Phylogenetic Analysis and Characterization of Plant, Environmental, and Clinical Strains of *Pantoea*

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Geetanchaly Nadarasah, candidate for the degree of Master of Science in Biology, has presented a thesis titled, *Phylogenetic Analysis and Characterization of Plant, Environmental, and Clinical Strains of Pantoea*, in an oral examination held on March 23, 2012. The following committee members have found the thesis acceptable in form and content, and that the candidate demonstrated satisfactory knowledge of the subject material.

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Abstract

Multihost bacterial pathogens are an increasing concern as more bacterial species are found to cause harm to humans. *Pantoea* is recognized as a multihost pathogen, colonizing various hosts including plants, insects, and humans; however it is unknown how these strains are related, and the extent of their specific host ranges. Multilocus sequence analysis (MLSA) on six housekeeping genes of *Pantoea* revealed that some species are mixed, and contain plant, clinical, and environmental strains, while other species groups are composed of only plant or only clinical strains. Comparative growth assays in maize, onion, and fruit flies revealed that all plant, clinical, and environmental strains are capable of colonizing both plant and animal hosts. *Pantoea* clinical strains had an overall greater growth rate within fruit flies in comparison to either plant hosts. The results of this work have shown that some *Pantoea* strains have a broad host range, while others are more host specific. The close relationship of plant and environmental strains to clinical strains and their ability to colonize plants and fruit flies with equal efficiency, highlights the potential for these strains to cause human infections. This work also suggests that strains causing human infections likely originate from the general environment.
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Dedication

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µL     microlitre
CF     cystic fibrosis
CFU    colony forming units
CIP    calf intestinal alkaline phosphatase
Exo    exonuclease 1
LB     lysogeny broth
MEGA   molecular evolutionary genetics analysis
MgSO4  magnesium sulfate
mL     millilitre
MLSA   multilocus sequence analysis
mM     millimolar
PCR    polymerase chain reaction
T3SS   type III secretion system
U      unit
Chapter 1: General Introduction

The role of the general environment in the emergence of new diseases has been the focus of many studies. Many plant pathogenic bacteria have been shown to cause human infections [1,2], indicating that environmental bacteria can evolve to exploit humans and animals as hosts. Bacterial genera like Burkholderia, which infects various plant species including onion, sorghum, rice, and velvet beans [3,4,5], is also a recognized human pathogen, causing chronic infections in patients with cystic fibrosis (CF) and granulomatous disease [6]. Other plant pathogenic bacteria that cause human disease include Serratia, Erwinia, and Pseudomonas, which have been implicated in pneumonia and septicaemia, chronic illness, and cystic fibrosis, respectively [7,8,9,10]. The reservoir for these pathogens has been shown to be in the rhizosphere, the zone around the roots of plants, and in the phyllosphere [2,11,12], both of which have a diverse composition of nutrients. As a result, infection of a human or animal host by these strains can occur by a scrape, ingestion, or contact with the skin or mucosal membranes [13].

Aside from direct contact, bacteria may also be transported from plants to indoor or clinical environments through the activities of carriers like flies and ants [14,15], providing bacteria with the opportunity to colonize a broader range of hosts. Those strains that thrive in their new environment, including those that are capable of exploiting a human host, will increase in the population, and may be returned back to the general environment through one of many routes. One route may be facilitated by the symptomology caused by the infection, which can promote bacterial shedding from the host [16]. Bacteria can move from human wastes to natural watersheds, where irrigation
of commercially relevant crops would lead to an increase in bacterial titre on plants and the general environment [17,18]. Insects may play a role in the return of pathogens back to the general environment. The common house fly, *Musca domestica*, has been reported as a carrier of many pathogenic bacteria including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis* [15], while the Pharoah ant, *Monomorium pharaonis*, is known to transport human pathogenic bacteria such as *Streptococcus* and *Salmonella* [19]. The possible association of plant pathogenic bacteria with humans through the environment could lead to them becoming adapted to humans over time. Likewise, the ability of these bacteria to return to the general environment, and possibly associate with plants, may result in the maintenance of the ability to colonize multiple hosts. The constant cycling of bacterial pathogens between environments and hosts may have significant implications for host specificity and the emergence of new infectious diseases.

**Host Specificity**

Host specificity encompasses the nature and extent of microbial adaptation to one or more hosts, with specialists and generalists as the two main categories [20]. Microbes that are generalists infect multiple hosts with little selectivity, while microbes that are specialists are more selective, being able to colonize one defined host or group of hosts [20]. The presence of bacteria in the environment, and their use of insects as possible vectors for transmission, provides a wider range of potential hosts to colonize, where the constant cycling of bacteria among multiple unrelated hosts in turn can lead to generalism [21]. To associate with their preferred host and cause disease a large set of pathogenicity
factors are often used by microbes, and these are known as host specificity factors [20]. Host-specific factors range from specialized attachment appendages to specialized secretion systems [22,23], and selectively target a particular feature of a host to initiate an association [24]. Interestingly, the presence of host specific genes can enhance host specificity. In the mutualist, *Vibrio fischeri*, the two-component sensor kinase, RscS, induces the Syp exopolysaccharide, resulting in the formation of biofilm and the colonization of the squid light organ [25,26]. The expression of rscS in a *Vibrio* strain deficient of the gene enabled host colonization [25], which shows that this gene is a host specificity factor.

**Specialists**

Specialists are organisms that have adapted to infecting one particular type of host or group of hosts. Over time, the repeated colonization of the same host may lead to a steady refinement of the pathogen genetic repertoire and the ability to exploit the host more efficiently and effectively [27]. Both plant and animal pathogens use host specificity factors for associating with their host, such as type III secretion system (T3SS) effectors [20]. T3SS is a protein appendage found commonly among Gram-negative bacteria [28]. Through this appendage, bacteria are able to attach to their host and release effector proteins to promote disease and infection [29]. The human-adapted serovars of *Salmonella enterica* use T3SS effector proteins: SseC, SseB, SseD, to facilitate the attachment of the secretion system to the phagosomal membrane of human cells [30]. The strong genetic similarities of sseD genes and sseD alleles to human cells show the host-specific binding of the T3SS and its strong association with humans [30]. GTP-binding
proteins, particularly Rho subfamily proteins, have also been implicated in the entry of many species of bacteria into eukaryotic cells. Rho subfamily proteins participate in various activities within the cell including endocytosis, apoptosis, and cell transformation [31]. Rho proteins of eukaryotic cells are known to be ADP-ribosylated by *Clostridium botulinum* C3-like transferases and they are also key targets of *Escherichia coli* cytotoxic necrotizing factors [31]. Through selective activation of the Rho proteins by bacterial agents, bacteria initiate an association with their host cells and gain entry into their host to promote disease [31].

Specialist bacterial pathogens have been proposed to be more evolutionarily favoured due to higher benefits than costs [32]. In a constant and narrow niche, specialist bacterial pathogens experience a higher probability of fixing beneficial alleles, a lower frequency of deleterious alleles exhibiting mutation-selection balance, as well as fewer deleterious alleles drifting to fixation [33]. More importantly, as a result of co-evolutionary processes between the bacterial pathogen and its host, specialist bacterial pathogens are able to achieve optimum virulence while maximizing their transmission potential [27]. Although specialization to one specific host may result in increased fitness, it comes at the cost of a lower ability to exploit other hosts.

**Generalists**

In contrast to specialists, generalists can infect and successfully colonize multiple hosts, but may not necessarily be able to cross species barriers [32]. However, having a broad host range comes at a cost. Although generalists are able to colonize a wider range of hosts, this impedes their maximal performance in any one host [33]. A broad niche
decreases the probability of fixing beneficial alleles and increases the frequency of harmful alleles (reduce mutation load) at mutation-selection equilibrium. On the other hand the narrow niche of specialists increases the rate of evolutionary response [33]. Despite the costs, many bacterial pathogens are generalists, and capable of exploiting a wide range of hosts.

Both *Burkholderia cepacia* and *Pseudomonas aeruginosa* are two generalist bacterial pathogens that have been studied extensively for their disease-causing capabilities. Both are ubiquitous in the environment, and can infect a broad range of hosts, including humans. *P. aeruginosa* has been found in soil and is known to cause soft rot in plants such as *Arabidopsis thaliana* and lettuce [34], but is also a major human pathogen that infects CF and immunocompromised patients [35,36]. Its presence in the general and clinical environments has raised questions about its virulence and pathogenic potential toward humans. Studies have examined the characteristics of environmental and clinical isolates of *P. aeruginosa*, and their virulence in a variety of hosts. CF strains have been shown to be more efficient in attaching to surfaces and forming biofilms in comparison to environmental strains, but have a lower planktonic cell growth rate, suggesting that the CF lung may be a more extreme environment [35]. Although clinical and environmental strains vary in their biofilm-forming capabilities, there is a high level of conservation in a core set of genes (~97%), which includes all known virulence factors. The conservation of virulence determinants suggests that selection for the maintenance of such traits exists in the environment. As a result, it is likely that environmental strains have the ability to cause human infections despite an expected low probability of encountering a human host. Environmental strains of *P. aeruginosa* also
have resistance to several antibiotics, with some strains showing ten-fold higher resistance in comparison to a reference clinical strain [37].

Like *P. aeruginosa*, *B. cepacia* is a bacterial pathogen found in soil, and known to infect both plants and humans [6]. Based on phenotypic and genotypic analyses, *B. cepacia* has been divided into nine phylogenetically differentiable species, which together constitute the *B. cepacia* complex [6]. Species like *Burkholderia pyrrocinia* are composed of a mixture of environmental and clinical isolates, while *Burkholderia ambifaria* contained only environmental strains [38]. *Burkholderia cenocepacia* showed evolutionarily distinct groups for agricultural and clinical strains [39]. A phenotypic analysis of the clinical and environmental strains across the *B. cepacia* complex showed that all were able to macerate onion tissue and colonize nematodes, albeit at varying levels [38]. Interestingly, clinical strains were found to have a higher level of antibiotic resistance than environmental strains [6].

Exploration of the pathogenicity of clinical and environmental strains of *Burkholderia*, as well as *Pseudomonas*, has revealed no difference in physical traits, such as optimal growth temperature and their ability to overcome phage infection [6,40]. Both clinical and environmental strains are also able to be both haemolytic and proteolytic to epithelial cells, encode T3SS genes, and quorum-sensing genes, which could be indicative of broader virulence potential [37].

Interestingly, analyses on *Stenotrophomonas* and *Aeromonas*, which are also known to infect multiple hosts, revealed similar results found in *Pseudomonas* and *Burkholderia*. Phylogenetic studies showed that there was no evolutionary separation of clinical and environmental strains, and that they had no difference in antibiotic resistance
Surprisingly, environmental strains of *Aeromonas* displayed a higher level of enterotoxicity and hemolysin production than clinical strains [42]; however, these characteristics could lead to a greater potential for human pathogenesis. Both *Stenotrophomonas* and *Aeromonas* are not known to be plant pathogens.

**Pantoea as a model system**

To understand the evolution of multihost pathogens and their potential impact on human health, model pathogen groups must be used, such as those that are known to naturally colonize a broad range of hosts and cause disease. *Pantoea* is ubiquitous in nature, colonizing plants, humans, and being found in the general environment. Many species of *Pantoea* are known to be plant pathogenic, and commonly found in diverse ecological niches including aquatic environments, soil, or sediments [43]. *Pantoea stewartii* subsp. *stewartii* causes Stewart’s vascular wilt disease of sweet corn, while *Pantoea agglomerans* is found to cause crown and root gall disease of gypsophila and beet [44,45]. Interestingly, the recent emergence of the *Pantoea* in humans has led to the realization that this genus represents a group of multihost pathogens with broad capabilities [43,46,47,48]. Many studies have investigated the pathogenicity of *Pantoea* in several plant hosts, and identified the possible pathogenicity factors involved during disease. The pathogenicity of *P. stewartii* appears dependent on the *hrp/wts* gene cluster, which directs the synthesis of a T3SS [29,46]. Additional work identified the involvement of a quorum-sensing (QS) system, which allows bacteria to monitor their population density by utilizing small, diffusible signals to orchestrate the expression of specialized gene systems for pathogenicity [49,50,51]. The QS system organizes the
timing and production level of the exo/capsular polysaccharide, stewartan, which significantly affects the degree of bacterial adhesion during in vitro biofilm formation and propagation within the plant host [50].

In recent years, however, *Pantoea* species have been frequently isolated from humans suffering from soft tissue or bone/joint infections following penetrating trauma by vegetation [47]. Given the ubiquity of *Pantoea* in the general environment, and the increasing number of human-related cases of *Pantoea*, several studies have examined how the different strains are related. Their ubiquity in the environment and their pathogenicity led to several studies examining their relationships using phylogenetics [52,53]. These studies have used multilocus sequence analysis (MLSA), a technique that provides an improved method for establishing relationships, and which involves the use of multiple protein-coding genes in phylogenetic reconstruction to neutralize the effects of horizontal gene transfer and homologous recombination [52]. In a recent study, MLSA was used to determine the identification of various *Pantoea* strains obtained from diverse origins. Most strains belonged to *P. agglomerans*, while others belonged to many diverse phylogenetic branches such as other *Pantoea* species and probable novel species [53]. A survey on the presence of *repA*, a common gene found on plasmids of plant pathogenic strains was also conducted, and it was shown that clinical strains also contained the *repA* gene [53], suggesting that clinical and plant-associated strains do not form distinct populations and may have similar virulence characteristics. These studies have pursued further analyses on phenotypic traits among plant and environmental strains. The maximal hourly growth rate of clinical strains at 37°C, human body temperature, was significantly less in comparison to plant strains within *P. agglomerans*, while at 24°C
there was no significant difference in maximal hourly growth rate among plant and clinical strains [43]. Although clinical strains have a lower maximal hourly growth rate at 37°C, their ability to grow at this temperature suggests their ability to exploit a human host.

These experiments along with the phylogenetic analyses prompted questions as to the host range and pathogenic capabilities of *Pantoea* strains. One study conducted a comparative virulence assay of five plant and five clinical strains using soybean plants and embryonated hen eggs as hosts. Interestingly, both plant and clinical strains of *P. agglomerans* developed stable epiphytic populations on soybean plants, while in the embryonated hen egg model similar levels of growth were observed [47]. However, the plant and clinical *P. agglomerans* strains were significantly less virulent than a phytopathogenic *P. ananatis* isolate in the hen egg model [47]. The ability of both plant and clinical strains to colonize a plant and a eukaryotic host suggests that the bacteria might possess similar virulence potential, where plant strains may have the capacity to infect humans. While this study and the several phylogenetic studies described above have provided some important insight in the pathogenicity of *Pantoea*, they have focused primarily on a few select strains, leaving many unanswered questions about the capabilities of this bacterial group.
Objectives

The first objective was to examine how a broad collection of plant, clinical, and environmental strains of *Pantoea* are related using a phylogenetic analysis through multilocus sequence analysis (MLSA) of six housekeeping genes. The clustering pattern of strains can be used to determine whether there is any evidence of host specialization in this opportunistic pathogen. The second objective was to analyze the colonization abilities of the various strains of *Pantoea* using maize, onion, and fruit flies as candidate hosts. Results from these growth assays provided a profile for each of the strains, possibly leading to a host specificity pattern that can be used to identify potential human pathogens.
Chapter 2: Phylogenetic Analysis and Characterization of Plant, Environmental, and Clinical Strains of *Pantoea*

*Abstract*

Strains of *Pantoea* have been found to colonize many different types of hosts such as insects, plants, and humans, but it is unclear how they are related and if they are virulent on hosts dissimilar to their origin of isolation. Phylogenetic analysis using multilocus sequence analysis (MLSA) on six housekeeping genes of *Pantoea* showed that some species within the genus contain a diverse array of plant, clinical, and environmental strains, while other species are more specific, containing only one type of strain. However, there is no distinct separation of the plant, clinical, and environmental strains. Comparative growth assays using three hosts: maize, onion, and fruit flies, showed that all strains were able to colonize hosts other than from which they were isolated. Some species groups within the phylogenetic tree had an equal ability to colonize all three hosts, while other species groups were more specialized. Overall, clinical strains had a significantly greater growth within fruit flies than in either of the two plant hosts. The results of this work show that some strains are diverse in their abilities to colonize different hosts, while others are more dedicated to colonizing one specific type of host. The close relationship of plant and environmental strains to clinical strains and their ability to colonize plants and fruit flies with equal efficiency, highlights the potential harm these strains may cause to humans while in the environment. A better understanding of the relationships and colonization abilities of the various strains may subsequently lead to the discovery of *Pantoea* strains that could be new potential human pathogens.
Introduction

Multihost bacterial pathogens colonize a wide range of hosts, with many being capable of crossing both interspecific and intraspecific boundaries. The frequent isolation of bacteria from humans following interactions with plants through open wounds or abrasions [48,54,55,56,57], or consumption of contaminated water [58] indicates a prominent role for the general environment as a reservoir for human pathogens. *Pantoea* is widespread in nature and is commonly known as a plant epiphyte, where it can be found in many diverse ecological niches such as soil and aquatic environments [59,60,61]. *Pantoea stewartii* subsp. *stewartii* is the causal agent of Stewart’s vascular wilt disease of sweet maize and maize [62] while *Pantoea citrea* is known to cause pink disease in pineapples [63]. Although common plant epiphytes and plant pathogens, several strains of *Pantoea agglomerans* produce antibiotics which are sold as commercial biological control agents against *Erwinia amylovora*, the fire blight pathogen of pear and apple trees [64,65], and *Pseudomonas syringae*, the basal kernel blight pathogen of barley [66].

