2009; Savio *et al.* 2011) (Skrodenyte-Arbaciauskiene *et al.* 2011) will provide additional insight to *Erwinia* spp. evolution and diversification. Future investigations into *E. tracheiphila* plasmid diversity and function (Smits *et al.* 2010a) (Shrestha *et al.* 2007) (Llop *et al.*) and sequencing of additional *E. tracheiphila* strains isolated from other *Cucurbita* and *Cucumis* ssp. (Rojas *et al.* 2010) will provide invaluable insight into factors determining host specificity and virulence.

**Methods**

**DNA Isolation**
A field isolate of *E. tracheiphila* was grown in nutrient broth for 4 days at 28°C. The culture was briefly centrifuged at 10,000 RPM and the pellet was washed with sterile PBS (pH=8.0) to remove residual media. Genomic DNA was isolated from the pellet using Promega Genomic DNA Wizard extraction kit. Sample identity was confirmed with partial 16S amplification and sequencing of isolated DNA using the following primer set: 530F (5’-GTGCCAGCMGCCGCGG-3’) and 1392R (5’-ACGGGCGGTGTGTRC-3’). In brief, 50 ng genomic DNA were added to a reaction mixture containing 1.0 µL of 10 µM forward primer, 1.0 µL of 10 µM reverse primer, and 12.5 µL of 2X GoTaq Master Mix (Promega). PCR cycling conditions were as follows: 4 minute initial denaturation at 94 °C followed by 28 cycles of 30 sec denaturation at 94 °C, 60 s annealing at 55 °C, and 90 s extension at 72 °C followed by a 7 minute final extension at 72 °C. 10 µL PCR product were treated with 2 µL ExoSAP-IT (USB) using a 37 °C incubation for 15 min, followed by an 80 °C incubation for 30 min and were submitted to the Penn State University Core Genomics Facility for bidirectional sequencing using Big Dye Chemistry on an Applied Biosystems 3730XL platform. A consensus sequence was generated using the forward and reverse.

### Sequencing and Assembly:

Genomic DNA was submitted for shotgun sequencing at the PSU Genomics Core Facility and 8 kb paired end (PE) sequencing at University of Hawaii using 454 Titanium Chemistry (Roche). In brief, 857,774 shotgun reads (~350 Mb) and 207,713 8 kb PE reads (~70 Mb) were generated, which represents approximately 85X coverage of the genome (estimated genome size ~ 5.0 Mb). Shotgun and paired reads were assembled into contigs and scaffolds using GS De Novo Assembler (Roche) to generate 300 contigs ranging in size from 200 bp to 200 kb (N50 contig length=22 kb), which were used to generate 27 scaffolds (4.7 Mb) ranging in size from 2 kb to 1.2 Mb (N50 scaffold size = 462 kb) (Table 5.9). While estimated read coverage across the majority of the scaffolds was consistently 60X, some scaffolds were present in substantially
higher coverages (200X or more) and possibly indicate the presence of high copy number plasmids, actively replicating phages, or emPCR artifacts.

Table 5-9. *E. tracheiphila* sequencing and assembly statistics

<table>
<thead>
<tr>
<th>Feature</th>
<th>Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contigs</td>
<td>1130, including 4.4Mb</td>
</tr>
<tr>
<td><code>n50</code> contig length</td>
<td>22 Kb</td>
</tr>
<tr>
<td>Average contig length</td>
<td>9.6 Kb</td>
</tr>
<tr>
<td>Largest contig size</td>
<td>102 Kb</td>
</tr>
<tr>
<td><code>n50</code> scaffold size</td>
<td>462 kb</td>
</tr>
<tr>
<td>Average scaffold size</td>
<td>174 Kb</td>
</tr>
<tr>
<td>Number of singleton reads (not integrated into assembly)</td>
<td>19025</td>
</tr>
<tr>
<td>Number of reads flagged as repeats (emPCR artifacts, not included in assembly)</td>
<td>10330</td>
</tr>
<tr>
<td>Number of assembled reads</td>
<td>1.1 million</td>
</tr>
</tbody>
</table>

Annotation of genomes

Both RAST (Aziz et al. 2008) and an automated annotation transfer tool at the Sanger Institute (*unpublished*) were used to annotate the draft genomes of *E. tracheiphila*. We used RAST results for the novel regions that were not automatically transferred from the reference genome *E. billingiae*. GenePRIMP was used to scan the intergenic regions of *E. tracheiphila*, *E. amylovora*, and *E. billingiae* for additional CDS and genes too degraded to be called by the RAST ab initio gene predictor (Pati *et al.* 2010). GenePRIMP putative gene annotations that 1) were < 50 AA long and 2) did not have detectable homologs in GenBank and/or 3) overlapped other coding regions were discarded. Each novel gene prediction was also curated with BLAST (Altschul *et al.* 1990) and FASTA (Pearson & Lipman 1988) results, and Pfam (Sonnhammer *et al.* 1997) and Prosite (de Castro *et al.* 2006) were used to identify protein motifs.