In recent years, however, *Pantoea* has been frequently identified in human infections with soft tissue or bone/joint infections following penetrating trauma by vegetation [48,54,55,56,57]. *P. agglomerans* bacteremia has also been described in association with the contamination of intravenous fluid [67], total parenteral nutrition [68], the anesthetic agent propofol [69] and blood products [70]. The increasing number of *Pantoea* species being isolated from plants, the general environment, and most importantly, humans has led to the reclassification of *Pantoea* as a group of opportunistic, ubiquitous, multihost pathogens, with an unknown virulence potential.
Previous studies have analyzed the relationships of plant, environmental, and clinical strains of *Pantoea* to evaluate their potential virulence potential. Through a multilocus sequence analysis (MLSA) on the different types of strains of *P. agglomerans*, plant, environmental, and clinical strains co-clustered, indicating that all different types of strains are closely related and may have similar host specificity and virulence [53]. One study used a host assay to evaluate host specificity and virulence, and compared plant and clinical strains of *P. agglomerans* to a plant strain of *Pantoea ananatis*. Both plant and clinical strains of *P. agglomerans* developed stable epiphytic populations on soybean plants, while in the embryonated hen egg model no difference in virulence was detected [47]. However, the five plant and five clinical *P. agglomerans* strains were significantly less virulent than a phytopathogenic *P. ananatis* isolate in the hen egg model [47]. Still, only a few strains were evaluated.

In this study, we used phylogenetic approaches and MLSA to evaluate the relationships between plant, clinical, and environmental strains of *Pantoea*. We then tested the colonization abilities of each strain in maize, onion, and fruit flies to determine their host ranges. Evaluation of the relationships and virulence of the different types of strains could lead to the detection of new potential human pathogens.

**Materials and Methods**

**Bacterial Strains**

A total of 128 *Pantoea* strains from clinical (56 strains), plant (33 strains), and environmental (39 strains) sources were analyzed in this study. Clinical strains were
obtained from the Regina General Hospital, Dr. Paul Levett at the Saskatchewan Disease Control Laboratory, St. Boniface General Hospital in Winnipeg, Texas Children’s Hospital, and Sunnybrook Hospital. The plant strains were obtained from New Zealand Culture of Plant Pathogens (NZCPP), Dr. Steven Lindow at Berkeley, and Dr. Gwyn Beattie at Ohio State. The environmental strains were acquired from sampling different sources in the environment around Saskatchewan. Each strain was plated onto rifampicin plates to select for rifampicin-mutant strains to reduce the potential for contamination.

*DNA extraction, amplification, and sequencing*

Bacterial strains were grown on Luria Bertani (LB) medium for 24 hours at 30 degrees Celsius. Genomic DNA was extracted and purified from 3 mL overnight cultures in LB medium using the Qiagen genomic DNA purification kit (Mississauga, Ontario). Polymerase chain reaction (PCR) amplification and sequencing of internal portions of the six housekeeping genes *fusA, leuS, pyrG, rpoB, gyrB*, and *16S rRNA* were performed using primers described previously [53]. Each PCR was performed in a total volume of 20 µL using 14.4 µL sterile water, 2.0 µL 10x Standard Taq Buffer, 0.2 µL Taq DNA polymerase (5 U/µL), 0.2 µL forward primer (50 µM), 0.2 µL reverse primer (50 µM), 2.0 µL dNTPs (200 µM each), and 1 µL (200-500 ng/µL) template DNA. PCR amplicons were then purified from the PCR mix by cleaning with Calf Intestinal Alkaline Phosphatase (CIP) and Exonuclease 1 (Exo) and incubated at 37 degrees Celsius for 30 minutes followed by an inactivation step at 85 degrees Celsius for 15 minutes. Primers were then premixed with clean PCR products and sent off for sequencing to Operon Sequencing, Huntsville, Alabama.
Data and sequence analysis

Sequences were aligned using Clustal W [71] to produce a concatenated tree. These sequences were compared with a selection of concatenated \textit{rpoB} and \textit{gyrB} sequences of \textit{Pantoea} species from GenBank, other genera within the \textit{Enterobacteriaceae} family, and a representative of \textit{Pseudomonas} as an outgroup to all strains. Gaps and errors in sequencing were then corrected using GeneDoc, and the alignment was transferred into Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 [72] to create a phylogenetic tree. A Neighbour-joining tree was constructed, with 1000 bootstrap replicates. Nucleotide divergence was determined using DnaSP program version 5.10 [73].

Plant growth assay

Maize seeds (Golden Bantam) were sown in potting soil, a mixture of soil and peat moss, and were grown in plant pots, where three seeds were sown per pot and were watered each morning. Seeds were sown 2-5cm (1-2”) deep in potting soil and were grown in 16 hour day cycles at 25 degrees Celsius. Plants, 3-4 weeks old plants with broader leaves, were used for inoculation. Overnight cultures of bacteria grown in rifampicin-LB broth were used for inoculating the maize plants. Optical densities of the cultured broths were used to calculate a final concentration of $10^7$ colony forming units (CFU)/mL in 10 mM MgSO$_4$. Bacterial suspensions were then inoculated (1 mL syringe) into the leaves of the three different plants per pot to provide three replicates. Nine spots per plant were inoculated on the leaves to provide three leaf tissue samples per replicate over day 0, day 1, and day 5. Samples of 1 cm radius from the inoculated leaves were
obtained right after inoculation (day 0) and on day 1 and 5. Samples were placed in 1.5 mL microcentrifuge tubes containing 300 µL of 10 mM MgSO₄ buffer and subsequently ground to release the bacteria. Homogenates were then serially diluted to 10⁻² CFU/mL in 90 µL of 10 mM MgSO₄ buffer. Samples were then plated onto rifampicin plates to select for the microbe of interest, and incubated overnight at 30 degrees Celsius. Bacterial counts were taken for each replicate, which were then used to determine the number of CFU per inoculated leaf tissue. Onion growth assays, using yellow onion, were performed as above in the same manner of maize assays.

**Fruit fly growth assay**

Optical densities of overnight cultures of bacterial strains grown in LB containing rifampicin were used to make 2 mL of 10⁻⁷ CFU/mL in 20 percent sucrose. Vials containing a single cotton puff were then soaked with the bacterial solution, and 27 adult flies were allowed to feed for 16 hours. Following feeding, flies were transferred to vials containing only Drosophila media. At each time point, three flies were sampled per replicate, and placed into a 1.5 mL microcentrifuge tube containing 300 µL of 10 mM MgSO₄ buffer. Flies were subsequently crushed and serially diluted to 10⁻² CFU/mL in 90 µL of 10 mM MgSO₄ buffer. Samples were then plated onto rifampicin plates to reduce bacterial contamination and incubated overnight at 30 degrees Celsius. Bacterial counts were used to calculate growth.

**Standardization and data analysis of growth assays**

Because initial doses varied between strains and across species groups, we standardized all growth assays to facilitate comparisons across hosts. To do this, we
converted all raw values obtained from each growth assay to log base two. The growth rate was then determined between day 0 and day 1, and day 1 and day 5, and these rates were then used to standardize all growth assays, assuming an initial dose of 10 CFU. SPSS version 17.0 was used to perform a two-way analysis of variance (ANOVA) on values obtained for each respectable growth assay [74]. Differences were found to be significant if p-values were <0.05.

Results

Genotypical analysis

Phylogenetic reconstruction by Neighbour-joining on the collection of 128 strains revealed that our collection contains at least ten different species groups, as defined by the location of marker sequences from type species. These include *Pantoea agglomerans*, *Pantoea eucalyptii*, *Pantoea conspicua*, *Pantoea brenneri*, *Pantoea anthophila*, *Pantoea stewartii*, *Pantoea ananatis*, *Pantoea dispersa*, *Pantoea eucrina*, *Pantoea calida*, and *Pantoea septica*. Several strains provided to us as *Pantoea* fell outside of the genus and were later identified as species of *Erwinia*, *Cronobacter*, *Escherichia*, and *Klebsiella* (Figure 1).

To validate the phylogenetic groupings, an analysis of nucleotide divergence was conducted to determine if the species groups were valid as predicted by phylogeny (Table 1). The average “within” species group percentage of nucleotide divergence is expected to be less than 1.5%, while the average “between” species divergence is expected to be greater than 1.5% [75]. Nucleotide divergence between the predicted species groups was
greater than 1.5%, except for *P. stewartii* and *P. ananatis* (1.474%) as well as *P. stewartii* and *P. brenneri* (1.136%). In some monophyletic groups, several strains were more divergent, and formed a sister group to the other strains. To test whether these may represent additional species, divergence was calculated between these strains and the rest of the group (Table 2). Interestingly, 17671 within *P. ananatis* had a nucleotide divergence of 2.577% and 06868 had a nucleotide divergence of 2.628% within the *P. eucrina* species group.
Figure 1. Neighbour joining tree based on the concatenated nucleotide sequences of \textit{fusA}, \textit{leuS}, \textit{pyrG}, \textit{rpoB}, \textit{gyrB}, and \textit{16S rRNA} of 128 \textit{Pantoea} strains. Bootstrap values after 1000 replicates are expressed as percentages where only those of 70\% and greater are shown. \textit{Pseudomonas} was included as an outgroup. (original in color)
After phylogenetic reconstruction, we mapped on the origin of isolation (plant, clinical, or environment) to determine whether there was any obvious association between phylogeny and origin of isolation. *P. agglomerans* is a mixed group having 18 plant, 10 clinical, and 29 environmental strains. There are two clear subgroups within this species, one composed of a mixture of plant, clinical, and environmental strains, while the other composed of only plant strains.

*P. eucalyptii* and *P. dispersa* are also mixed, with *P. eucalyptii* having plant, clinical and environmental strains, and *P. dispersa* having two clinical and one plant strain from the collection. In contrast, *P. conspicua, P. brenneri, P. eucrina, P. calida, and P. septica* all contain only clinical strains, while *P. anthophila, P. stewartii*, and *P. ananatis* are made up of plant strains. There is some correlation between species group, and location of isolation, with some species being composed of only clinical strains, only plant strains, or mixtures.
Table 1. Nucleotide divergence expressed in percent among the different species groups within the phylogenetic concatenated tree.

<table>
<thead>
<tr>
<th></th>
<th>Pantoea agglomerans</th>
<th>Pantoea eucalyptii</th>
<th>Pantoea brenneri</th>
<th>Pantoea stewartii</th>
<th>Pantoea ananatis</th>
<th>Pantoea dispersa</th>
<th>Pantoea eucrina</th>
<th>Pantoea calida</th>
<th>Pantoea septica</th>
<th>Erwina billingiae</th>
<th>Escherichia coli</th>
<th>Klebsiella</th>
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<tr>
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<td>na</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
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<td>1.864</td>
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<td>1.474</td>
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<td>Pantoea septica</td>
<td>3.518</td>
<td>2.697</td>
<td>2.768</td>
<td>2.269</td>
<td>2.725</td>
<td>3.663</td>
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<td>4.021</td>
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Table 2. Comparison of nucleotide divergence, in percent, among unique strains within certain phylogenetic species groups.

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<tr>
<th>Comparisons</th>
<th>Nucleotide Divergence</th>
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<tr>
<td>SP04013 vs. <em>Pantoea eucalyptii</em></td>
<td>0.61%</td>
</tr>
<tr>
<td>17671 vs. <em>Pantoea ananatis</em></td>
<td>2.58%</td>
</tr>
<tr>
<td>06868 vs. <em>Pantoea eucrina</em></td>
<td>2.68%</td>
</tr>
<tr>
<td>BB957621B2 vs. <em>Pantoea calida</em></td>
<td>0.07%</td>
</tr>
<tr>
<td>TX4 and TX3 vs. <em>Pantoea septica</em></td>
<td>1.43%</td>
</tr>
</tbody>
</table>
Growth Assays

Quantitative growth assays were conducted for all plant, clinical, and environmental strains using maize, onion, and fruit flies as hosts. Strains in the mixed *P. agglomerans* and *P. eucalyptii* groups had no statistical difference in growth in the maize, fruit fly, and onion hosts. All plant, environmental, and clinical strains in *P. agglomerans* grew to approximately $10^5$ CFU by day 5 in all three different hosts. In contrast, plant strains of the species group *P. eucalyptii* had a greater growth in maize plants, reaching approximately $10^3$ CFU by day five, while clinical strains had a greater growth within fruit flies, reaching approximately $10^5$ CFU by day 5. Interestingly, environmental strains within *P. eucalyptii* reached approximately $10^4$ CFU by day 5 in both the maize and fruit fly hosts.

Species groups containing only one type of strain, like *P. stewartii*, *P. ananatis*, *P. eucrina*, and *P. calida* had no significant difference in the ability of their strains to colonize the three different hosts. *P. stewartii* and *P. ananatis*, which contain plant strains, grew better in maize and onion plants, reaching approximately $10^5$ CFU by day 5 (Refer to Appendix A). Surprisingly, there was no significant difference in their ability to colonize fruit flies. Similarly, *P. calida* and *P. eucrina*, species groups containing only clinical strains, were able to grow better in fruit flies attaining $10^4$ CFU by day 5, but were able to colonize plant hosts equally as well (Refer to Appendix A). Interestingly, both pairs of groups are monophyletic.

*P. brenneri*, *P. dispersa*, and *P. septica* are composed almost exclusively of clinical strains, and exhibit a preference for the fruit fly host. *P. brenneri* grows to $10^4$ CFU by day 5 in flies, but only to $10^2$ in the plant hosts ($p=0.001$). *P. brenneri* also
contains only clinical strains, which show significantly greater growth in fruit flies than
in either of the two plant hosts (p=0.001) (Figure 2). *P. dispersa* is a smaller group
comprising two closely related clinical strains, M1657A and M1657B, and the plant
strain, 625. The two clinical strains grow to significantly higher densities in fruit flies
than the plant strain, reaching approximately $10^5$ CFU by day 5 (Figure 3). Surprisingly,
both clinical strains grew equally well in the two plant hosts. This is also seen for *P.
septica*, which is composed of only clinical strains. *P. septica* has a greater growth in fruit
flies, reaching $10^4$ CFU by day 5, in comparison to maize and onion plants, $10^3$ CFU by
day 5, which is an almost two fold decrease in growth (p<0.001) (Figure 2). Interestingly,
all clinical strains had equal growth in both plant hosts.

Non-*Pantoea* groups were included within the phylogenetic tree for comparison,
but were also included in our quantitative growth assays. Both *Erwinia* and *Cronobacter*
contain only plant strains, while *Escherichia* and *Klebsiella* contain only clinical strains.
For *Erwinia*, a significant difference in growth can be seen in the two hosts (p=0.017),
where plant strains grow better in maize ($10^3$ CFU by day 5) than in the fruit fly hosts,
($10^2$ CFU by day 5). Clinical strains of *E.coli* and *Klebsiella* grew equally well in all three
different hosts. Both *E. coli* and *Klebsiella* were able to colonize the two plant hosts
equally well, but were able to grow to higher densities in fruit flies, reaching
approximately $10^3$ CFU by day 5.
Figure 2. Comparative growth assay of clinical strains across *Pantoea septica*, and *Pantoea brenneri*. All clinical strains overall within the species *Pantoea septica*, and *Pantoea brenneri* have a significantly greater growth in fruit flies in comparison to both maize and onion plants (p<0.05). The growth, number of colony forming units (CFU), was determined per leaf disk for maize and onion hosts and per fruit fly for the fruit fly host. (n=33 for maize, n=32 for fruit fly, and n=31 for onion for all clinical strains, df=2, F=30) (original in color)
Figure 3. Comparison of growth within hosts among strains of *Pantoea dispersa*. The two clinical strains within *Pantoea dispersa* had a significant difference in their ability to colonize maize plants ($p=0.000$), fruit flies ($p=0.053$), and onion plants ($p=0.001$) in comparison to the plant strain. No significant difference in growth can be seen in the two clinical strains to colonize both the maize and onion plants ($p=1.000$). The growth, number of colony forming units (CFU), was determined per leaf disk for maize and onion hosts and per fruit fly for the fruit fly host. ($n=2$ for clinical strains in all three hosts, while $n=1$ for plant strain, df=2, $F=3.7$) (original in color)
For most groups within the phylogenetic tree, clinical strains show a greater ability to colonize fruit flies in comparison to plant and environmental strains. An overall comparison of clinical strains from all the different groups further affirmed a significantly greater growth in fruit flies in comparison to maize and onion. To evaluate the ability of all clinical strains to colonize fruit flies, clinical strains were separated into their respective origins of isolation: blood, urine, and external. Interestingly, all three clinical groups: blood, urine, and external, had had an approximate ten-fold greater growth in fruit flies than in maize and onion plants ($p=0.001$), with a significant difference of $p=0.000$, $p=0.004$, and $p=0.008$ for blood, external, and urine respectively (Figure 4). Conversely, an analysis of all plant and environmental strains in their ability to colonize the different hosts revealed no difference in growth. Overall, these analyses show that despite their origin of isolation all plant, environmental, and clinical strains were able to colonize maize, onion, and fruit flies, and clinical strains are able to colonize fruit flies more efficiently than plant hosts (Figure 5).
Figure 4. Overall analysis of growth across clinical strains. All clinical groups: blood, external, and urine had an approximately ten fold greater growth (p=0.001) in fruit flies than in maize and onion plants. The growth, number of colony forming units (CFU), was determined per leaf disk for maize and onion hosts and per fruit fly for the fruit fly host. (n=25 for maize, n=24 for fruit flies, and n=24 for onion for blood, external, and urine, df=4, F=2.65) (original in color)
Figure 5. Multihost colonization of the 128 plant, clinical, and environmental strains. Plant, clinical, and environmental strains show an ability to colonize all three hosts: corn, fruit fly, and onion. Different levels of growth were achieved by day five but environmental and plant strains were able to colonize fruit flies while clinical strains were able to colonize both plants as effectively as the environmental and plant strains studied. The growth, number of colony forming units (CFU), was determined per leaf disk for maize and onion hosts and per fruit fly for the fruit fly host. (n=3 for environmental, clinical, and plant for all three hosts) (original in color)
Discussion

MLSA of all 128 strains indicated that the majority of strains belonged to *P. agglomerans*, while others belonged to other *Pantoea* species or other *Enterobacteriaceae*.

Both *P. agglomerans* and *P. eucalyptii* are mixed groups containing plant, clinical, and environmental strains, which suggests that plant, clinical, and environmental strains are closely related and may therefore have similar characteristics and virulence potential. This could imply that plant and environmental strains may have the ability to cause human disease, and human strains may have the ability to be pathogenic towards plants and the environment. Similar results were obtained by previous studies that used MLSA to evaluate the relationships between plant, clinical, and environmental strains of *P. agglomerans* [43]. Interestingly other species groups like *P. septica* contain only clinical strains, and may be specialized to colonizing animal or human hosts. It is also possible that these strains are still capable of infecting other organisms, like plants, but are more frequently isolated from the clinical environment due to higher persistence in the environment, or specific characteristics that promote more efficient dispersal.

Species groups established by phylogeny were well supported by our analysis of nucleotide divergence. Most comparisons between species groups led to nucleotide divergences greater than 1.5%, suggesting that the species groups predicted by the phylogeny are supported. The plant pathogenic group, *P. stewartii*, and the clinical pathogenic group, *P. brenneri*, had a nucleotide divergence of less than 1.5%. The low divergence could reflect horizontal gene transfer between the two species groups, resulting in a higher overall phylogenetic relatedness. The constant cycling of pathogens
in the environment could allow clinical and plant strains to interact and possibly exchange genetic information, leading to a more generalist lifestyle in the environment.

*P. ananatis* strain 17671 and the *P. eucrina* strain 06868 both had nucleotide divergences greater than 1.5%, suggesting that they are not similar to the strains within their respective species group as shown through the phylogeny. These likely represent new *Pantoea* species, but this would have to be validated through biochemical tests.

Growth assays using plants and insects were especially informative in evaluating the potential pathogenicity of each strain. Strains within the mixed groups, *P. agglomerans* and *P. eucalyptii*, had no significant difference in their ability to colonize all three hosts. The ability of plant and environmental strains to have similar virulence potential and close relatedness to clinical strains further affirms the potential of plant and environmental strains to colonize humans. The equal ability of all plant, clinical, and environmental strains within *P. agglomerans* and *P. eucalyptii* to colonize fruit flies suggests that they are possible human pathogens. Similarly, in another study, plant and clinical strains of *P. agglomerans* were able to colonize soy bean plants and embryonated hen eggs with equal growth [47]. Interestingly, a plant strain of *P. ananatis* was able to attain a significantly higher mortality rate in an embryonated hen egg in comparison to clinical strains of *P. agglomerans* [47]. The ability of a plant strain to be more virulent than a clinical strain indicates that these strains can become problematic if they encounter a suitable human host.

*P. dispersa* displayed different virulence potential between its two clinical strains and one plant strain, with the two clinical strains colonizing the fruit fly better than the two plant hosts and the plant strain colonizing the two plants hosts more efficiently than
the fruit fly host. Both *P. brenneri* and *P. septica* had a significantly greater ability to colonize fruit flies in comparison to either maize or onion plants, which suggest that these clinical species groups may be more specialized to colonizing animal hosts than plant hosts. This suggests that some strains within some species groups of *Pantoea* may have some level of host specificity, and phylogeny is not a good predictor of host specificity.

Although mammalian models were not used in this study, fruit flies have been shown to mimic the human immune response [76,77]. Our quantitative growth assays revealed that despite the assumption that plant strains could colonize only plant hosts and clinical strains only human hosts, all plant, clinical, and environmental strains can colonize maize, onion, and fruit flies, albeit at varying levels. Although all strains were unable to grow to high titres in all three different hosts, the ability of *Pantoea* strains to grow to levels higher than the initial dose shows their versatility, and potential to exploit a wide range of hosts. It is possible that through constant exposure to these hosts, plant, clinical, and environmental strains might be able evolve the necessary host specific pathogenicity factors that would enhance their growth in one or more hosts. *Pantoea* would have to possesses a wide range of pathogenicity factors that would give it the ability to establish; however, aside from the plant pathogenicity factors, including exopolysaccharide production, type III secretion system effectors, toxins, and adhesins, the pathogenicity determinants that allow it to colonize fruit flies are unknown [78,79]. Fruit flies have closely related innate immune system pathways to humans and similar mechanisms of recognition of microbial invaders [80]. The ability of plant and environmental strains to colonize fruit flies as well as they colonize plant hosts shows the potential for these strains to cause human infections.
Phylogenetic analysis reaffirms the idea that plant and environmental strains of *Pantoea* retain human pathogenic potential, based on the fact that they are closely related to clinical strains. Growth assays demonstrate that origin of isolation does not indicate host range. The ability of plant and environmental strains to have close relationships with clinical strains and have an equal ability to colonize both plants and fruit flies demonstrates the versatility of *Pantoea*, and suggests that the pathogenicity of these strains is greater than previously assumed.
Concluding Remarks

A thorough understanding of the relationship between plant, environmental, and clinical strains within *Pantoea* is critical for determining their ability to emerge as serious human pathogens. By analyzing the relationships of a diverse collection of strains, this study has increased not only our understanding of how these different types of strains are related, but also their potential virulence in various hosts. Phylogenetic analysis revealed that plant, clinical, and environmental strains are closely related and thus may have similar characteristics and virulence potential. Some species of *Pantoea* are mixed, and contain strains isolated from humans, plants, and the general environment while others are composed of strains isolated from only humans or only plants. Interestingly, analysis of nucleotide divergence revealed the presence of potentially new species of *Pantoea*, but also highlighted cases of possible exchange of genetic information between different species. The broad scale evaluation of colonization ability of the different species in our collection showed that growth in fruit flies seems to be good indicators of clinical relevance. Fruit flies and humans share a high level of conservation of intracellular pathways within their respective innate immune systems, in addition to having similar methods to overcome pathogen invasion. The close relatedness of plant and environmental strains to clinical strains, and their ability to colonize and grow in the fruit fly affirms that these strains have human pathogenic potential. Lastly, this work sets the foundation for the identification of host specificity factors and determinants that allow *Pantoea* to colonize different hosts.
Future Directions

The understanding of the relationships of plant, clinical, and environmental strains across many species of *Pantoea* and their virulence in different hosts is clear but the actual virulence factors involved during pathogenicity are not well known. Investigation into the specific pathogenicity factors involved during disease will help to understand how these different types of strains are able to colonize multiple hosts and if there is further similarities among the different type of strains. A genetic screen using transposon mutagenesis using fruit fly larvae will assist in achieving the characterization of genes involved in pathogenesis.
List of References


63. Stewart FC (1897) A bacterial disease of sweet corn. New York State Agricultural Experiment Station Bulletin 130: 422-439.


Appendices

Appendix A. Comparative growth of all species groups.

Figure 1. Growth of plant, environmental, and clinical strains within *Pantoea agglomerans* in maize, fruit fly, and onion hosts. (original in color)
Figure 2. Comparative growth assays of plant, environmental, and clinical strains within *Pantoea eucalyptii* in maize and fruit fly. (original in color)
Figure 3. Comparative growth of clinical strains within *Pantoea brenneri* in maize, fruit fly, and onion hosts. (original in color)
Figure 4. Comparative growth of plant strains within *Pantoea stewartii* in maize, fruit fly, and onion hosts. (original in color)
Figure 5. Comparative growth analysis of plant strains within *Pantoea ananatis* in maize, fruit fly, and onion. (original in color)
Figure 6. Comparative growth of plant and clinical strains within *Pantoea dispersa* in maize, fruit fly, and onion hosts.
Figure 7. Comparative growth of clinical strains within *Pantoea eucrina* in maize, fruit fly, and onion hosts. (original in color)
Figure 8. Comparative growth analysis of clinical strains within *Pantoea calida* in maize, fruit fly, and onion. (original in color)
Figure 9. Growth of clinical strains within *Pantoea septica* in maize, fruit fly, and onion hosts. (original in color)
Figure 10. Comparative growth of plant strains within *Erwinia* in maize and fruit fly hosts. (original in color)
Figure 11. Growth of clinical strains within *Escherichia coli* in maize, fruit fly, and onion hosts. (original in color)
Figure 12. Comparative growth of clinical strains within *Klebsiella* in maize, fruit fly, and onions hosts. (original in color)
Appendix B. Characterization of mutants.

Table 1. Characterization of mutants generated through transposon mutagenesis of *Pantoea ananatis* strain 15320.

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<th>Name</th>
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<th>E-value</th>
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</tr>
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<tr>
<td>0_506</td>
<td>hypothetical protein (<em>Actinobacillus ureae</em> ATCC 25976)</td>
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<tr>
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<td>BAK12886.1</td>
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<td>0_587</td>
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<tr>
<td>19_5b</td>
<td>yahK gene product, Zn binding alcohol dehydrogenase (<em>Pantoea ananatis</em> LMG 5342)</td>
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<tr>
<td>20_7a</td>
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**Appendix C. Information regarding the strains used in this thesis.**

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<th>Patient Information</th>
<th>Extra Information</th>
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<td>Environment</td>
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<td>7612</td>
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<td>Origin</td>
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<td>Extra Information</td>
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<td>11 year old female</td>
<td>Received from Dr. Paul Levett, Saskatchewan Disease Control Laboratory, Regina, Saskatchewan</td>
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<td>B014130</td>
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<td>11 year old male</td>
<td>Received from Dr. Paul Levett, Regina, Saskatchewan</td>
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<td>B015092</td>
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<td>9 years old female</td>
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<td>56 year old female</td>
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<td>Groin</td>
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<td>Origin</td>
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<td>Extra Information</td>
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<td>BB350028A</td>
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<td>Blood culture, fever</td>
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<td>BB350028B</td>
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<td>BB834250</td>
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<td>BB957621A1</td>
<td>Clinical, human</td>
<td>CAPD dialysate, peritonitis</td>
<td>Male</td>
<td>Received from St. Boniface General Hospital, Winnipeg, Manitoba</td>
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<td>BB957621A2</td>
<td>Clinical, human</td>
<td>CAPD dialysate, peritonitis</td>
<td>Male</td>
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<td>CAPD dialysate, peritonitis</td>
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<td>Clinical, human</td>
<td>CAPD dialysate, peritonitis</td>
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<td>Strain</td>
<td>Origin</td>
<td>Isolated Host</td>
<td>Patient Information</td>
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<td>Male</td>
<td>Received from St. Boniface General Hospital, Winnipeg, Manitoba</td>
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<td>Origin</td>
<td>Isolated Host</td>
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<td>F9026</td>
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<td>H42501</td>
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<td>Plant</td>
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<td>Plant</td>
<td>Type strain</td>
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<td>Received from David Coplin, Columbus, Ohio</td>
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<td>Plant</td>
<td>Type strain</td>
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<tr>
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<td>Origin</td>
<td>Isolated Host</td>
<td>Extra Information</td>
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<td>SP01202</td>
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<tr>
<td>Strain</td>
<td>Origin</td>
<td>Isolated Host</td>
<td>Patient Information</td>
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<td>SP03383</td>
<td>Environment</td>
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<td>Isolated Host</td>
<td>Patient Information</td>
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<td>SP05091</td>
<td>Environment</td>
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<td>Isolated in Saskatchewan by summer students of Dr. John Stavrinides</td>
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Appendix D. Insects as alternative hosts for phytopathogenic bacteria.

Note: This appendix has been republished, with permission, from Nadarasah, G., and Stavrinides, J. Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiology Reviews*. 35(3): 555-575.

Insects as alternative hosts for phytopathogenic bacteria

Geetanchaly Nadarasah and John Stavrinides

Running Title
Non-plant hosts for plant pathogenic bacteria

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Keywords
plant pathogens, bacteria, host, insects, pathogenicity, transmission, vector, multi-host, host specificity, evolution

69
Abstract

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

Plant pathogenic bacteria are responsible for some of the most devastating losses of major agricultural crops and vital fruit trees, causing millions of dollars in damage annually. Their agricultural and economic impact has afforded them significant attention over the last 30 years, resulting in enormous strides in the exploration of their epidemiology and specialized disease strategies. Research of plant pathogenic bacteria has not only seeded our understanding of the genetics of disease, epidemiology, and the factors contributing to emerging infectious diseases, but has also led to the development of effective control and prevention measures for many plant diseases [81,82]. More recently, however, there has been a shift in the exploration of the plant pathogens to a broader community level, which moves beyond the traditional single host-single pathogen model to a wider and more encompassing view of the evolution and ecology of plant pathogenic bacteria. Much of this research has expanded the field into a new direction, and has resulted in the unearthing of the hidden ecology and true pathogenic potential of many bacteria that have long been considered strict and very dedicated phytopathogens.