Transmembrane domains, signal sequences, and rRNA genes were identified with TMHMM (Krogh *et al.* 2001), SignalP (Dyrløv Bendtsen *et al.* 2004), and BLASTN (Altschul *et al.* 1990),
respectively. ISFinder was also used to detect Insertion Sequence (IS) elements in the genomes (Siguier et al. 2006). Manual curation was done with these novel regions using Artemis (Rutherford et al. 2000) and Artemis Comparison Tool (ACT) (Carver et al. 2005).

**Comparative Analysis with Sequenced Erwinias ssp.**

WebACT and DoubleACT were used to generate figures and compare whole genomes and specific regions of *E. tracheiphila* and other bacteria. Amino acid similarity was compared with the Needle program from the EMBOSS package (Rice et al. 2000). Pairwise Average Nucleotide Identity between the genomes was calculated using in-house scripts following ANI definition of (Konstantinidis & Tiedje 2005).

**Phylogenetic Analysis**

Detection of Laterally Transferred and Phage-related genes

Protein-coding genes of *E. tracheiphila* genome were used as queries in BLASTP search against *nr* database. Homologs with an E-value below $10^{-4}$ were kept (up to 50,000 matches per gene). Using the results of these BLAST searches, candidates for horizontal gene transfer were identified in DarkHorse program (min_align_coverage=0.7; LPI score < 0.85) (Podell & Gaasterland 2007). Individual genes identified from DarkHorse or from manual inspection were analyzed phylogenetically. Alignments of a amino acids of representative sample of all top Blastp hits were aligned in MEGA. The appropriate substitution model for each alignment was chosen with ProTest (Abascal et al 2005). The alignment was then run in RaxML with 100 bootstrap replicates on the CIPRES server (www.phylo.org) and the .tre file visualized in FigTree (Rambaut 2007).

To identify presence of gene homologs in phage pan-genome, protein-coding genes of *E. tracheiphila, E. amylovora CFBP 1430* and *E. billingiae Eb661* genomes were used as queries in BLASTP search against Viral RefSeq database. Hits with E-value below $10^{-10}$ and 70% alignment for both query and subject sequences were kept. CRISPRFinder was used to scan for *cas* genes (Grissa et al. 2007).
**Supplemental Material**

**Table 5-10.** Supplemental table of Darkhorse Output of HGT candidates with COG functional assignments

Preliminary identification of potential HGT candidates identified through Darkhorse with an lineage probability index (LPI) cutoff set at LPI < 0.85, where lower LPI means the CDS is less like other *Erwinia* genes and more likely to be an HGT candidate. All hypothetical genes with no hypothesized function were excluded from the table, as were 24 ribosomal genes. Darkhorse is Blastp based and would not be able to identify HGT candidates from closely related bacteria, and is likely to miss HGT candidates obtained from other enterobacteriaceae, such as chitin-associated proteins and attachment structures discussed in the text. The presence of enterobacterial phages and IS elements provides a possible mechanism explaining the presence of the HGT candidates from enterobacteriaceae present in the *E. tracheiphila* genome that do not appear in the table. Genes discussed in the text are starred.

<table>
<thead>
<tr>
<th>query_id</th>
<th>norm_ LPI</th>
<th>E value</th>
<th>Functional</th>
<th>COG</th>
<th>Annotated Function</th>
<th>species</th>
</tr>
</thead>
</table>
| ETR2251  | 0.036     | 1.00E-71| Functional | Amino acid transport and metabolism | serine A D-3-phosphoglycerate dehydrogenase | *Saccharomyces cerevisiae FostersO*
| ETR1047  | 0.427     | 2.00E-43| Functional | Carbohydrate transport and metabolism | dTDP-4-dehydrorhamnose 3,5-epimerase | *Pseudonocardia sp. P1*
| ETR1789  | 0.432     | 5.00E-62| Functional | Nucleotide transport and metabolism | Uracil phosphoribosyltransferase | *Streptomyces albus J1074*
| ETR0899  | 0.441     | 9.00E-85| Functional | Inorganic Ion Transport and Metabolism | Siderophore biosynthesis protein | *Paenibacillus mucilaginosus KNP414*
| ETR1277  | 0.441     | 5.00E-55| Truncated  | Carbohydrate transport and metabolism | * Extracellular endoglucanase | *Bacillus subtilis B556*
| ETR2385  | 0.441     | 4.00E-27| Functional | Coenzyme transport and metabolism | Thiamine biosynthesis protein | *Streptococcus pseudopneumoniae IS7493*
| ETR2455  | 0.441     | 1.00E-94| Functional | General Function Only | PhnP Metal-dependent hydrolases of the beta-lactamase superfamily I | *Paenibacillus sp. HGF7*
| ETR2456  | 0.441     | 7.00E-126| Functional | Carbohydrate transport and metabolism | ABC-type sugar transport system, periplasmic component | *Paenibacillus sp. HGF7*
| ETR1313  | 0.472     | 6.00E-35| Functional | General Function Only | Probable acetyltransferase | *Candidatus Nitrospira defluvii*
| ETR3543  | 0.472     | 7.00E-59| Functional | DNA replication, recombination, and repair | D12 class N6 adenine-specific DNA methyltransferase | *Desulfurispirillum indicum SS5*
| ETR1048  | 0.517     | 2.00E-49| Functional | Carbohydrate transport and metabolism | UDP-glucose 4-epimerase | *Arthrobacter platensis NIES-39*
| ETR1049  | 0.517     | 5.00E-98| Functional | Carbohydrate transport and metabolism | putative NDP-hexose methyltransferase protein | *Arthrobacter platensis NIES-39*
| ETR2503  | 0.658     | 1.00E-54| Functional | Cell envelope biogenesis | TonB-dependent receptor | *Bilophila sp. 4_1_30*
| ETR0128  | 0.659     | 2.00E-44| Pseudo    | General Function Only | Competence protein CoiA | *Pseudogulbenkiania sp. NH8B*
<table>
<thead>
<tr>
<th>Accession</th>
<th>E-value</th>
<th>Description</th>
<th>Functional Category</th>
<th>Comments</th>
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</thead>
<tbody>
<tr>
<td>ETR0338</td>
<td>0.659</td>
<td>3.00E-109</td>
<td>Functional</td>
<td>Transcriptional regulator, Cro/CI family</td>
</tr>
<tr>
<td>ETR1785</td>
<td>0.659</td>
<td>1.00E-08</td>
<td>Functional</td>
<td>D-Lactate dehydrogenase</td>
</tr>
<tr>
<td>ETR3017</td>
<td>0.659</td>
<td>7.00E-24</td>
<td>Functional</td>
<td>Response regulator receiver domain protein (CheY-like)</td>
</tr>
<tr>
<td>ETR3367</td>
<td>0.659</td>
<td>9.00E-116</td>
<td>Functional</td>
<td>* Isopenetyl-diphosphate delta-isomerase, FMN-dependent</td>
</tr>
<tr>
<td>ETR3370</td>
<td>0.659</td>
<td>4.00E-97</td>
<td>Functional</td>
<td>* Geranylantrantransferase (farnesylphosphate synthase)</td>
</tr>
<tr>
<td>ETR3372</td>
<td>0.659</td>
<td>8.00E-94</td>
<td>Functional</td>
<td>* 3-oxoacetyl-[j-acarrier protein] reductase</td>
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<tr>
<td>ETR3376</td>
<td>0.659</td>
<td>2.00E-140</td>
<td>Functional</td>
<td>* Putative cytochrome P450 hydroxylase</td>
</tr>
<tr>
<td>ETR0451</td>
<td>0.66</td>
<td>3.00E-70</td>
<td>Pseudo</td>
<td>Carbohydrate transport and metabolism</td>
</tr>
<tr>
<td>ETR0604</td>
<td>0.66</td>
<td>1.00E-50</td>
<td>Functional</td>
<td>n-acetylmuramoyl-1-alanine amidase</td>
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<td>ETR0833</td>
<td>0.66</td>
<td>3.00E-87</td>
<td>Functional</td>
<td>Manganese ABC transporter, ATP-binding protein SitB</td>
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<tr>
<td>ETR0834</td>
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<td>2.00E-81</td>
<td>Functional</td>
<td>Manganese ABC transporter, inner membrane permease protein SitC</td>
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<td>ETR0900</td>
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<td>1.00E-59</td>
<td>Functional</td>
<td>Methionyl-tRNA synthetase-related protein</td>
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<td>ETR0901</td>
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<td>FAD-dependent oxidoreductase</td>
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<td>ETR1045</td>
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<td>1.00E-104</td>
<td>Functional</td>
<td>Glucose-1-phosphate cytidlyltransferase</td>
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<td>ETR1185</td>
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<td>1.00E-154</td>
<td>Functional</td>
<td>miA B tRNA-i(t)A37 methylthiotransferase</td>
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<tr>
<td>ETR1539</td>
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<td>6.00E-64</td>
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<td>1.00E-51</td>
<td>Functional</td>
<td>Uncharacterized protein ImpD</td>
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<tr>
<td>ETR1766</td>
<td>0.66</td>
<td>2.00E-28</td>
<td>Pseudo</td>
<td>NAD(P)/flavin oxidoreductase</td>
</tr>
<tr>
<td>ETR2957</td>
<td>0.66</td>
<td>3.00E-32</td>
<td>Pseudo</td>
<td>Acetyltransferase</td>
</tr>
<tr>
<td>ETR3009</td>
<td>0.66</td>
<td>7.00E-63</td>
<td>Functional</td>
<td>gcp gcp YgjD/Kae1/Qri7 family, required for threonylcarbamoyladenosine (t6A) formation in tRNA</td>
</tr>
<tr>
<td>ETR3371</td>
<td>0.66</td>
<td>3.00E-147</td>
<td>Functional</td>
<td>* Cytochrome P450</td>
</tr>
<tr>
<td>ETR3373</td>
<td>0.66</td>
<td>1.00E-14</td>
<td>Functional</td>
<td>* Uncharacterized p450-system 3fe-4s ferredoxin</td>
</tr>
<tr>
<td>ETR3374</td>
<td>0.66</td>
<td>2.00E-146</td>
<td>Functional</td>
<td>*Putative cytochrome P450 hydroxylase</td>
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<td>ETR3375</td>
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<td>6.00E-158</td>
<td>Functional</td>
<td>* Cytochrome P-450</td>
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<td>2.00E-29</td>
<td>Pseudo</td>
<td>Plant-induced nitrilase, hydrolyses beta-cyano-L-alanine</td>
</tr>
<tr>
<td>ETR3774</td>
<td>0.66</td>
<td>2.00E-132</td>
<td>Truncated</td>
<td>xopAG putative avrgf1 family effector/xanthomonas outer protein</td>
</tr>
</tbody>
</table>