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

There are numerous potential interactions that can result from the association between a microbe and an insect, all of which are defined by the relative effects on the fitness of the individual organisms (referred to as symbionts). Mutualisms may form between the two organisms, where both derive a benefit from their interaction. Mutualisms may be defensive, where the microbe provides protection to the insect host [83], or nutritional, where the microbe supplements the diet of the insect host with key nutrients [84]. Parasitisms may also develop between microbe and insect, where the microbe benefits by extracting nutrients from its host at a cost to its host. In the case of the latter, the microbe may cause disease in the insect, usually marked by a condition that impairs or disrupts normal host functioning and physiology, and is often associated with specific symptomology. A commensalism describes the association between insect and microbe where the microbe benefits and the insect is unaffected. Commensalisms likely characterize many of the interactions that exist in the natural environment, but are most likely to go unnoticed. Both commensalistic and parasitic symbioses can range from highly specific to non-specific, with the development of more specific interactions being favoured in cases where specialist microbes encounter specialist insects recurrently over long periods of time, and more general interactions in cases where generalist or transient insects encounter specialist bacterial pathogens (or vice versa).
Phytopathogenic bacteria have evolved to harness insects as vectors to effect their dissemination and delivery directly onto or into their preferred plant hosts. These partnerships can be either commensalistic or slightly parasitic to the insect, but in either case, the insect performs as a living carrier that transmits the microbe to its final (definitive) host. Many of these symbioses are highly specific, and are categorized by the ability of the bacterium to replicate in and move through its vector. The tendency to replicate within the insect vector is described as either propagative or non-propagative, while the tendency of the microbe to move through its vector can be described as circulative or non-circulative [85]. In circulative non-propagative transmission, the microbe is ingested by its insect vector as it feeds on the host plant, after which it migrates into the midgut or hindgut epithelium, and is then released into the haemolymph of the insect [85]. The microbe then enters into the salivary glands, and can be inoculated to healthy plant hosts via the saliva while the insect feeds [86,87]. In this case, the microbe does not replicate in its host vector. In contrast, circulative propagative transmission occurs when a microbe is able to replicate within its insect vector, and spread to other organs within the insect. The microbe crosses the membrane to enter into the haemolymph and is later instigated into the saliva to thereby be released from the insect to cause new infection in the host plant [88]. Non-circulative and non-propagative microbes are those which generally form a physical association with the insect, and are subsequently mechanically transmitted to the plant host (infection by an insect stylet that is coated with a pathogen, for example [89].

Over the last decade, the exploration of phytopathogenic bacteria and their interactions with insects has expanded beyond the traditional phytopathogen-vector
relationship to include cases where phytopathogens exhibit entomopathogenic associations. Most of these relationships have been characterized only recently, and represent a new paradigm in bacterial-insect interactions. Certainly, this has not lessened the focus on the traditional plant-microbe or vector-microbe association, but has provided additional breadth to the biology, ecology, and evolution of phytopathogenic bacteria. This review will examine the alternative associations of phytopathogenic bacteria with insects, focusing on the genetics and ecological relevance of those insects that can serve as either their primary host or vector. Also examined is one special interaction where the microbe exploits a single insect as both host and vector, and explore the possibility that this unusual interaction represents a transitionary phase in the evolution of phytopathogen-insect interactions.

Insects as vectors for phytopathogenic bacteria

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

*Xylella fastidiosa* is xylem-restricted, fastidious phytopathogen that causes citrus variegated chlorosis and Pierce’s Disease of grape [90,91]. *X. fastidiosa* is transmitted between plant hosts exclusively by xylem-feeding sharpshooter leafhoppers (Hemiptera, Cicadellidae) and spittlebugs (Hemiptera, Cercopidae) [92,93], which deliver the bacteria directly into the plant. Leafhoppers use their piercing and sucking mouthparts to penetrate the water-conducting xylem vessels of host plants to access the xylem sap, and if infected, extravasate *X. fastidiosa* through their food canal injecting the bacteria directly into the xylem vessels of the plant [94]. Once inside, the bacteria multiply and spread from the site of infection to colonize the xylem and form a biofilm [91,95,96,97]. From there, the bacteria spread to adjacent uncolonized xylem vessels, possibly through the pit membrane [91], resulting in the physical obstruction of water flow through plant tissues, causing leaf, shoot, and eventually, plant death [98].

The infiltration of key insect vectors into important grape and citrus farming areas of North America prompted a dramatic increase in the exploration of the epidemiology of *X. fastidiosa* and the role of insect vectors in pathogen dispersal [91,97,99,100,101]. The relatively recent introduction of the efficient leafhopper vector, the glassy-winged sharpshooter, *Homalodisca vitripennis*, and the blue-green sharpshooter, *Graphocephala atropunctata* (Signoret) into California resulted in Pierce’s Disease becoming a more aggressive and prevalent disease; however, because *X. fastidiosa* lacks vector-species specificity, as seen with many other phytopathogenic bacteria [102], nearly all sharpshooter species are able to transmit *X. fastidiosa*, albeit with differing transmission efficiencies [91]. Although both insect vectors are capable of transmitting *X. fastidiosa*,
the glassy-wing sharpshooter is often seen as a more efficient vector than the blue-green sharpshooter [99]. Transmission efficiency may be linked to feeding site preference since the blue green sharpshooter is known to have a preference for feeding on young tissue and leaves, while the glassy-wing prefers both young tissue and mature woody parts of the plant [100]. Linked to this is the fact that *X. fastidiosa* is found to be disproportionately dispersed within symptomatic plants, an attribute that may influence the acquisition of the pathogen, depending on the tendency of specific insect species to feed on tissues that may have lower bacterial concentrations [99]. Acquisition efficiency was significantly higher from plants which had a higher bacterial load, thus implying a direct correlation among bacterial concentration and vector transmission efficiency [99,103].

Following ingestion, the bacteria become localized to the insect foregut where they multiply and grow [104]. The pathogen can be transmitted immediately after acquisition [91,94,105], indicating that bacterial multiplication in the foregut of the insect vector is not vital for pathogen transmission, and that *X. fastidiosa* is a noncirculative vectored phytopathogen [94,99,105]. Although *X. fastidiosa* may propagate through non-circulative propagative transmission, the bacterium cannot be passed from parent to offspring, as neither transovarial (immature egg to adult) transmission nor transstadial (mature egg to adult) transmission have been observed for this bacterium [106,107]. In addition, infected new born nymphs generally lose their infectivity after molting their foregut cuticular lining [105].

The interaction of *X. fastidiosa* with its insect vectors appears to be determined by the *rpf* locus (regulation of pathogenicity factor) [108]. *X. fastidiosa* uses cell-to-cell
signalling mediated by a small diffusible signalling molecule known as DSF (Diffusible Signalling Factor) [91]. The production of DSF is dependent on the gene \textit{rpfF}, which has characteristics similar to long-chain fatty acyl CoA ligases [109,110,111]. Mutations in \textit{rpfF} caused a deficiency in the ability of the bacteria to form a biofilm in the insect host, despite being taken up from the plant [91]. Suprisingly, \textit{rpfF} mutants are hypervirulent in grape plants [108]. Likewise, mutation of a second locus, \textit{rpfC}, does not impair the ability of \textit{X. fastidiosa} to colonize the insect, but does alter its ability to be transmitted to new host plants [91]. It has been proposed that this is due to \textit{rpfC} mutants being stronger biofilm formers than the wildtype strain, which reduces the number of planktonic cells that can be released from the insect during feeding. For plant virulence, mutations in the \textit{rpfC} gene cause \textit{X. fastidiosa} to become deficient in longitudinal migration along the xylem vessel, resulting in lower growth and spread in grape stems than the wild type strain [91].

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

\textit{Pantoea stewartii and the flea beetle}

Stewart’s disease (or Stewart’s wilt) of corn, caused by the bacterium \textit{Pantoea stewartii}, (formerly \textit{Erwinia stewartii}) causes significant yield loss in dent and sweet corn as a result of leaf blighting [112]. The development of Stewart’s wilt has two
distinct symptomologies: wilt and leaf blight. In both cases the manifestation of disease initially begins once the bacterium has successfully invaded the leaf tissue through lesions produced by the flea beetle [112]. Upon entry, \textit{P. stewartii} multiplies within the leaves producing yellowish, water-soaked lesions or streaks that eventually elongate and later coalesce along the leaf veins of corn leaves and soon become necrotic [113,114]. The bacterium colonizes the xylem vessels, where their production of large amounts of bacterial exo/capsular polysaccharide (EPS), also known as stewartan, restricts the flow of free water, causing wilting, and this can be followed by a general browning and water-soaking of the stalk tissue [112,115,116].

The successful infection of corn plants by \textit{P. stewartii} appears dependent on the \textit{hrp/wts} gene cluster, which directs the synthesis of a type III secretion system [29,117]. Through transposon mutagenesis, Frederick \textit{et al.} (2001) identified the \textit{wtsE} gene, which encodes a 201-kDa protein which is strikingly similar to DspE in \textit{E. amylovora} and the protein AvrE found in \textit{P. syringae} pv. tomato, both of which have been implicated in virulence [118,119]. Additional work on \textit{P. stewartii} pathogenesis identified the involvement of a quorum-sensing (QS) system, which allows bacteria to monitor their population density by utilizing small, diffusible signals and to orchestrate the expression of specialized gene systems for pathogenicity [50,51,120]. Studies conducted by Koutsoudis \textit{et al.} [50] suggested a possible functional corollary between bacterial biofilm development and xylem colonization similar to that described for \textit{X. fastidiosa} infections of grape vine. From their research they recognized that QS system organized the timing and level of EPS produced, which significantly affects the degree of bacterial adhesion during \textit{in vitro} biofilm formation and propagation within the plant host. Moreover, their
microscopic studies revealed that \textit{P. stewartii} colonizes the xylem of corn with spatial specificity rather than by arbitrary growth to fill the lumen of the xylem, as seen with \textit{X. fastidiosa}.

\textit{X. fastidiosa} is disseminated among suitable host plants via a specific insect vector – the corn flea beetle, \textit{Chaetocnema pulicularia} – which acquires the pathogen while feeding on infected corn plants [113]. The pathogen becomes localized along the alimentary tract of adult corn flea beetles [121], where it remains for the entire duration of the insect’s life [112]. The beetles overwinter in the soil of grassy areas near agricultural fields for the duration of the winter season, and although colder winter temperatures reduce beetle survivorship, many beetles still survive to transmit the disease [112,114]. With the spring thaw, the beetles exit their dormancy stage and begin to feed, and deposit the pathogen into the feeding wounds via their feces, allowing \textit{P. stewartii} to enter the veins of corn leaves and cause disease [112,114].

Beetles that feed on infected tissue acquire the bacterium and promote the spread of the pathogen throughout the season [112]. The colonization of corn flea beetles by \textit{P. stewartii} appears to be mediated by a type III secretion system (T3SS) that is distinct from that used for colonizing the plant host [122]. Recently, Correa \textit{et al}. [123] studied a mutant strain of \textit{P. stewartii} DC283, which had a mutation in the \textit{ysaN} gene, a component of the second T3SS apparatus. They discovered that the beetles were able to acquire both the mutant and wildtype strains of \textit{P. stewartii} equally, but the \textit{ysaN} mutant did not persist like wildtype, and declined in frequency four days following acquisition. Through the use of confocal laser scanning microscopy, Correa \textit{et al}. (2008) demonstrate that \textit{P. stewartii} persists in the hindgut lumen of beetles, but does not invade the gut cells. \textit{P.}
*P. stewartii* is capable, however, of invading cells of Malphigian tubules that protrude from the gut of beetles. Correa *et al.* (2008) indicate that this supports previous studies that indicate that the most likely route of bacterial transmission is through insect frass. This is also supported by observations by Correa *et al.* (2008), who have observed flea beetles clustering together in small groups on maize leaves under growth chamber conditions, which they suggest would promote *P. stewartii* infiltration into plant tissues.

*P. stewartii* utilizes the corn flea beetle as a vector to disperse to corn plants, and this interaction appears to be facilitated at least in part by a T3SS. By extension, this would implicate the involvement of specific type III secreted effectors, which likely interact with host substrates to facilitate bacterial colonization of the insect. Although the actual mechanism of how *P. stewartii* colonizes their insect vector is not understood fully, it appears that the phytopathogen has adapted to the beetle, which promotes its dissemination.

*Serratia marcescens* and the squash bug

*Serratia marcescens* is a phloem-resident pathogen that causes cucurbit yellow vine disease of pumpkin (*Cucurbita moschata* L.) and squash (*Cucurbita pepo* L.), which is characterized by wilting, phloem discoloration, and yellowing foliage [124,125,126]. Recent studies have shown that *S. marcescens* produces a biofilm along the sides of the phloem tissues of the plant once inside its host, blocking the transport of water and nutrients and eventually causing the plant to wilt and die [127,128]. A genetic screen to identify genes that modulate biofilm formation in *S. marcescens* revealed the
involvement of fimbrial genes, as well as an oxyR homolog, a conserved bacterial transcription factor having a primary role in oxidative stress response [129].

*S. marcescens* is transmitted by the squash bug, *Anasa tristis* (DeGreer), which is commonly found throughout the U.S. as well as between Canada and Central America [130]. The squash bug feeds on its plant host using piercing-sucking mouthparts, which penetrate intracellularly through the tissue, and are directed towards the vascular bundles of the plant vasculature [131]. The visible signs and extent of feeding damage to squash plants correlate with the number and size of bugs, as well as the amount of time each bug spends on the plant and on the feeding site [131]. Long term feeding on the fruit leads to fruit collapse, while leaf feeding induces isolated necrotic lesions [131]. Early experiments revealed the presence of starch granules in the gut of *A. tristis*, which are only found in the cytoplasm of plants, suggesting that the squash bugs ingest the intracellular contents of plant cells [132]; however, experiments in which squash bugs were allowed to feed on plants having safranin-stained xylem fluid found the red dye in the gut of the insects, suggesting that xylem is also a food source for the insects [131]. Surprisingly, squash bug feeding damage extends beyond the xylem vessels and into the phloem. Areas adjacent to the spongy mesophyll of the leaf and the cells of the palisade and epidermal layers of leaves also exhibit signs of localized feeding-induced injury [131,133,134]. Extensive feeding on the stem can damage the vascular tissue of the plant, thereby resulting in the wilt of the leaf apical to the feeding site, or wilt of the entire plant if it is a seedling [131,133,135]. Heavy feeding can cause leaves to turn black and soon become crisp [130].
Early studies examined the colonization of the squash bug by *S. marcescens*. Wayadande *et al.* [94] initially hypothesized that *S. marcescens* shared a similar relationship with its insect vector as seen between *X. fastidiosa* and sharpshooters, where the bacteria are localized to the foregut of the insect vector and are released through the food canal during successive feeding bouts; however, upon examination of the foregut of adult and nymph squash bugs allowed to feed on bacteria-infiltrated squash cubes, the foregut cibaria of the infected insects were found to be clear of any bacteria-like structures. From their results, Wayadande *et al.* concluded that the ability of *A. tristis* to transmit *S. marcescens* after molting indicated that the hemocoel, and not the gut, acts as a possible site of retention for the infectious bacteria. This is in contrast to work showing that *S. marcescens* is pathogenic once introduced into the haemolymph of *A. tristis* [94,136,137].

The incubation time (or latent period) of *S. marcescens* was shown to be very short, with some adults being capable of transmitting the bacterium one to two days after initial acquisition [136]; however, adult squash bugs upon bacteria-infiltrated squash fruit cubes, were noted to transmit the bacterium only sporadically to squash plants within a twenty one day testing period [94]. This short latent period coupled with an irregular transmission patterns are indicative of a noncirculative mode of transmission [105,136]. Despite its noncirculative association, *S. marcescens* overwinters in the dormant insect vector, a strategy that protects the pathogen against low winter temperatures, ensuring a high survival rate and thus successful transmission to plants in the following season [138].
**Erwinia tracheiphila and the cucumber beetle**

Bacterial wilt is a serious threat to commercial melon and cucumber production in some parts of the world, including parts of North America. Bacterial wilt is caused by the bacterium, *Erwinia tracheiphila*, which is transmitted by both the striped cucumber beetles (*Acalymma vittata*) and spotted cucumber beetles (*Diabrotica undecimpunctata*) [139]. These beetles are attracted to their host by cucurbitacins, a group of secondary plant metabolites that are commonly found within the plant family Cucurbitaceae [140]. Cucurbitacins are bitter toxic compounds [141], which are known to accumulate in cucumber beetles and confer protection against predation [142,143], but have detrimental effects on most invertebrate and vertebrate herbivores [144,145]. The preferred plant hosts of *E. tracheiphila* are wild and cultivated cucurbits, including muskmelon, pumpkin, gourd, and squash, with cucumbers being the most susceptible hosts [146].

Mechanical wounding of the plant tissue is necessary for bacterial infection, since the bacterium cannot infect the cucumber plant through the normally found openings (stomates and hydathodes) of a plant [139]. While feeding on infected cucurbits with their piercing and sucking mouthparts, the cucumber beetle acquires *E. tracheiphila*, which then migrates to the insect gut epithelium. While infected beetles feed on healthy cucurbit plants, bacteria are deposited on the leaves via beetle fecal droppings, which leach into the lesions created by the feeding beetles [147]. *E. tracheiphila* can only migrate toward a wound providing there is a sufficient aqueous film on the leaf surface [139], although the cucumber beetles’ stylet can also become infected with the pathogen, providing a direct, mechanical method of infection [147]. Once inside the plant, *E. tracheiphila* spreads to the xylem vessels, multiplies, and infects all parts of the plant. As
the bacterium multiplies in the xylem, the efficiency of water transport is reduced to less than one-fifth of normal water flow, resulting in extensive plugging of the vessels and the subsequent wilt of the plant [146].