**Organisms:**
- Neisseria meningitidis CU385
- Nitrobacter hamburgensis X14
- Rhizobium loti R7A
- Burkholderia dolosa AUO158
- Agrobacterium tumefaciens F2
- Ralstonia solanacearum Po82
- Burkholderia gladioli BSR3
- Rhizobium etli IE4771
- Cupriavidus necator N-1
- Burkholderia dolosa AUO158
- Burkholderia sp. CCGE1002
- Ralstonia solanacearum Po82
- Rhizobium etli CNPAF512
- Agrobacterium tumefaciens F2
- Rhizobium etli CNPAF512
- Achromobacter xylosoxidans AXK-A
- Acidovorax citrulli AAC01-1
<p>| ETR3869  | 0.66 | 8.00E-149 | Functional | Signal transduction mechanisms | Sensory transduction system regulatory protein | Rhizobium leguminosarum bv. trifolii WSM2304 |
| ETR3963  | 0.66 | 2.00E-19  | Functional | Plasmid | Addiction module toxin, RelE/StbE family protein | Achromobacter xylosoxidans C54 |
| ETR0118  | 0.708 | 2.00E-07  | Functional | Transcription | Putative transcriptional regulator | Thiomonas sp. 3As |
| ETR1276  | 0.8  | 3.00E-93  | Truncated | Carbohydrate transport and metabolism | * Expansin | Saccharophagus sp. JAM-R001 |
| ETR1714  | 0.8  | 3.00E-33  | Functional | Translation, Ribosomal structure, and biogenesis | Translation initiation factor 1 | Idiomarina sp. A28L |
| ETR1902  | 0.8  | 4.00E-39  | Pseudo | Amino acid transport and metabolism | soxA sarcosine oxidase, alpha subunit | Halomonas sp. TD01 |
| ETR1985  | 0.8  | 9.00E-18  | Pseudo | General Function Only | Glyoxalase/bleomycin resistance protein/dioxygenase | Salinisphaera shabanensis E113A |
| ETR2283  | 0.8  | 2.00E-52  | Functional | Translation, Ribosomal structure, and biogenesis | trimB rRNA (guanine46-N7)- methyltransferase | Salinisphaera shabanensis E113A |
| ETR2807  | 0.8  | 6.00E-100 | Functional | Cell motility and secretion | outG general secretion pathway protein | Shewanella pealeana ATCC 700345 |
| ETR3158  | 0.8  | 0         | Pseudo | Cell envelope biogenesis | xopAM xopAM xanthomonas outer protein am | Xanthomonas vesicatoria ATCC 35937 |
| ETR3444  | 0.8  | 5.00E-172 | Functional | Energy production and conversion | adhC S-(hydroxymethyl)glutathione dehydrogenase | Glaciecola sp. 4H-3-7+YE-5 |
| ETR3960  | 0.8  | 5.00E-29  | Functional | Plasmid | HigB toxin protein | Methylophaga aminisulfidivorans MP |
| ETR0025  | 0.801 | 0         | Functional | Lipid transport and metabolism | ToxR-activated gene TagA | Aeromonas veronii B565 |
| ETR0375  | 0.801 | 3.00E-175 | Functional | Inorganic Ion Transport and Metabolism | hemE Uroporphyrinogen III decarboxylase | Aeromonas veronii B565 |
| ETR0499  | 0.801 | 2.00E-173 | Functional | Amino acid transport and metabolism | dctA Aerobic C4-dicarboxylate transporter for fumarate, L-malate, D-malate, succinate | Aeromonas veronii B565 |
| ETR1223  | 0.801 | 1.00E-124 | Functional | Lipid transport and metabolism | suCd Succinyl-CoA ligase [ADP-forming] alpha chain | Aeromonas veronii B565 |
| ETR1289  | 0.801 | 8.00E-66  | Functional | Defense Mechanisms | Organic hydroperoxide resistance protein | Acinetobacter calcoaceticus PHEA-2 |
| ETR2011  | 0.801 | 0         | Functional | Intracellular trafficking | Putative transport protein | Aeromonas veronii B565 |
| ETR2806  | 0.801 | 5.00E-43  | Functional | Cell motility and secretion | General secretion pathway protein G | Aeromonas veronii B565 |
| ETR2823  | 0.801 | 1.00E-16  | Functional | Energy production and conversion | ATP synthase C chain | Aeromonas veronii B565 |
| ETR2843  | 0.801 | 1.00E-114 | Functional | Carbohydrate transport and metabolism | 2-deoxy-D-gluconate 3-dehydrogenase | Aeromonas veronii B565 |
| ETR2944  | 0.801 | 1.00E-21  | Pseudo | General Function Only | Glyoxalase family protein | Aeromonas veronii B565 |
| ETR3123  | 0.801 | 1.00E-53  | Functional | Amino acid transport and metabolism | argR Arginine pathway regulatory protein | Aeromonas veronii B565 |
| ETR3187  | 0.801 | 7.00E-33  | Functional | Translation, Ribosomal structure, and biogenesis | ArgR, repressor of arg regulon | Aeromonas veronii B565 |
| ETR3314  | 0.801 | 2.00E-70  | Pseudo | Transcription | SSU ribosomal protein S18p | Aeromonas veronii B565 |
| ETR0377  | 0.802 | 0         | Functional | Amino acid transport and metabolism | proP L-Proline/Glycine betaine transporter ProP | Pseudomonas putida S16 |
| ETR0398  | 0.802 | 0         | Pseudo | General Function Only | abc transporter related protein | Pseudomonas putida S16 |
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**Functionality Overview:**