Although little is known about the interaction between *E. tracheiphila* and the cucumber beetle, there appears to be some level of coevolution. The bacterium is able to overwinter in the digestive tract of its vector, and is able to escape through the fecal droppings, without any adverse impact on its insect vector. Although the insect is left with the burden of carrying the bacterium for the rest of its life, its association with the pathogen does not drastically affect its lifestyle. The existence of any coevolutionary processes leading to the formation of the interaction between *E. tracheiphila* and the cucumber beetle is still unknown.

*Erwinia amylovora and pollinators*

*Erwinia amylovora* is the causal agent of fire blight of apple and pear, a detrimental bacterial disease of rosaceous plants, infecting primarily significant pear and apple varieties [148,149]. The effects of *E. amylovora* on apple and pear trees have been catastrophic, causing the death of blossoms, shoots, limbs, and at times, entire trees [65]. The primary infection site of the pathogen in fire blight disease is through tree blossoms [148,150,151], which begins with bacterial colonization of the stigma, reproduction on the stigmatic surface, migration along the length of the style, and eruption into the host tissue via the nectarthodes [149,152]. Stigmas, which are borne on the ends of the style, have been demonstrated to be the principal site of epiphytic colonization by *E. amylovora* [65,150,152,153,154,155]. Despite the generalization that aerial surfaces of plants like
stigmatic surfaces are unreceptive to bacterial growth due to exposure to ultraviolet radiation and varying osmotic pressure, stigmas provide *E. amylovora* with a nutrient-rich, protected, and hydrated environment for growth [65]. Micrographs showing *E. amylovora* growing mostly within the large intracellular spaces between the secretory papillae of stigmas have reaffirmed this [153,154].

*E. amylovora* has a non-specific association with pollinating insects that travel from tree to tree collecting nectar [65], including honey bees, *Apis mellifera* (family *Apidae*), which have been shown to be extremely efficient vectors [156]. To investigate which species of insects were able to transmit *E. amylovora*, Emmett and Baker (1971) inoculated various insects with *E. amylovora* and transferred the insects to apple and pear blossom trusses and evaluated rates of tree infection. Several insects were able to transmit the bacterium and induce infections in blossoms and shoots, although it appeared that larger species of insects, like bees, were more efficient in transmitting the pathogen to blossoms in comparison to smaller species of insects, such as anthomyiid flies. Larger insects were able to infect more trusses and more flowers per truss, possibly due to their ability to carry more inoculum, as well as their larger overall migration distances [156].

There have been no conclusive studies demonstrating that the bacterium enters and colonizes insects; rather, there is overwhelming evidence that *E. amylovora* adheres to the external surfaces of its insect vectors and is subsequently transmitted to healthy plants mechanically. In one experiment by Hildebrand (2000) *Aphis pomi* was surface contaminated with fluorescent *E. amylovora* through exposure of a thin lawn sprayed with the bacterium. Over several consecutive days, aphids were crushed, plated, aliquoted, and bacterial presence analysed by PCR. Results revealed florescent bacteria
on the legs, cornicles, proboscis, and on the antennae of the aphids [157]. Persistence of bacteria on insect surfaces has been shown to be at least seventy two hours on *Aphis pomi* [158], nine days on the flesh fly *Sarcophaga carnaria* [159], five days on the green lacewing (*Chrysoperla carnea*) [157], and up to twelve days on some aphid species, likely facilitated by the exopolysaccharide capsule of the bacteria [157].

Because there is no evidence of bacterial internalization by insects, overwintering of *E. amylovora* appears to be within the canker on its host plant. Once spring emerges and temperatures are favourable, bacteria ooze from the cankers, and cause an infestation of the blossom [160]. This process is contrary to *P. stewartii*, *S. marcescens*, and *E. tracheiphila*, which overwinter in their specific dormant insect vector to protect themselves from the harsh winters.

*Candidatus Liberibacter and citrus psyllid*

*Candidatus* Liberibacter is a phloem-limited phytopathogenic bacterium that causes huanglongbing disease (HLB) or citrus greening on citrus fruits around the world [161,162]. *Ca. Liberibacter* has a semi-specific symbiotic relationship with two different psyllid insect vectors: *Diaphorina citri* Kuwayama [163] and *Trioza erytreae* (del Guercio) [164]. *D. citri* is the principal vector in Asia, Brazil, and Florida, while *T. erytreae* transmits *Liberibacter* in Africa [162]. *D. citri* has been in existence in Brazil for over 60 years [165,166] and in Florida since 1998 [167]; however, HLB appeared in both locations simultaneously. The psyllid has also been reported in areas of Texas in 2001 [168] as well as in several other countries in the Caribbean basin [169].
HLB has been divided into Asian and African strains based on the influence of temperature and host symptoms. In Asia the bacterium of HLB has been characterized as Candidatus Liberibacter asiaticus (Las), where it infects the majority of citrus cultivars and causes extensive economic loss by limiting the lifespan of infected trees [170,171,172,173]. Las is heat tolerant, and can produce HLB symptoms at temperatures above thirty degrees [171,174], whereas the African species, Candidatus Liberibacter africanus, is heat-sensitive and does not cause symptoms above thirty degrees [171,174]. Recently, a new species of Candidatus Liberibacter was identified, which was unique from the other three species because it caused disease in solanaceous plants, and was vectored by a different psyllid species, Bactericera cockerelli [175]. B. cockerelli is a polyphagous phloem feeder, which can productively reproduce on a wide variety of host plant species, but has predominantly been a pest of potato (Solanum tuberosum L.) and tomato (Solanum lycopersicon L.) [175,176,177].

Las-carrying T. erytreae and D. citri psyllids are efficient vectors of HLB, which carry the bacteria in the haemolymph and salivary glands [178,179]. Work by Hung et al., (2004) demonstrated that infected nymphs, which are barely mobile, quickly develop into Las-carrying adults with the capability to fly and transmit the pathogen to other citrus plants. They show that Las cannot be detected at all in first instars, suggesting that first instars are incapable of carrying the pathogen. Second instars, however, do carry the pathogen, but at extremely low titre. Psyllids are therefore able to bear the bacterium in either adult or nymphal stages, but not as first instars. Hung et al. (2004) also demonstrate that bacterial titre increases with each instar, suggesting that the pathogen replicates during vector metamorphosis [171], and can therefore be considered
propagative [162]. In a separate study, HLB was found to be present at a higher infection frequency in eggs, first instars, and second instars isolated from potato host plants than from those isolated from tomato [175]. Psyllids from potato were found to have a fixed concentration of bacteria from the first instar stage to the adult phase, whereas those isolated from tomato had very low titres at the egg and first instar phase, which increases considerably in the second instar stage and becomes fixed at the third instar period [175]. This suggested that Ca. Liberibacter is vertically transmitted, but its rate is dependent on the host plant from which it was isolated. This is in direct conflict to previous reports that Las persists in the adult insect vector for twelve weeks and does not directly transmit Las to its offspring [171].

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

*Pectobacterium* and the fruit fly

*Pectobacterium carotovorum* (formerly *Erwinia carotovora*) is member of the Enterobacteriaceae [180] and the causal agent of the tuber-borne lethal potato blackleg disease [181]. The pathogen produces pectolytic enzymes, which target plant cell walls and lead to their complete destruction [182]. Production of these exoenzymes is
controlled by a global regulatory mechanism, and more specifically, the expI gene [183]. expI mutants are deficient in exoenzyme production, and are completely avirulent as they can neither break down the plant tissue nor multiply within potato plants [182,183,184]. expI has a general signalling function, and directs the synthesis of a signal molecule that is involved in cell density-dependent control of exoenzyme genes in P. carotovorum. Pirhonen et al. (1993) demonstrated this hypothesis, through extracellular complementation of the defect in exoenzyme production, where the diffusible signal molecule produced by ExpI-proficient cells can be recognized by the mutant and subsequently used to activate exoenzyme gene expression.

In addition to causing disease in potato, P. carotovorum also has another suitable host: the fruit fly, Drosophila. In 2000, Basset et al. (2000) identified a strain of P. carotovorum, Ecc15, which provoked a systemic immune response in Drosophila larvae following natural ingestion [185,186]. Feeding of larvae with living Ecc15 resulted in them having high expression of antimicrobial peptide genes in their fat body, which is functionally analogous to mammalian liver [187]. Although this bacterial strain did not appear to be pathogenic to its insect vector, its ability to induce a systemic immune response implied that it may have infectious properties that can be recognized by the Drosophila innate immune system [188]. Out of the sixteen Ecc strains tested by Basset et al. (2003) [188], only three were found to have the ability to infect Drosophila larvae by natural infection. Such a result enabled Basset et al. to hypothesize that there may be specific genes that permitted Ecc15 to associate with its insect vector [185].

Using a genetic screen, Basset et al. in 2003 identified two genes that are required by P. carotovorum to cause infection in Drosophila. One gene, evf, enabled persistence in
the host [188], and was controlled by the *hor* gene – a key regulator capable of conveying signals from various environments to effectors involved in both plant pathogenesis and *Drosophila* infection [188]. Transfer of the *evf* gene to non-infectious *Pectobacterium* strains or to other enterobacteria was found to improve the ability of the bacterium to survive in the gut of *Drosophila* and trigger an immune response [188]. The fact that the gene *evf* was found in only a few *P. carotovorum* suggested that this gene had been acquired recently through horizontal gene transfer [188]. When the *evf* gene was overexpressed in *P. carotovorum*, bacteria were able to colonize the apical side of the gut epithelium and at times to spread to the body cavity [188].

The expression of the *evf* gene results in the accretion of bacteria in the anterior midgut and radically influences gut physiology [189]. It was suggested that *evf* could disrupt the peritrophic membrane, which is a chitinous membrane that outlines the insect vector’s gut and averts bacteria from coming into the gut cells [188]. Basset *et al.* (2003) also proposed that *evf* could allow the propagation of bacteria in this environment, or produce a toxin which could disrupt the physiology of the gut cells. Recent crystal structure data of Evf shows it to be an α/β protein having a novel fold and intricate topology, with evidence for a palmitoic acid being covalently linked to the 209 cysteine residue of the Evf protein through an association with a thioester linkage, suggesting that Evf may be targeted to membranes [190]. Palmitoylation, a post-translational modification that increases the affinity of soluble proteins for lipid membranes [191,192], is necessary for biological activity as shown by the abolishment of Evf function following mutation of the key cysteine residue required for palmitoylation [190]. Surprisingly, Evf was found to be present in the cytoplasm, not in the periplasm [189], but was shown to
bind to model membranes and promote aggregation. In subsequent studies, Quevillion-Cheruel et al. (2009) showed that the overexpression of the Evf protein promoted bacterial accumulation in the gut in an arrangement typical of an organized community, as seen in a biofilm, and suggest that the ability of the Evf protein to be able to amass bacteria may be due to its capacity to interact with and promote aggregation of vesicles. Quevillion-Cheruel et al. (2009) concluded that the function of the Evf protein must be related to post-translational modification, where the biological function of the evf gene may be more directed towards membrane anchoring of the protein.

One Ecc strain, Ecc1401, which did not possess the evf gene, was able to induce an immune response in Drosophila, suggesting there may be additional factors involved in host association. P. carotovorum can effectively spread from plant to plant via Drosophila, and although Drosophila may not be its intended carrier, there is evidence for adaptation of the bacterium to this host that results in efficient bacterial association, retention, and ultimately, dispersal.

**Insects as primary hosts for phytopathogenic bacteria**

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

*Dickeya dadantii and the Pea Aphid*

*Dickeya dadantii* (formerly *Erwinia chrysanthemi*) is a member of the *Enterobacteriaceae* and the agent of soft rot disease of a wide range of economically important crops, including potatoes and maize [193]. Disease develops following movement of the pathogen from the stem base throughout the tissues, producing a brown staining of the vascular tissues, and occasionally necrosis and hallowing of the stem [194]. *D. dadantii* causes the rapid disruption of parenchymatous tissues, principally induced by the use of its pectic enzymes, and accelerates the disease process with cellulases, iron assimilation, a type III secretion system, exopolysaccharides, motility, and proteins involved in resistance to plant defences [195,196,197,198,199]. Despite its long history as a typified plant pathogen, *D. dadantii* strain 3937 was shown to be a pathogen of the pea aphid, *Acyrthosiphon pisum* [195]. Grenier and colleagues (2006) determined that *A. pisum* aphids that had ingested *D. dadantii* eventually succumbed to their infection, with the minimum infectious dose of *D. dadantii* being calculated as fewer than ten bacterial cells [195]. Recent genome sequencing of the *D. dadantii* strain 3937 revealed the presence of four genes encoding homologues of insecticidal toxins, which were hypothesized to contribute to the pathogenicity of the bacterium in the aphid [195,200]. These homologues were later found to be able to complement the *cyt* family of genes from *Bacillus thuringiensis*, which encode haemolytic toxins [201]. Gut proteases were hypothesized to cleave and activate the *D. dadantii* Cyt toxins in the aphid, resulting
in pore formation in the insect gut membrane, and leading to bacterial invasion of the aphid and eventual death; however, the Δcyt mutant retained virulence, suggesting that other virulence genes or factors are involved [195,202]. The cyt-like toxins may therefore be involved in the early colonization of the aphid digestive tract, which is consistent with what is known for the *B. thuringiensis* homologues [203].

In a later study, Costechareyre and colleagues [204] found that the four coregulated cyt genes are expressed in response to high osmolarity. They suggest that this is because *D. dadantii* is commonly found in the low osmolarity intercellular fluids of its host plant, where toxin synthesis is likely not necessary; however, a high concentration of sucrose is prevalent in the phloem sap, which would trigger toxin production if bacteria are internalized in a phloem-feeding insect gut, like that of aphids. Further exploration revealed that cyt gene expression is repressed by both hns (histone-like nucleoid structuring protein) [204] and vfmE, a regulator of plant cell wall-degrading enzymes [205], since both hns and vfmE mutants retained the pathogenicity of wildtype. PecS, a regulator of pectinases and cellulases [206] appeared to regulate cyt gene expression since pecS mutants were found to be non-pathogenic when ingested by the aphid. Mutants of the GacA, OmpR and PhoP regulators, which are involved in plant pathogenesis [207,208,209] and do not appear to affect Cyt toxin production, had reduced virulence in the aphid. The Cyt toxins, which are expressed under very specific conditions, are therefore only part of the suite of virulence factors used by *D. dadantii* for causing disease in aphids.

The relatively low minimum infectious dose of *D. dadantii* required for aphid infection could suggest high infectious rates for aphids overall, as this low density can be
easily acquired from plant surfaces, or from feeding on contaminated vascular tissues [204,210,211]. It is unclear whether \textit{D. dadantii} is transmitted readily to healthy plants by the aphids, or whether this represents a more opportunistic or general association.

\textit{Erwinia aphidicola and the Pea Aphid}

\textit{Erwinia aphidicola}, a member of the \textit{Enterobacteriaceae}, has been identified as the causal agent of leaf spot disease of common bean (\textit{Phaseolus vulgaris}), and chlorosis and necrosis of pea (\textit{Pisum sativum cv. Tirabeque}) [212,213]. In addition to causing plant disease, \textit{E. aphidicola} also exhibits pathogenicity toward the pea aphid, \textit{A. pisum}. Harada \textit{et al.} (1997) [214] initiated the study of a mysterious bacterium, called bacterium X, which was found to infect the gut of insects that had been kept aseptically. The bacterium, which was later identified as \textit{E. aphidicola}, could grow productively in the aphid gut, inhibiting post-final ecdysis and resulting in mortality of the adult insect.

Harada and Ishikawa (1997) were able to note that the bacterium produced extracellular polysaccharides when they were left to grow in a medium containing sucrose, trehalose or their component monosaccharides. This capsule may not be essential to cellular function, but it may allow certain saprophytes to attach to areas where there is an abundance of nutrients, or allow certain pathogens to avoid engulfment by phagocytes [215]. The capsule layer may have been contributing to the attachment and colonization of the pathogen in the aphid gut. The cause of aphid mortality following colonization was proposed to be due to the aseptic conditions of the aphid gut, since normal gut colonizers would help maintain \textit{E. aphidicola} densities in check [215]. Still there are likely many
genetic factors that contribute to the colonization of the gut, yet these still remain unexplored.