- **Functional**: Functions include translation, ribosomal structure, amino acid transport, metabolism, cell motility and secretion, energy production and conversion, carbohydrate transport, chaperones, ribonucleotide transport, and metabolism.

- **Pseudo**: General function only.

- **Transcription**: Putative transcriptional regulator.

- **Translation**: Ribosomal structure.

- **Cellulosis**: Cellulose synthesis.

- **Secretion**: Secretion of proteins.

- **Energy Production**: Energy production and conversion.
<p>| ETR       | 0.802 | 1.00E-46 | Pseudo | Postranslational modification, protein turnover, chaperones | * TypeIII Chaperone SheF | Pseudomonas syringae pv. glycinea str. race 4 |
| ETR       | 0.802 | 2.00E-45 | Functional | Cell motility and secretion | no data | Pseudomonas syringae pv. maculicola str. M6 |
| ETR0865   | 0.803 | 2.00E-49 | Functional | Nucleotide transport and metabolism | YcfF/HinT protein: a purine nucleoside phosphoramide | Vibrio anguillarum 775 |
| ETR1790   | 0.803 | 1.00E-60 | Functional | Transcriptional Regulator | Transcriptional regulator, MarR family | Photobacterium angustum S14 |
| ETR0037   | 0.804 | 0       | Functional | Translation, Ribosomal structure, and biogenesis | Translation elongation factor LepA | Vibrio parahaemolyticus RIMD 2210633 |
| ETR0390   | 0.804 | 1.00E-87 | Functional | Transcription | Transcription antitermination protein NusG | Vibrio parahaemolyticus RIMD 2210633 |
| ETR0444   | 0.804 | 8.00E-104 | Functional | Carbohydrate transport and metabolism | kduD 1 2-deoxy-D-glucuronic acid 3-dehydrogenase | Vibrio parahaemolyticus RIMD 2210633 |
| ETR0508   | 0.804 | 0       | Functional | Amino acid transport and metabolism | ilvD Dihydroxy-acid dehydratase | Vibrio parahaemolyticus RIMD 2210633 |
| ETR0683   | 0.804 | 2.00E-148 | Functional | Translation, Ribosomal structure, and biogenesis | tRNA(Cytosine32)-2-thiocytidine synthetase | Vibrio parahaemolyticus RIMD 2210633 |
| ETR0898   | 0.804 | 6.00E-25 | Functional | Energy production and conversion | GCN5-related N-acetyltransferase | Vibrio harveyi 1DA3 |
| ETR1175   | 0.804 | 7.00E-135 | Pseudo | Carbohydrate transport and metabolism | chitinase [Vibrio sp. RC341]gi|260840080|gb|EEX66684.1| | Vibrio sp. RC341 |
| ETR1423   | 0.804 | 3.00E-89 | Functional | DNA replication, recombination, and repair | Endonuclease III | Vibrio parahaemolyticus RIMD 2210633 |
| ETR1731   | 0.804 | 7.00E-37 | Functional | Postranslational modification, protein turnover, chaperones | Glutaredoxin 1 | Vibrio mimicus SX-4 |
| ETR1791   | 0.804 | 0       | Functional | Secondary metabolites biosynthesis, transport and catabolism | Peptide synthetase | Vibrio nigripulchritudo ATCC 27043 |
| ETR1802   | 0.804 | 1.00E-173 | Functional | Amino acid transport and metabolism | metE metE 5-methyltetrahydropteroylglutamate--homocysteine methyltransferase | Vibrio parahaemolyticus RIMD 2210633 |
| ETR1812   | 0.804 | 6.00E-67 | Functional | Coenzyme transport and metabolism | Molybdenum cofactor biosynthesis protein MoaC | Vibrio cholerae HE48 |
| ETR1826   | 0.804 | 2.00E-18 | Functional | Cell motility and secretion | upp Uracil phosphoribosyltransferase | Vibrio furnissii CIP 102972 |
| ETR1953   | 0.804 | 8.00E-102 | Functional | Nucleotide transport and metabolism | * F17 fimbral protein precursor | Vibrio parahaemolyticus RIMD 2210633 |
| ETR2032   | 0.804 | 7.00E-100 | Functional | Translation, Ribosomal structure, and biogenesis | truA rRNA pseudouridine synthase A | Vibrio parahaemolyticus RIMD 2210633 |
| ETR2156   | 0.804 | 2.00E-53 | Functional | Coenzyme transport and metabolism | Queuosine biosynthesis QueD, ispG 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphostate synthase | Vibrio cholerae HE39 |
| ETR2448   | 0.804 | 1.00E-179 | Functional | Lipid transport and metabolism | Cysteine desulfurase | Vibrio parahaemolyticus RIMD 2210633 |