\textit{Pantoea stewartii and the Pea Aphid}

\textit{P. stewartii}, the Stewart’s wilt pathogen, which normally associates with its flea beetle vector, was recently found to exploit the pea aphid as a host. In 2010, a study by Stavrinides \textit{et al.} (2010) showed that \textit{P. stewartii} DC283 (DC283) was pathogenic toward the pea aphid, as aphids fed a single dose of DC283 began to accumulate bacteria in their gut, with titres reaching $5 \times 10^8 \text{ cfu}$, and aphid death following within 72 hours. To identify the specific genetic determinants that are involved in pathogenesis, and more specifically, those that contribute to lethality of the aphid, a transposons mutagenesis screen was conducted. A single locus, which was termed \textit{ucp1} (you cannot pass), was identified that appeared to be essential for aggregation and pathogenicity of DC283. \textit{ucp1}-proficient bacteria formed aggregates in the crop and hindgut, whereas the \textit{ucp1} mutant did not. Aggregates of \textit{ucp1}-expressing bacteria were suggested to be more resilient, accumulating in the crop and hindgut until the point of barricading the flow of honeydew. This result coincides with the structural and functional features of \textit{ucp1}, which included several predicted transmembrane domains, suggesting membrane localization and possible substrate or matrix binding capabilities.

Six potential homologs of Ucp1 were identified in the draft genome of DC283, all of which were found to share a highly conserved N terminus, but an entirely non-homologous C terminus [216]. The conserved N terminus contains the transmembrane domains, and prediction of protein localization places the hypervariable C terminus
facing the extracellular environment. Based on this, *ucp1* was proposed to function as a Microbial Surface Component Recognizing Adhesive Matrix Molecules (MSCRAMM) - a family of adhesion proteins utilized by animal pathogens to bind to proteinaceous components of the eukaryotic host cell to permit pathogenesis [217]. To lend credence to this hypothesis, all seven related genes were expressed in *E. coli*, and each line fed to aphids. Only *ucp1* was necessary and sufficient for pathogenicity in the aphid, whereas the other lines were avirulent like control lines. In addition, *E. coli* lines expressing the protein exhibited the same aggregation phenotype as seen for wildtype DC283, suggesting that this protein was necessary and sufficient for this phenotype. It was unclear, however, if this protein was involved in direct binding to an aphid gut receptor, and whether the other six related proteins could bind to other matrix molecules in different hosts. The drastic variability seen in the potentially exposed C terminus of all seven proteins could be the direct result of genetic shuffling or pathoadaptation imposed by host immune pressures [216,218,219,220]. Alternatively, it was proposed that the C terminus of Ucp1 does not bind to eukaryotic proteins, but instead to other exposed Ucp C termini of nearby cells, thereby promoting the linking of the structures to produce a bacterial matrix. Although its precise function is unclear, the *ucp1* locus appears essential for the pathogenicity of *P. stewartii* in pea aphids.

**One insect for all occasions**

Many insects are efficient vectors of phytopathogenic bacteria, transporting them to candidate host plants in the environment. In these associations, the bacterium is not often considered to harm its vector, although there may be a reduction in the fitness of the
insect as a result of carriage [216]. These interactions can be characterized as commensalistic or slightly parasitic depending on the specific insect-bacterium association. In contrast, some insects have been shown to function as a primary host for bacteria, and are exploited by these pathogens as equivalents to their plant hosts. In cases where the insect is exploited as a primary host, the association is effectively parasitic, with the fitness of the bacteria increasing at a cost to the insect. These bacteria exhibit entomopathogenic characteristics, utilizing specific virulence factors to overcome insect host defences, propagate, and disperse. But, what if an insect can function as both a primary host and a vector for a given phytopathogen?

Although there is a tendency for us to categorize the interactions among organisms into discrete groups, the general biology and ecology of many phytopathogens and their interactions with other organisms in the environment are still rather nebulous. More recent studies of phytopathogens have uncovered unique interactions with insects, including one case where the insect appears to serve as a suitable primary host, as well as a vector. This particular association raises several interesting issues, including the difficulty of reconciling the commensalistic and pathogenic life stages of the pathogen. Pathogens are known to reach a virulence optimum, which maximizes their aggressiveness and transmission potential [210]. High levels of aggressiveness may result in the death of the host insect before the bacterium is able to disperse to other hosts; thus, natural selection will favour a reduction in the aggressiveness of the pathogen to permit dispersal of the bacterium and thereby increase bacterial fitness [221]. To overcome this trade-off, the bacterium can exploit its insect host maximally by replicating rapidly, yet this would come into direct conflict with the dynamics of association between a typical
phytopathogen and vector. For this last example, the application of the fundamental concepts of host-microbe associations is complicated by the fact that the insect can function as both a host and vector. It is an especially exciting interaction for the simple idea that it may represent a transitional stage in the evolution of phytopathogen-vector and phytopathogen-host associations.

**Pseudomonas syringae and the Pea Aphid**

*Pseudomonas syringae* is a phytopathogenic bacterium noted for its diverse interactions with different plant species. Although many strains are known to cause disease on various plants, many epiphytic strains have also been identified [222]. *P. syringae* propagation onto and between host plants involves rain splash-mediated inoculation from infected to uninfected plants, facilitated by the aggressive epiphytic and aggregation capabilities of *P. syringae*. *P. syringae* has also been shown to disperse via precipitation [223].

*P. syringae* was considered a very strict phytopathogen, capable of infecting a variety of different plants [224]; however, a recent study by Stavrinides *et al.* (2009) demonstrated that some strains of this species have entomopathogenic potential. The bean strain, *P. syringae* pv. *syringae* B728a (B728a), which is an aggressive epiphyte and pathogen of bean, was shown to exploit the pea aphid as a suitable alternative primary host. Within 36-48 hours of ingesting B728a, aphids succumb to infection with growth of up to $3 \times 10^6$ colony forming units (cfu) per aphid. In contrast, ingestion of the tomato strain *P. syringae* pv. *tomato* DC3000 (DC3000) by aphids, results in bacterial titres of $1 \times 10^9$ cfu/aphid, with no evidence of disease, and aphid survivorship remaining
unaffected beyond 72 hours. This suggests the presence of strain-specific virulence factors contribute to the colonization of the aphid by B728a.

Whole genome comparisons of DC3000 and B728a identified toxin complex (tc) genes in both strains whose homologues have been implicated in insect association [225]. The tc genes present in DC3000 appeared degenerate, with mobile genetic elements and deletions disrupting the reading frame, whereas the orthologs in B728a were intact. These genes were strong candidates for explaining the virulence of B728a and the avirulence of DC3000. Mutation of two of the B728a tc genes does not attenuate virulence, indicating that these genes were not the primary virulence determinants for B728a in the aphid. To identify those genetic factors that were involved in aphid colonization, Stavrinides et al. (2009) performed a mutagenesis screen to identify mutants that had reduced or abolished virulence. Multiple hypovirulent B728a mutants were recovered, including one that was defective in the fliL gene, which is required for flagellar formation. The fliL mutant was completely avirulent, growing to tires of $4 \times 10^7$ cfu/aphid, which were not lethal to the aphid, much like the avirulent DC3000 wildtype strain. To identify the phenotypic effects of the fliL mutation, various motility assays were undertaken. A swarming assay revealed that the fliL mutant was incapable of swarming, a type of movement commonly seen in bacteria that allows for coordinated movement over a solid or semi-solid surface. It is unclear, however, if it is swarming specifically that is required for virulence in the aphid, or whether there are pleiotropic effects, where motility regulates virulence factors.

In exploring the pathogenicity of B728a toward the aphid, Stavrinides et al. (2009) noted that infected aphids exhibited some very unusual behaviors. After the onset
of severe disease, aphids would discontinue feeding and commence to wander around, depositing and moving honeydew behind them. Stavrinides et al. (2009) hypothesized that the honeydew that was passing through the aphids contained B728a. Using a simple culturing method, they found that viable B728a was present in the deposited honeydew at incredibly high doses, suggesting that the epiphyte propagates in the aphid, and then is redeposited back onto plant surfaces; however, since many of these feeding experiments were performed under artificial conditions, Stavrinides et al. (2009) attempted to demonstrate that the aphid could indeed become infected by feeding on plants that were colonized epiphytically by B728a. Aphids were introduced onto plants that had been surface-inoculated with B728a, and after a feeding period, aphids were harvested and screened for the presence of B728a. Aphids were shown to acquire the bacteria, which likely colonized the digestive tract, multiplied, and were then excreted in the aphid honeydew. The acquisition of the bacterium by the aphids most probably occurs via stylet-mediated plant host probing, which occurs when aphids land on a plant and attempt to determine if it is a suitable plant host [210,226,227]. Infection by epiphytic bacteria may occur during this process, where aphids repeatedly push their stylet through the host tissue, pushing down any surface bacteria, and then ingesting those bacteria while sampling plant fluids. Under this model, aphids acquire the pathogen during probing of an epiphytically-colonized plant host, with the ingested bacteria subsequently colonizing and propagating within the aphid. The bacteria escape from the aphid via the honeydew, and are deposited back onto the plant surface where they are given an opportunity to reassociate with their plant host. At this point, the aphid functions as a vector.
To determine the amount of inoculum deposited on the plant surface by infected aphids, Stavrinides et al. (2009) introduced infected aphids onto host plants, and bacteria densities quantified following a feeding period. The phyllosphere was shown to be inoculated with up to $2 \times 10^7$ phytopathogenic bacteria per square centimeter per aphid, suggesting that the aphid is an excellent culturing vessel for this phytopathogen. They propose that because honeydew is carbohydrate rich, the deposition of bacteria in a suspension of nutrients may enable *P. syringae* to enhance its survival and perseverance on the surface of the leaf. Certainly, because B728a is pathogenic to the aphid, successful deposition onto the leaf would have to occur quickly, and before the death of the aphid. In the case of DC3000, however, the bacteria do not kill the aphid, making this association more consistent with a true vectoring relationship.

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

**Aphids and Insect Defenses**

It is particularly interesting that many of the interactions between insects and bacteria described above have involved the aphid – one of the most destructive
agricultural insect pests[215]. These insects are able to incapacitate plants as sap feeders, pollutive excreters, toxifiers, and as well indirectly as vectors of viral diseases [215]. Their close association with a variety of plants predisposes them to a diversity of epiphytic and phytopathogenic bacteria [216]. Several studies have highlighted the general affinity of the members of the Enterobacteriaceae for aphids, many of which colonize the aphid gut [215,228]. So are aphids more susceptible to pathogen attack than other insects?

Insects have evolved specific behaviours that allow them to avoid predation, environmental stressors, and pathogens, but when these stressors bypass the defensive behaviours, insects must rely on the physical defences, such as those provided by their protective cuticle or gut pH level for defense [229,230,231,232,233]. If these barriers are also breached, immunological defence mechanisms such as clotting, encapsulation, phagocytosis, and the synthesis of antimicrobial substances come into play [233,234,235]. Analysis of the recently sequenced genome of the pea aphid has revealed that aphids do have defense mechanisms found universally in other arthropods, including the JAK/STAT and Toll signalling pathways, which are involved in both development and immunity; however, several essential genes involved in innate immunity of arthropods are absent from the genome, including the IMD signalling pathway, c-type lysozymes, defensins, and PGRPs. The absence of these genes may be due to an inability to locate homologues due to the large evolutionary distance between aphids and the taxa from where such genes are well studied [233], or due to aphids possessing an alternative, yet equally effective immune response. There is little evidence for the latter. It was also suggested that unlike Drosophila whose source of food is constantly contaminated with a
diverse array of microbes, aphids would only encounter entomopathogens and bacteria in the phloem sap of plants very rarely, therefore not requiring a more developed defense arsenal [236]; however, through probing of plants, aphids have been shown to contact and ingest a diversity of epiphytic bacteria, both pathogenic and non-pathogenic [210,216,233]. Another possibility is that aphids invest in terminal reproduction when faced with an immune challenge in contrast to spending extensive amounts of energy attempting to defend themselves [233,236]. Indeed, stabbed aphids generated more offspring than those who were untreated [236], although this is also seen in crickets, [237], waterfleas [238], and snails [239,240], which appear to have more developed immune systems [233]. Interestingly, the secondary endosymbionts such as Hamiltonella defensa, which provides protection against the parasitoid wasp, Aphidius ervi, and Regiella insecticola, which protects against fungal pathogens [233], persist within the haemolymph and are detected and managed by the aphid immune system.

**Evolution of alternative associations**

Bacterial phytopathogens have been, up to now, considered just that – bacteria that are capable of colonizing, reproducing, and disseminating from only plant hosts; however, it is now very evident that these plant pathogenic bacteria have the ability to exploit insects with which they share an overlapping niche as alternative primary hosts. Many interesting questions arise from this including those relating to general ecology, pathogenic potential, and host-specific virulence factors. For example, the ability of a microbe to associate intimately with two hosts across two different kingdoms likely compels an evolutionary struggle for the microbe, which must evolve host-specific
strategies for associating with each of its hosts. A phytopathogen will undergo adaptation of its overall aggressiveness toward its plant host in order to achieve its fitness optimum, but this optimum may be different in the insect vector or host, requiring the pathogen to achieve an intermediate multi-host optimum (Figure 2). The X. fastidiosa rpfF gene, which is required for insect association causes a reduction in plant virulence [91,108], illustrating that there are tradeoffs associated with multi-host associations.

Aside from the complexities of multi-host associations, the directionality of host association is also an interesting paradigm. In the majority of interactions described above, insects serve as either vectors or alternative hosts for phytopathogenic bacteria, and in cases where the insect presently serves as a vector, the phytopathogen uses the insect cavity as a transport vehicle for moving to its next plant host. But how did these relationships develop? The association of phytopathogens and insects may have begun with insects feeding transiently on plant tissues colonized by phytopathogens. Internalized bacteria survived the conditions of the insect cavity as well as immunological defenses to be dispersed successfully to a new host (Figure 3). The reiteration of this process over evolutionary time would have selected for those bacterial variants whose fitness increased as a result of this interaction – namely, those that were capable of surviving in the insect, were less immunogenic, and/or had higher replication and dispersal as a result of associating with the insect. This would have resulted in many of the interactions that exist today between phytopathogens and their vectors; however, did this association begin so pleasantly? Phytopathogens may have first evolved entomopathogenicity and began colonizing insects as alternative hosts following recurrent encounters over the course of evolution. These interactions may have then
converted to a more benign association, where the entomopathogen became substantially reduced in virulence, but capable of maintaining its association with specific insect hosts long enough to ensure its dispersal. In any host-microbe relationship, the specific tradeoffs endured by each partner will dictate the strength and overall success of the association. In the associations where phytopathogens are transmitted by vectors, by definition, there needs to be a very low cost to the insect for carrying the bacterium; however, there may often be a slight cost to carriage that can destabilize the success of the association [241].

It is interesting to note that many of the phytopathogens shown to have alternative insect associations are in the Enterobacteriaceae [242] – a group that generally associates with animal and insect hosts. So, did these phytopathogens evolve entomopathogenicity, or were they, in fact, insect-associated microbes that evolved phytopathogenicity, but still retain their ancestral insect-association lifestyle? If these bacteria were once insect-associated, either entomopathogens or insect commensals, they may have evolved phytopathogenic capabilities after repeated deposition on plants over evolutionary time (Figure 4). An increase in fitness that result from repeated encounters with their own insect hosts or other insects in the environment would have contributed to the maintenance of the determinants necessary for insect association. Many of the enteric plant pathogens described here seem to retain their ancestral gut-associating capabilities. Phytopathogenic Enterobacteriaceae including, Erwinia, Dickeya, Serratia, and Pantoea, retain relatively tight pathogenic and non-pathogenic associations with herbivorous- and plant-associated insects [215,243,244,245]. For example, Erwinia amylovora is pathogenic to the olive fly and Western flower thrips [243,246], but survives 12 days on
aphids, and at least 5 days in association with the green lacewing [157], while *D. dadantii, P. stewartii,* and *E. aphidicola* have been shown to be pathogenic to the pea aphid, colonizing the gut and causing death [195,215,216]. Similarly, the colonization of *Drosophila* by *P. carotovorum* results in a host defence response, characterized by the production of antimicrobials [185]; however, bacterial persistence is enabled by the bacterial gene, *evf,* which enhances survivorship of the bacteria in the gut by preventing insect excretion [188]. This gene was suggested to be acquired recently through horizontal gene transfer, possibly suggesting that insect persistence is an acquired and not an ancestral capability. Certainly, there may be issues of host specificity that also come into play, where there may be another true host of *P. carotovorum* in which the bacteria can persist without the *evf* gene. In contrast, genomic comparisons of the enteric phytopathogen *Pectobacterium atrosepticum* and several enteric animal pathogens revealed the acquisition of many different plant-associated pathogenicity islands by *P. atrosepticum,* including a T3SS, and genes for agglutination, adhesion, and phytotoxin biosynthesis [211]. These islands share homology to genes from other plant-associated bacteria, suggesting acquisition through horizontal gene transfer from phytopathogens. The *P. atrosepticum* genome does not show obvious signatures of having undergone new niche adaptation, suggesting that it has only gained new capabilities through incremental gene loss and gain. The identification of interactions between these phytopathogens and plant-associated insects could indicate that their ancestral gut associations have remained an integral component of their lifecycle, and the evolution of plant pathogenicity may have followed from frequent insect-mediated deposition on plants (Figure 4).
In the association between *P. syringae* and the pea aphid, the strain B728a exhibits pathogenicity toward the pea aphid, with infection resulting in aphid death in less than 36 hours. The pathogen replicates in the aphid, and is then deposited onto the plant via the aphid honeydew, making the aphid an efficient vector for the phytopathogen. In most microbe-vector associations, the bacterium is not pathogenic toward the vector, since this would reduce the likelihood of being transmitted to the next plant host; however, since the aphid is already plant associated, the microbe can replicate maximally without having to offset the cost of killing the host. In this somewhat atypical interaction, the aphid can be used as both a primary host and vector (Figure 1), which raises interesting questions about the evolutionary directionality of host association. Could this interaction represent a transitional state in the evolution of insect-microbe interactions, where B728a began as being only vectored by the aphid, but not causing its death, and gradually moved toward entomopathogenicity? Or is it attenuating in virulence as it is becoming more adapted to the aphid, perhaps to a strict vectoring association? The ability of the related tomato pathogen *P. syringae* pv. tomato DC3000 to replicate within, but not cause death of the pea aphid, would suggest that B728a has moved toward entomopathogenicity (Figure 5). The pea aphid is not known to feed on tomato plants, and would therefore be unlikely to encounter DC3000, supporting the idea that the entomopathogenicity of B728a is an acquired trait. This exciting prospect lends itself to further exploration of this interaction, including the identification and characterization of the specific genetic determinants required for this relationship. The analysis of the genetics of this interaction is presently underway, and will yield important insight into the evolution and ecological relevance of these alternative associations.
Conclusions and future developments

Our knowledge of the general ecology of phytopathogenic bacteria has begun to expand beyond their immediate interactions with plants, to encompass the other ecological players in the environment. There is increasing evidence that plant pathogenic bacteria have evolved specific and non-specific associations with insects, which they exploit as delivery vehicles or as primary alternative hosts. Specific bacterial genetic determinants have been identified that lend credence to the notion that these associations are not incidental, but have evolved with recurrent encounters, followed by natural selection. While many of these studies have provided an incredible wealth of information on the genetics and pathology of bacterial association, their ecological relevance remains ambiguous. It is certain, however, that a better understanding of phytopathogen epidemiology will require a better understanding of the nature of specific interactions and associations with other organisms in the environment.

Acknowledgements

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Table 1. Properties of bacterial pathogens.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Family</th>
<th>Genome size (MB)</th>
<th>Plant hosts</th>
<th>Insect hosts</th>
<th>Nature of insect association</th>
<th>Plant pathogenicity factors</th>
<th>Insect pathogenicity factors</th>
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<tr>
<td>Candidatus Liberibacter</td>
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<td>Citrus</td>
<td>Psyllid</td>
<td>Vector</td>
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<td>-</td>
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<td>Pea aphid</td>
<td>Host</td>
<td>hrp/hrc</td>
<td>cyt</td>
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<td>Vector</td>
<td>amylovoran, levan, hrp/hrc</td>
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<td>~4</td>
<td>Bean, pea</td>
<td>Pea aphid</td>
<td>Host</td>
<td>hrp/hrc</td>
<td>-</td>
</tr>
<tr>
<td>Erwinia tracheiphila</td>
<td>Enterobacteriaceae</td>
<td>~4</td>
<td>Cucumber, melon</td>
<td>Cucumber beetle</td>
<td>Vector</td>
<td>-</td>
<td>-</td>
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<td>Pantoea stewartii</td>
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<td>Maize</td>
<td>Flea beetle, aphid</td>
<td>Vector/ host</td>
<td>hrp/wts</td>
<td>ysa (ysaN), ucp1</td>
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<td>Vector</td>
<td>rpfC</td>
<td>rpfF</td>
</tr>
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**Figures**

Figure 1. The role of the aphid as both vector and alternative primary host for the plant pathogen *Pseudomonas syringae* pv syringae B728a (B728a). Acquisition of B728a by aphids occurs through feeding on plants colonized epiphytically by bacteria (yellow dots). The plant pathogenic bacteria replicate within infected aphids, and are excreted in globules of honeydew, which fall onto the plant surface. Infected aphids may wander to other plant hosts, vectoring the bacteria in the process. Shortly after infection with B728a, the host aphid, which has been used as a mass replication vessel by the bacteria, succumbs to sepsis. Adapted from Stavrinides *et al* (2009). (original in color)
Figure 2. A bacterium having multiple hosts must achieve a balance with both its hosts to achieve optimal fitness. The lifecycles of some phytopathogens include insect vectors, which function to transmit the bacteria to new plant hosts (middle circle). During their association with the plant (top), phytopathogens utilize plant-specific strategies for colonizing and causing disease in plant tissues, eventually reaching a virulence optimum that maximizes its fitness. This optimum, however, may conflict with the strategies used for colonizing, persisting, and being transmitted from the insect vector, necessitating a balance that maximizes the fitness of the pathogen with both hosts. (original in color)
Figure 3. Plant-first model. Phytopathogenic bacteria may have evolved alternative associations with insects following either transient interactions with generalists (left), which may move to an unsuitable plant host for the pathogen, or through interactions with specialized insects that feed on a limited set or subset of plants that overlap with the preferred hosts of the pathogen. (original in color)
Figure 4. Insect-first model. Present-day phytopathogens that associate with insects may have had ancestral associations with insects, but following deposition onto plant hosts via their plant-associating insect hosts, genetic exchange with other plant pathogens found within the phyllosphere or rhizosphere may have led to the evolution of phytopathogenicity. (original in color)
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Appendix E. Insights into cross-kingdom plant pathogenic bacteria.

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Insights into cross-kingdom plant pathogenic bacteria

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Abstract

Plant and human pathogens have evolved pathogenicity and virulence factors to successfully exploit their respective hosts. Phytopathogens utilize specific determinants that help to breach reinforced cell walls and manipulate plant physiology to facilitate the disease process, while human pathogens use determinants for exploiting mammalian physiology and overcoming highly developed adaptive immune responses. Emerging research, however, has highlighted the ability of seemingly dedicated human and plant pathogens to cause plant and human disease, respectively. Such microbes represent an interesting paradigm in the evolution of cross-kingdom pathogenicity, and the benefits and tradeoffs of exploiting multiple hosts with drastically different structures and physiologies. This review will examine common phytopathogens that exhibit human disease, and human pathogens that have phytopathogenic capabilities, with discussion on the evolution of cross-kingdom pathogenicity. We illustrate that while cross-kingdom pathogenicity appears to be maintained, the directionality of host association (plant to human, or human to plant) is difficult to determine. Cross-kingdom pathogens have important implications for the emergence of infectious diseases, and the role of plants as potential pathogen reservoirs.
Introduction

Plant pathogenic bacteria are responsible for major economical losses in agricultural and farming industries worldwide, prompting massive research efforts to understand their ecology, pathology and epidemiology. Studies of many agriculturally relevant pathogens like *Pseudomonas syringae*, *Xylella fastidiosa*, *Erwinia amylovora*, and *Xanthomonas campestris* [247,248,249,250] have revealed an extensive specialization to plant systems, including a wealth of plant-specific pathogenicity determinants such as type III secretion systems, plant hormone analogs, and enzymes that target plant-specific cell wall components [247,251,252,253]. Despite this, we often neglect the fact that the interactions of phytopathogenic bacteria are not confined to plants, and may include other organisms in the environment. Recent studies have begun to show that many plant pathogens have the capacity to colonize other hosts outside of the plant kingdom, including insects, animals, and humans [254] [255].

Much like our perceptions of plant pathogens, human pathogens are studied primarily for their detrimental impact on human health. Pathogens such as *Escherichia coli*, *Listeria monocytogenes*, and *Campylobacter jejuni* possess a diverse set of genetic factors that enable pathogenicity, ranging from specialized secretion systems to toxins and adhesins, all of which are involved in manipulating or circumventing the human immune system [256,257,258]. We often consider these human pathogenic bacteria to be exclusive animal pathogens, causing disease and epidemics, yet the constant interaction of human carriers with their environment predisposes these pathogens to alternative niches, which includes free-living states, as well as the potential association with non-human hosts. Not surprisingly, we have begun to discover that some animal pathogens,
which are known to cause serious human diseases, also have plant pathogenic capabilities. The epidemiology and disease strategies of these pathogens, whether considered primarily a plant or human pathogen, are of particular interest from the perspectives of both the biology and evolution of cross-kingdom pathogenesis.

The disease strategies used by cross-kingdom pathogens to infect unrelated hosts are of particular interest since plant and animal hosts have distinctive physical barriers and defense responses. While specialists may have evolved dedicated factors for overcoming the physical barriers and innate defenses in a certain host, a cross-kingdom pathogen would require a diverse library of genes and disease strategies to overcome the specific obstacles of each of its hosts. This would either necessitate a suite of pathogenicity factors to enable attachment, disease development, and dispersal for each host, or a universal disease strategy in which similar subsets of pathogenicity factors are used for all hosts. Although signatures of coevolution may not be as pronounced for cross-kingdom pathogens, host adaptation will still proceed through incremental nucleotide substitutions, genetic rearrangements, or acquisition of genetic elements involved in virulence or host specificity [259]. The identification of these genetic determinants in cross-kingdom pathogens with both human- and plant-pathogenic potential can provide a better understanding of the evolution of phytopathogenicity, as well as the role of plants as potential reservoirs for clinically relevant bacteria.

This review will examine various bacterial pathogens that exhibit cross-kingdom pathogenicity, where humans and plants are both potential hosts. *Pantoea, Burkholderia, Salmonella, Serratia* and *Enterobacter*, and *Enterococcus* are six genera that have brought insight into cross-kingdom pathogenicity, and the versatility of bacteria (Table
First, we examine the pathogenicity of *Pantoea* and *Burkholderia*, which are commonly regarded as plant pathogens, but have been shown to cause human disease. We then examine what is known about the disease strategies of the human pathogens *Salmonella*, *Serratia*, *Enterobacter* and *Enterococcus*, which have been shown to cause plant disease. We highlight what is known about the disease determinants and strategies for each pathogen in each of its hosts, and identify those examples where specific determinants are used in multiple hosts. Finally, we address the evolutionary means by which plant pathogens may evolve to be pathogenic to humans, as well as the possible routes of microbes that can lead to the evolution and maintenance of cross-kingdom pathogenicity.

**Plant pathogens that infect humans**

Plant pathogens have evolved a repertoire of pathogenicity factors that are used to invade plant host cells, facilitate disease development, and eventually promote pathogen dispersal [260]. Surprisingly, some of these plant pathogens cause disease in humans, and are frequently isolated from human infections in the nosocomial environment. The genus *Pantoea*, for example, currently includes seven species (*Pantoea agglomerans*, *Pantoea ananatis*, *Pantoea citrea*, *Pantoea dispersa*, *Pantoea punctata*, *Pantoea stewartii*, and *Pantoea terra*) [261,262,263,264,265,266,267] that are known to cause plant disease. *P. agglomerans* causes crown and root gall disease of gypsophila and beet [261,266], *P. ananatis* causes bacterial blight and dieback of Eucalyptus [262], brown stalk rot of maize [268], and stem necrosis of rice [269], and *P. citrea* is the causal agent of pink disease in pineapple [267]. The type III secretion system (T3SS) appears to be an
essential plant pathogenicity factor for many species of *Pantoea*, enabling efficient colonization and onset of disease through the injection of bacterial effector proteins that disrupt defense signalling in host cells [270]. *Pantoea agglomerans* pvs. *gypsophilae* and *betae* are known for using a T3SS for causing galls on their host plants [271]. While *P. agglomerans* pv. *betae* can induce gall formation on gypsophila and beet plants, *P. agglomerans* pv. *gypsophilae* is only capable of causing gall formation on gypsophila. Host range is determined in part by the type III effector protein, PthG, which is recognized by the beet plant resulting in a defense response, but functions as a virulence factor in gypsophila [271]. Studies with transgenic *Nicotiana tabacum* plants expressing PthG suggest that the effector may interfere in the plant auxin signalling pathways, resulting in higher observed auxin and ethylene levels, and subsequent blockage of root and shoot development [271]. The manipulation of plant-specific hormones is likely directly responsible for callus/gall formation, and highlights the specialization of this pathogen to plant systems.

Despite this specialization to plants, species of *Pantoea* have also been discovered to be pathogenic to humans. Now classified as an opportunistic human pathogen, *P. agglomerans* was implicated in a large U.S. and Canadian outbreak of septicaemia caused by contaminated closures on infusion fluid bottles [272]. *P. agglomerans* has since been associated in the contamination of intravenous fluid, parenteral nutrition, blood products, propofol, and transference tubes, causing illness and even death [273,274,275,276,277]. *P. agglomerans* has also been obtained from joint fluids of patients with synovitis, osteomyelitis, and arthritis [263], where infection often occurs following injuries with wood slivers, plant thorns, or wooden splinters [54,263,278,279]. This is also true for *P.*
ananatis, P. septica, and P. dispersa, which are known for causing disease in onion and sugar cane plants, but have also been implicated in bacteremic infections, septicaemia, and leukemia, respectively [280,281,282]. Phylogenetic studies examining the relationship between the plant isolates and the clinical isolates have shown that they are indistinguishable, and their potential pathogenicity in plant and animal hosts is unclear [47].

Much like Pantoea, species of Burkholderia are also recognized phytopathogens, but are ubiquitous in the terrestrial and aquatic environment. Burkholderia species, including *Burkholderia cepacia, Burkholderia cenocepacia, Burkholderia ambifaria*, and *Burkholderia pyrrocinia* were initially identified as inhabitants of agricultural soil, with some, like *Burkholderia glathei* being found in fossil soils in Germany [283]. Given their ubiquity in the terrestrial environment, it is not surprising that some strains, like *Burkholderia plantarii* and *Burkholderia gladioli* have been shown to be pathogenic to onion, rice, gladiolus and iris [283]. Similarly, *Burkholderia glumae*, the most important bacterial pathogen of rice in Japan, Korea and Taiwan, was shown to carry a plant T3SS, which is essential for its ability to cause plant disease [284]. An analysis of the proteins regulated by HrpB, which activates the expression of T3SS genes, identified 34 extracellular proteins that were secreted, with 21 of 34 having putative HrpB-binding sequences in their upstream regulatory regions [285]. Another set of 16 proteins were recognized to be secreted via a type II protein secretion system (T2SS) [285]. Mutants lacking either the T2SS or the T3SS produced toxoflavin, but were less virulent to rice panicles, indicating the importance of these proteins in plant pathogenicity [285]. Likewise, *Burkholderia pseudomallei* possesses multiple T3SS gene clusters, one of
which, T3SS2, showed high similarity to the T3SSs in phytopathogenic Xanthomonas spp. and Ralstonia solanacearum [286]. Arabinose-negative strains of B. pseudomallei are more virulent in tomato plants, and these strains appear to carry a T3SS, suggesting that these two phenotypes are linked to virulence in plants [287]. Other species, like B. cepacia, have strains that are specialized to plant roots and have been found in the maize rhizosphere [288], while others are pathogenic to onion, and cause severe rots using a cocktail of plant-specific enzymes following biofilm formation [289].