| ETR2462   | 0.804 | 2.00E-161 | Functional | Amino acid transport and metabolism | glnB glnB Nitrogen regulatory protein P-II | Vibrio parahaemolyticus RIMD 2210633 |
| ETR2472   | 0.804 | 4.00E-48 | Functional | Postranslational modification, protein turnover, chaperones | Single-stranded DNA-binding protein | Vibrio cholerae C6706 |
| ETR2615   | 0.804 | 5.00E-63 | Functional | DNA replication, recombination, and repair | | |</p>
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References


Chapter 6

Conclusions

The research described here takes a systems biology approach to understanding interactions in a co-evolved pathosystem comprising the wild gourd *Cucurbita pepo* ssp. *texana*, the bacterial wilt pathogen *Erwinia tracheiphila*, the striped cucumber beetle vector *Acalymma vittatum*, and *Zucchini Yellow Mosaic Virus* (ZYMV). This work was prompted by the notable sparsity of basic or applied knowledge about *E. tracheiphila*, and began by specifically addressing the mechanisms underlying epidemiological patterns in experimental fields where instances of co-infection in the same host are significantly lower than would be expected by chance. From that observation, we first tested \(^1\) how pathogen effects on host phenotype indirectly affect vector foraging behavior and disease exposure; \(^2\) whether there are direct interactions between ZYMV and *E. tracheiphila* in a shared host plant; \(^3\) quantitative dynamics of beetle colonization by *E. tracheiphila* using molecular methods; and \(^4\) genomic comparisons to understand microbial evolution and disease emergence.

Pathogen infection can change host phenotype in ways that directly alter a host's contact with transmitting vectors, and can also indirectly alter a host’s exposure to other pathogens. Here, I describe how volatile organic compounds released from the leaves and flowers of healthy and pathogen-infected wild gourd plants mediate exposure to the bacterial wilt pathogen through vector attraction, and how viral infection induces a host volatile phenotype that reduces exposure to foraging beetles. *A. vittatum* acquire *E. tracheiphila* by feeding on symptomatic, wilting foliage of infected plants, and transmit the pathogen when infective frass falls on leaf herbivory wounds or floral nectaries of healthy plants. Feeding preference tests showed that beetles will
preferentially feed on leaves of either *E. traceiphila* or *ZYMV*-infected seedlings relative to healthy seedlings, but in field plantings virus-infected plants have less beetle damage and lower incidence of wilt disease than healthy plants. In both field and greenhouse experiments, I found that infection with *E. traceiphila* induces the emission of a unique volatile blend from symptomatic wilting foliage relative to asymptomatic foliage on infected plants, healthy foliage, and *ZYMV*-infected foliage, and that floral volatile emission is suppressed by both *E. traceiphila* and *ZYMV* infection relative to healthy plants. Behavioral assays show beetles are more attracted to volatiles from wilting leaves relative to healthy foliage, but prefer flowers on healthy plants relative to viral or bacterial infected plants. Taken together, *E. traceiphila*-induced volatiles provide a mechanism by which beetle vectors are more attracted to symptomatic wilt-infected foliage relative to non-symptomatic leaves on the same plant, healthy foliage, or virus-infected leaves through inducing the release of a unique volatile blend from wilting leaves. Beetle vectors are more attracted to the increased number of flowers and higher concentration of volatiles per flower on healthy relative to infected plants, suggesting a plausible ecological mechanism through changes in host-plant volatile phenotype affect interactions with beetle vectors for both infected and healthy hosts. *ZYMV* reduces exposure of virus infected host plants to *E. traceiphila* through suppressing floral volatile emission and not inducing foliar volatile release.