Interestingly, B. cepacia that causes onion rot can also cause life-treating pulmonary infections in individuals with chronic granulomatous disease, or cystic fibrosis (CF) [290]. Between 1980 and 1990, B. cepacia was associated with a number of cases of “cepacia syndrome” in CF treatment centres and social gatherings, where CF patients became infected by other B. cepacia-carrying individuals, with many of the infections being fatal [283,291,292,293,294]. Recently, fatal infections were also observed in non-CF patients in reanimation wards in Europe and North America [295]. Investigation into the ability of various strains of B. cepacia to penetrate airway barriers revealed that all three of the B. cepacia strains tested circumvented the mechanical barriers of mucus and ciliary transport to penetrate the airway epithelium [296]. Different strains of B. cepacia use distinct invasion pathways and virulence determinants, which may account for differences in virulence strains [296].

Several of the pathogenicity determinants for different species of Burkholderia in human hosts have been determined. For example, B. pseudomallei, which infects tomato plants, contains a complete pqsA–pqsE operon, which is highly similar to the genes responsible for the synthesis of the virulence-associated signalling molecule 2-heptyl-3-
hydroxy-4(1H)-quinolone (PQS) found in *Pseudomonas aeruginosa* [297]. Introduction of the *B. pseudomallei* *hhqA* and *hhqE* genes into *P. aeruginosa* *pqsA* and *pqsE* mutants, promoted the restoration of PQS production and virulence. Likewise, the presence of a capsular polysaccharide has been implicated in the pathogenicity of *Burkholderia* toward humans. Parental strains of *B. mallei* with the capsule were highly virulent in hamsters and mice, while capsule mutants were avirulent in both animal models [298].

**Human pathogens that infect plants**

Our human-centric view of disease often leads to sweeping assumptions that human pathogenic bacteria are devoted to human hosts. Species of *Salmonella*, *Serratia*, *Enterobacter*, and *Enterococcus* are commonly considered problematic human pathogens that are frequently found in the nosocomial environment, and which cause food poisoning, general infections, and septicaemia [299,300,301,302] (Table 1). Relatively recent studies, however, have begun to uncover that these human-pathogenic bacterial species are also accomplished phytopathogens that are capable of causing disease in a wide variety of plant hosts.

*Salmonella* is the causal agent of many human, animal, and bird diseases worldwide, including gastroenteritis and typhoid fever, and results in approximately 1.4 million human illnesses and 600 deaths annually in the United States [300]. Successful host infection by *Salmonella* appears dependent on many pathogenicity determinants, including two T3SSs and a large suite of secreted effectors that function to mediate both intercellular and intracellular survival in the host [303]. Other virulence factors include the *flhD* gene, which regulates the production of the flagellum, is essential for the full
invasive potential of the bacterium [304]. Other virulence factors like flavohemoglobin protect against nitric oxides, and mediate bacterial survival in macrophages after phagocytosis [305].

Despite its clear adaptation to survival in human hosts, *Salmonella* has also been isolated from the phyllosphere of tomato crops [306]. *S. enterica* levels on wild tomato (*Solanum pimpinellifolium*) were lower than on domesticated tomato cultivars (*Solanum lycopersicum*), with *S. enterica* preferentially colonizing type 1 trichomes. Plants irrigated with contaminated water had larger *S. enterica* populations than plants grown from seeds planted in infected soil; however, both routes of contamination resulted in detectable *S. enterica* populations in the phyllosphere, suggesting that *S. enterica* has the ability to associate with plants and has adapted to survive in the phyllosphere. *agfA* and *agfB* were identified as being involved in enabling *S. enterica* to associate with plants [307]. *agfA* mutants were unaffected in their ability to attach or colonize alfalfa sprouts, whereas *agfB* mutants showed reduced colonization ability. This suggests that *agfB* alone plays a role in plant attachment [307].

*Salmonella* is not only capable of colonizing the phyllosphere; it is also capable of infecting the plant *Arabidopsis*, and causing death of plant organs [308,309]. Inoculation of *Salmonella* in *Arabidopsis* via shoot or root tissues resulted in chlorosis, wilting, and eventually death of the infected tissues within seven days [308]. The specific virulence factors involved are not yet known; however, its plant pathogenicity, much like its human pathogenicity appears dependent, in part, on the flagellum. Fili, a protein needed for flagellar assembly has been shown to be essential for plant pathogenesis, possibly through its involvement in a specialized protein export pathway [310]. The Fili protein is
similar to the HrpB6 protein of the rice pathogen *Xanthomonas*, which mediates interactions between *Xanthomonas* and its host [310]. Secretion of proteins may therefore be integral for both human and plant association for *Salmonella*, although whether the same proteins are used for pathogenesis, is still unknown.

*Serratia marcescens* is a human pathogen commonly found in the respiratory and urinary tracts of humans, and is responsible for approximately 1.4% of nosocomial infections in the United States [301]. Through transposon mutagenesis, genes involved in lipopolysaccharide (LPS) biosynthesis, iron uptake, and hemolysin production were discovered to be essential for bacterial virulence in the host *Caenorhabditis elegans* [311]. Further studies have shown that purified protease proteins of *S. marcescens* administered to the lung tissue of guinea pigs and mice produced pneumonia symptoms and haemorrhaging, which was similar to those animals with acute *Serratia* pneumonia [312]. Yet, despite its animal virulence factors, *Serratia* has also been found to be a common phytopathogen. *S. marcescens* is recognized as a phloem-resident pathogen that causes cucurbit yellow vine disease of pumpkin (*Cucurbita moschata* L.) and squash (*Cucurbita pepo* L.), which is marked by wilting, phloem discoloration, and yellowing of foliage [124,125,126]. Recent studies have shown that *S. marcescens* produces a biofilm along the sides of the phloem vessels, blocking the transport of nutrients and eventually causing the plant to wilt and die [127,128]. A genetic screen to identify genes that modulate biofilm formation in *S. marcescens* revealed the involvement of fimbrial genes, as well as an *oxyR* homolog – a conserved bacterial transcription factor having a primary role in oxidative stress response [129]. The involvement of these plant-specific genes in human pathogenicity has not been determined.
Another bacterium that has also shown cross-kingdom pathogenesis is the Gram-negative bacterium *Enterobacter cloacae*, an important nosocomial pathogen responsible for bacteremia, lower respiratory tract infections, skin and soft-tissue infections, as well as urinary tract infections [299]. Recently, an *E. cloacae* infection of the bloodstream was traced back to contaminated human albumin [313]. *E. cloacae* synthesize a Shiga-like toxin II-related cytotoxin, which was implicated in an infant case of haemolytic-uremic syndrome [314]. Studies have shown that the concentration of the outer membrane protein (OmpX) produced by *E. cloacae* during infection influenced the ability of the bacterium to invade rabbit intestinal tissue [315]. Overproduction of OmpX led to a 10-fold increase in the invasiveness of *E. cloacae* in rabbit intestinal enterocytes *in situ*, whereas the mutant and wildtype strain were unable to effectively invade the same tissue [315]. Interestingly, OmpX shares a high amino acid similarity to the virulence proteins PagC and Rck of *Salmonella typhimurium* as well as the virulence-associated Ail protein of *Yersinia enterocolitica*. Although there is evidence that *E. cloacae* has evolved to colonize the human host, it has also been identified as a plant pathogen of macadamia (*Macadamia integrifolia*), and the causal agent of grey kernel disease [316]. The onset of grey kernel disease affects not only the quality of the kernels produced by the plant, but results in grey discoloration and a foul odour [316]. *E. cloacae* also causes bacterial soft rot disease in dragon fruit (*Hylocereus* spp.) [264], bacterial leaf rot in *Odontioda* orchids [317], and is also responsible for internal yellowing disease in papaya [316]. Again, the specificity of the virulence factors used in each of these hosts is not known.

Cross-kingdom pathogenesis is not limited to Gram-negative bacteria. Enterococci are part of the normal intestinal flora of humans and animals, but are also
important pathogens responsible for serious infections, especially in immunocompromised patients [302]. With increasing antibiotic resistance, enterococci are recognized as nosocomial pathogens that can be challenging to treat. The genus Enterococcus includes more than 17 species, but only a few can cause local or systemic clinical infections including urinary tract and abdominal infections, wound infections, bacteremia, and endocarditis [318]. Clinical isolates of Enterococcus faecalis have been found to produce hemolysin – a virulence factor that leads to the lysis of red blood cells [319]. Hemolytic strains exhibit multiple drug resistance more frequently than non-hemolytic strains, while strains isolated from fecal specimens of healthy individuals display a low (17%) incidence of hemolysin production [319]. The salB gene of E. faecalis has also been shown to be a virulence factor, since it increases bacterial adherence to extracellular matrix (ECM) proteins and promotes biofilm formation during infection [320]. The surface protein Esp was shown to contribute to the colonization and persistence of the bacterium in the urinary tract [321]. E. faecalis has also been shown to exhibit pathogenicity toward insects in addition to human hosts, where the extracellular gelatinase of E. faecalis was found to destroy the host defence system through the degradation of inducible antimicrobial peptides in insect hemolymph and in human serum [322]. Similarly, a putative quorum-sensing system gene (fsrB) and a serine protease (sprE) were shown to play an important role in mammalian and nematode models of infection [302].

E. faecalis is not only capable of infecting mammalian and nematode hosts; it also can infect the leaves and root tissue of Arabidopsis thaliana, causing plant death seven days after inoculation [302]. The manifestation of disease initially begins once the
bacterium has successfully attached itself to the leaf surface, where upon entry of the leaf tissue through the stomata or wounds, *E. faecalis* multiplies and colonizes the intercellular spaces of the plant host and causes rotting and disruption of the plant cell wall and membrane structures. The phytopathogenicity of *E. faecalis* appears to involve some of the same genetic determinants involved in animal pathogenesis, including the quorum sensing system (*fsrB*) and a serine protease (*sprE*) [302]. The quorum-sensing mutant (Δ*fsrB*) exhibited an attenuated virulence in the *A. thaliana* root model, since fewer bacteria were able to attach to the root surfaces, and biofilm formation was greatly reduced. Similarly, the serine protease mutant (Δ*sprE*) no longer caused plant death, suggesting that this gene plays an important role in *E. faecalis* plant pathogenesis [302]. *E. faecalis* clearly uses a general disease strategy that allows it to use the same virulence factors for exploiting two different hosts.

**Evolutionary models**

Cross-kingdom pathogenicity represents an intriguing balance between the costs and benefits of bacterial generalization versus specialization. While there are benefits to specializing on one host, and evolving to exploit its subtle vulnerabilities, the availability of this host would limit the success of the pathogen, thereby favouring host diversification. Conversely, a broad host range would ensure host availability for the pathogen, but at the significant cost of having to utilize a universal, and likely suboptimal infection strategy for exploiting that host. When the alternative host is from a different kingdom, the dynamics become even more intriguing, since the disease strategy is likely to be quite different. The evolution of cross-kingdom pathogenicity and its origins can be
described using several simplistic models. In the context of the examples described here, one model describes the evolution of a successful animal pathogen that acquires the ability to cause disease in a plant host (human to plant). If we presuppose that human pathogens are readily acquired from the environment and are already adapted to mammalian hosts [323], there are several ways in which these animal pathogens may acquire plant-pathogenic potential. Deposition of animal pathogens back into the environment, such that they are introduced near or onto plants recurrently may facilitate the exchange genetic information with other organisms in the environment, and the gain of a selective advantage that enables persistence. One primary route to this general environment is through the natural strategies used by bacteria to ensure dissemination from their infected host. Symptoms such as diarrhea that result from an infection provide the bacteria with an escape route from the human host into the environment [324] (Figure 1). Released bacteria move from human wastes to natural watersheds, with subsequent reuse of these sources for irrigation of commercially relevant crops increasing bacterial titres in the phyllosphere [308,309]. But, the movement of human pathogens to the general environment may also occur through indirect routes. The Pharoah ant (*Monomorium pharaonis*) is able to transport human pathogenic bacteria including *Salmonella*, *Staphylococcus*, and *Streptococcus* within a nosocomial setting [325], and there have been reports of the common house fly, *Musca domestica*, carrying *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus* [326] (Figure 1). These insects function as transports for human pathogenic bacteria, moving them from the clinical setting to the general environment [255], and likely onto plants.
Acclimation to a plant host would occur over extended periods of time, and would be accelerated with repeated cycling of pathogens from humans to the general environment.

The second model that can account for the evolution of the cross-kingdom pathogens has plant pathogenicity as the ancestral state \[327\], with established plant pathogens having evolved the ability to exploit humans as alternative hosts. The simplest mode of transmission is direct contact with an epiphytically colonized or diseased plant by humans, whether by ingestion, contact with the skin or other mucosal membranes, scrapes or abrasions, providing the bacteria with a direct route to a potential host. Several plant pathogens that cause opportunistic human infections are associated with commercially relevant crop plants. Species of *Pantoea* are found on beets \[261,266\], maize \[268\], rice \[269\], and pineapple \[267\]. In the case of *Pantoea*, human infections occur frequently following abrasions caused by rose thorns, splinters, and arthritis, suggesting that these plant-associated strains can lead to human infection directly (Figure 1) \[54,263,278,279\]. Similarly, *Burkholderia* infects various plant species including onion, rice, sorghum and velvet beans \[283,291,328\], and may also have a direct route to humans. Indirect transfer to humans or even to the nosocomial environment may be mediated by other organisms like ants and flies, which have been implicated as carriers of many bacterial species \[325,326\], and may provide environmental isolates with a direct route to other environments and hosts.

Although there is evidence to support both models of evolution, it is difficult to establish evolutionary directionality for each pathogen. The ability of human pathogens to exploit plants as alternative hosts is of particular significance from the perspective of host jumps and host-specific adaptation, since plants would ultimately serve as an
extensive reservoir for clinically relevant bacteria. Some plant endophytes, which are organisms that live inside of plants often asymptptomatically, have been shown to exhibit human pathogenic potential. Species like *Morganella morganii*, *Klebsiella pneumoniae*, *Pantoea agglomerans* and *Streptomyces* sp., have clinical relevance, but have been more closely investigated for their association with plants [329,330]. *M. morganii* is a phosphate solubilising bacterium [331], *K. pneumoniae* a nitrogen-fixing endophyte [332], *P. agglomerans* a gall-forming phytopathogen, and *Streptomyces* sp. a plant endophyte and plant pathogen [333,334,335]. Many of these animal-pathogenic endophytes have been shown to be latent plant pathogens, where after establishing a symbiotic mutualistic relationship with their plant host, the bacterium infects and causes severe disease within the plant host possibly due to environmental changes [336]. It has also been observed that an organism that is an endophyte on one plant species may have the potential to cause disease in a different plant species [337]. Yet, many human pathogens have been found to occur naturally in the rhizosphere [329], and it has been suggested that the mechanisms necessary for surviving in the rhizosphere are similar to those necessary for causing human infection [2]. Contributing to the maintenance of these cross-kingdom pathogens may be anthropogenic factors. Certain species with human and plant pathogenic potential, such as *Pantoea* and *Burkholderia cepacia* are also commonly used as biocontrol agents against *Erwinia amylovora* and *Rhizoctonia solani*, respectively [338,339]. The extensive use of these bacteria as biocontrol agents on commercially relevant crops can lead to broad dispersal in the environment, increasing their populations dramatically and providing them with greater opportunity for interaction with other microbes. Horizontal gene transfer with other plant and human pathogenic bacteria in the
environment can lead to extensive exchange of host-specific virulence factors or niche-
specific determinants, creating rapidly-evolving populations that may exhibit oscillations
between host-associative and free-living states.

**Conclusion**

Advances in molecular genetics coupled with the exploration of the pathogenic
potential of seemingly dedicated pathogens is beginning to reveal that many
phytopathogenic bacteria are capable of exploiting human hosts, and many human
pathogens are capable of exploiting plant hosts. The constant cycling of pathogens from
the general environment to human environments may help to maintain cross-kingdom
pathogenicity, with plants serving as intermediate hosts or reservoirs for human
pathogens. The ability of these cross-kingdom pathogens to maintain their population
levels in a variety of environments increases their pan genome and evolutionary potential,
ultimately making these pathogens significant from the perspective of emerging and
remerging infectious diseases.

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Figures

Figure 1. Cycling of cross-kingdom pathogens between plants and humans. Movement of human-associated bacteria (Salmonella, Serratia, Enterobacter, Enterococcus) into the general environment may occur through wastewater, with additional microbial input coming from agricultural and livestock runoff. Irrigation using contaminated water, along with vectoring by insects can lead to inoculation of plant surfaces or plant soils. Movement of potential human pathogenic bacteria from plants is also likely facilitated by insects, which can disperse bacteria into the general environment. The route back to humans is less clear, although some pathogens like Burkholderia and Pantoea can cause direct infections following cutaneous lesions or injuries from thorns or splinters. (original in color)