While *ZYMV* – infected plants experience reduced exposure to foraging beetle vectors, virus infected plants may also be less susceptible to subsequent infection with *E. traceiphila* through induction of non-specific immune responses. Systemic acquired resistance (SAR), characterized by accumulation of PR proteins and enhanced cell lignification is often observed in plants induced with biotic or abiotic stresses, and are less susceptible to subsequent pathogen challenge. Salicylic acid (SA) is a defense signaling hormone that has an essential role in the induction of SAR. Here, I show that *E. traceiphila* suppresses SA induction in host plants soon after inoculation and after symptoms develop. This finding is similar to several other bacterial
pathogens that have been shown to secrete effector proteins that specifically inhibit SA signaling. SA levels in ZYMV-infected plants are significantly higher than E. tracheiphila or mock-infected plants. Despite elevated SA levels in ZYMV – infected plants, both ZYMV - infected and healthy plants exhibit the same rates of infection with E. tracheiphila in controlled inoculation experiments. Products of the octadecanoid pathway (JA, LA, LNA) were measured in undamaged and herbivore damaged healthy, E. tracheiphila, and ZYMV – infected plants. JA is induced in all three treatments after herbivory, and patterns of induction of JA, LA, and LNA suggest that induced herbivore defenses mediated by the octadecanoid pathway are not the determining factor affecting beetle preference for pathogen – infected (either E. tracheiphila or ZYMV) leaves compared to healthy leaves in dual choice feeding tests.

To better understand the relationship between E. tracheiphila and A. vittatum, we developed a qPCR method using Taqman probes specific to an E. tracheiphila outer membrane protein (OmpA) and 18S as a normalizing gene to examine the acquisition and retention parameters of E. tracheiphila. I found acquisition of E. tracheiphila to average ~80%, but long-term retention increases significantly for longer exposure (24hr) compared to shorter exposure (3hr) time. E. tracheiphila exhibits complex colonization dynamics of the beetle digestive tract: Large amounts of bacteria are observed in both beetle and frass immediately after an acquisition access period. At 5 days post acquisition, median bacterial levels have are the same in the whole insect but significantly less in frass. By 28 days post acquisition, the median bacterial ratio in the whole beetle is again not different, but there is significantly more bacteria shed in frass. From these data, I infer that colonization of the insect in a persistent manner is inefficient. The bacteria that do colonize then enter a growth and colonization phase where little bacteria is shed. By 28 days, there is significantly more bacteria being shed in frass compared to the amount of bacteria shed at 5 days. Inoculation experiments with frass collected from groups of beetles 0, 5, and 28 days after an acquisition access period showed that higher bacterial concentrations observed in
frass at 0 and 28 days post AAP correlates with higher inoculation success in wild gourd seedlings. Results from several fitness assays suggest that *E. tracheiphila* does not affect *A. vittatum* fecundity or longevity, and that *E. tracheiphila* is therefore a facultative, neutral commensal within *A. vittatum*.

An absence of genetic sequences from *E. tracheiphila* hinders further work understanding bacterial interactions with hosts and vectors. To address this, we used 454 Titanium chemistry to sequence an *E. tracheiphila* culture isolated from wild gourd planted at the PSU Rock Springs Research Farm to 60-80X coverage and conducted a comparative analysis with other closely related, plant associated enteric bacteria. I found that the closest sequenced relative to *E. tracheiphila* is the non-pathogenic rosaceous floral epiphyte and symbiont *E. billingiae*, although *E. tracheiphila* expresses many virulence traits shared with *E. amylovora*. The *E. tracheiphila* genome is characterized by significant inversions and rearrangements, and an influx of proliferating insertion sequences and replicating phage that likely encourage massive genomic recombination. Horizontal gene transfer analysis detects the presence of many putative HGT events for functions important for both plant virulence and insect colonization. *E. tracheiphila* is also undergoing genome reduction through pseudogenization, although notably, core biosynthetic and metabolic pathways are conserved, suggesting *E. tracheiphila* is reducing towards a facultative, niche-adapted species that will retain much of the metabolic machinery of free living enterobacterial species. The inclusion of the *E. tracheiphila* genome greatly reduces the size of the hypothesized core *Erwinia* genome. My analysis identifies likely determinants influencing plant and insect interactions and suggests directions for future functional analyses.

In an applied setting, this research has direct applications for the design of sustainable pest control strategies in agricultural systems. Leaf volatiles provide an herbivore-specific cue that could supplant previous insecticide-laced diabroticite traps that emulate floral volatiles, and attracted many non-target floral pollinators that are often inadvertently attracted to the floral
blend baited traps. A more quantitative understanding of bacterial colonization dynamics of insect vectors and the publication of the *E. tracheiphila* genome sequence will also offer novel avenues for basic and applied research (Bocsanczy *et al.* 2008). While many of the results presented in this dissertation are idiosyncratic to the *Cucurbita* pathosystem, larger questions are raised. This research provides a framework for approaches to other complex pathosystems, where multiple herbivores and pathogens are co-circulating (Knops *et al.* 1999). Genetic homogeneity characteristic of modern agriculture (Stukenbrock & McDonald 2008) and the development of farmland in formerly wild areas of domesticated plant and animal populations is creating unprecedented spillover and opportunities for host jumps and emergence of new disease threats (Conn *et al.* 2002). Understanding how pathogens interact in complex ecosystems and the molecular and ecological factors driving their emergence will be key to creating effective prevention and control strategies.

Understanding mechanisms of microbial persistence in environmental reservoirs as important drivers of disease dynamics (Leroy *et al.* 2005; Snitkin *et al.* 2012) is being recognized as an increasingly important factor driving some disease outbreaks, and a potential target for pathogen control. For example, enterobacterial disease agents have historically been researched in the context of a pathogenic agent to the plant or animal host on which the bacterium produces disease. Many recent outbreaks of enteric food-borne pathogens were transmitted through contaminated plant material and the ability of food-borne enteric pathogens like *Salmonella typhimurium* (Schikora *et al.* 2008), and virulent strains of *Escherichia coli* (Cooley *et al.* 2003) to persistently colonize crop plants and create disease outbreaks with important public health consequences is now widely recognized (Brandl 2006). However, bacterial persistence outside of its disease host, either in the environment or in an alternative host, are increasingly understood as integral to disease cycles and transmission to new hosts (Holden, Pritchard, & Toth, 2008). For obligately insect-borne diseases, interactions with vectors determine disease dynamics.
(Hinnebusch 1996; Hinnebusch et al. 2002; Sarkar et al. 2010; Zhang et al. 2011). Detailed understanding mechanisms of persistence in vectors, how pathogens are shed and healthy hosts exposed, and ecological factors mediating vector attraction to infected and healthy hosts, will all contribute to successful containment strategies.

High throughput sequencing technology is providing especially rapid development in the field of microbial ecology, evolution, and pathogenomics and providing the unprecedented opportunity to make vast contribution towards understanding the ecology and evolution of non-model organisms and systems (Earl et al. 2008). These are leading to great advances in disease ecology, where the molecular and genetic processes underlying ecological interactions can now be affordably described, greatly increasing our understanding of host-microbe interactions (Bäckhed et al. 2005; Broderick et al. 2006) for bacteria of ecological (Ffrench-Constant et al. 2003; Hofmann et al. 2005), public health (Chain et al. 2004), and agricultural interests (Kay 2009).

References


VITA

Lori R. Shapiro

EDUCATION

Ph.D. in Entomology 2007-2012
Pennsylvania State University
B.S. Biology, B.S. Ecology, B.S.E.S. Entomology
Certificate in International Agriculture 2001-2006
University of Georgia

PUBLICATIONS

Ecology Letters July 2012
“Pathogen effects on vegetative and floral odors mediated vector attraction and host exposure in a complex pathosystem”

FELLOWSHIPS and AWARDS

• University Fellowship 2007-2008
• National Science Foundation Graduate Fellowship 2008-2010
• USDA Microbial Genomics Training Fellowship 2010-2012

FIELD EXPERIENCE

2007-2011

• Field work at Russell E. Larson Experimental Farm at Rock Springs, PA to document interactions between wild gourd Cucurbita pepo ssp. texana, the striped cucumber beetle Acalymma vittatum, and several endemic diseases in a field setting Oct-Nov 2009

• Survey of the wild gourd Cucurbita argyrosperma in the Mexican states of Jalisco, Michoacán, Guerrero, and Oaxaca in collaboration with researchers from Universidad Nacional Autonoma de Mexico (UNAM)

OUTREACH and VOLUNTEER 2007-2012

Participation in over 40 separate science education and outreach events on the PSU campus and in the surrounding community. Events and responsibilities included: Coordinating the Entomology department’s display at Exploration Days, leading Frost Entomology Museum insect biology programs for visiting school groups, leading laboratory activities for Women in Science and Engineering summer camps, conducting conservation and education programs at local nature centers, libraries, and elementary schools