

Xylella fastidiosa Vector Transmission Biology

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Research on the transmission biology of *Xylella fastidiosa* Wells et al. has not been continuous and can be divided into three periods, each driven by a unique set of factors. An epidemic of Pierce's disease of grapevine in the 1930s and 1940s led to characterization of the disease and the identification of xylem sap feeders as vectors of its etiological agent (*X. fastidiosa*). The findings by Japanese researchers in 1967 (Doi et al., 1967; Ishiie et al., 1967) that "mycoplasma-like organisms" were associated with yellows diseases of plants led to further explorations of the etiology of Pierce's disease and the identification of a bacterium, *X. fastidiosa*, as its causal agent that immediately resulted in additional work characterizing its transmission biology. The third period was prompted by two epidemics (in Brazil and California) and the advent of molecular tools, which have led to new findings since 2000. In addition, work on *X. fastidiosa* transmission biology has been based largely on and strongly influenced by the use of Pierce's disease of grapevines as a model system; the use of other systems will be important to challenge current paradigms and will certainly yield unexpected results. Although some of the important findings on *X. fastidiosa* transmission that are decades old are briefly discussed in this chapter, readers are strongly encouraged to study the original publications by authors such as Severin (1949, 1950), Freitag (1951), and Purcell (1981, 1989), among others. Those reports are rich in details and are considered classics for a reason.

This chapter reviews the biology of *X. fastidiosa* transmission, focusing on a discussion of research findings since the 1940s, rather than providing a complete literature review. Two related chapters in this book cover aspects of *X. fastidiosa* transmission, and the focal topics of those chapters are not discussed here. Chapter 13 proposes a hypothesis for *X. fastidiosa* transmission based on vector probing behavior, while Chapter 14 covers the transmission ecology of the strain causing citrus variegated chlorosis. Several reviews on *X. fastidiosa* address topics not covered here. Some of those focus on the pathogen itself

(Chatterjee et al., 2008a), vector ecology and disease epidemiology (Hopkins and Purcell, 2002; Redak et al., 2004), transmission ecology and disease management (Almeida et al., 2005a), and comparative genomics (Moreira et al., 2004).

Pathogen Biology

Newton Pierce's 1892 report in the U.S. Department of Agriculture's Vegetable Pathology Bulletin No. 2 marked the beginning of scientific research on diseases caused by the bacterium *X. fastidiosa* (Pierce, 1892). Pierce, a bacteriologist by training, was assigned to California to study the destructive "Anaheim disease" or "California vine disease." Although Pierce was not able to identify the etiological agent of the disease, his work carefully addressed different hypotheses that led to the conclusion that a microscopic infectious agent, most likely a microorganism, caused Anaheim disease. He also suggested that insect vectors could be implicated in pathogen spread. Despite his efforts and those of others, the epidemic had devastating consequences on the grape industry in southern California. A 1939 report by D. G. Milbrath in a California Department of Agriculture bulletin named the disease "Pierce's disease" (Gardner and Hewitt, 1974). It is intriguing that although Weimer (1937) found bacterium-like bodies associated with alfalfa dwarf, a disease also caused by *X. fastidiosa*, the agent of these diseases was thought to be a virus until 1971 (Hopkins and Mortensen, 1971). The causal agent of Pierce's disease and other diseases likely caused by the same organism, as determined through biological assays, was recognized to be a bacterium in 1973 (Goheen et al., 1973; Hopkins and Mollenhauer, 1973). Axenic culturing of the bacterium was first reported in 1978, when Koch's postulates were fulfilled for Pierce's disease (Davis et al., 1978); it was named *X. fastidiosa* a decade later (Wells et al., 1987). Most of the work during that period assumed that *X. fastidiosa* was a cohesive group of bacteria causing disease in a wide range of plants. This assumption remained unchallenged until researchers began using DNA-based genotyping methods to compare *X. fastidiosa* isolates colonizing different host plants (Chen et al., 1992).

There are at least four major genetic groups of *X. fastidiosa*, currently divided into subspecies (Schaad et al., 2004; Schuenzel et al., 2005). The subspecies proposal has been largely

accepted by the community conducting research on this bacterium; however, the term “strain” is sometimes used as a synonym. This taxonomical proposal is based on a limited number of American isolates, however. *X. fastidiosa* causes disease in pears in Taiwan (Leu and Su, 1993; Leu et al., 1998), although little is known about that disease and the pear strain will most likely be assigned to another species once more data are available (Mehta and Rosato, 2001). A potentially new subspecies was recovered from trees in the southwestern United States (Randall et al., 2009). In addition, there is one published report of *X. fastidiosa* from Kosovo (Berisha et al., 1998), although it is not clear whether the bacterium is established in Europe. Importantly, studies of *X. fastidiosa*'s genetic diversity have been extremely biased toward strains causing disease in plants of economic importance, certainly limiting our understanding of the true diversity of this organism. *X. fastidiosa* has been shown to colonize a large number of plant species, albeit with different degrees of multiplication and movement within the xylem-vessel network and host symptomatology (Purcell and Saunders, 1999). The paradigm that *X. fastidiosa* is a generalist pathogen with a wide host range is being challenged by an alternative hypothesis that specific subspecies cause disease in a limited number of plant species while still being capable of colonizing many other species as harmless endophytes.

The colonization of xylem vessels by *X. fastidiosa* appears to be a consequence of successive migration events from one vessel to another mediated by the degradation of pit membranes. Movement between vessels is essential for bacterial survival. In some hosts, cells may multiply locally but do not move and eventually infections die out (Purcell and Saunders, 1999). New vessels are invaded by a few colonizing individuals, eventually leading to the formation of a large colony that inhibits water flow upward through the plant (Newman et al., 2003). When enough vessels are clogged, a poorly understood physiological plant response occurs, usually resulting in leaf scorching or plant stunting. Details of these processes are outside the scope of this chapter.

Identification of Insect Vector Taxa

The epidemic of Pierce's disease of grapevines associated with alfalfa dwarf in California's Central Valley during the 1930s and 1940s led to several breakthroughs. Epidemiological data suggestive of the involvement of insects in pathogen spread resulted in the identification of sharpshooter leafhoppers (Hemiptera: Cicadellidae) as vectors of the etiological agent of Pierce's disease (Hewitt et al., 1942). That finding was followed by reports showing that different species of xylem-sap-sucking sharpshooters were vectors of *X. fastidiosa* (Frazier and Freitag, 1946; Hewitt et al., 1946). In addition to several species of sharpshooters, the finding that xylem sap feeding was associated with pathogen transmission led Severin (1950) to test spittlebugs (Hemiptera: Cercopidae) as vectors in the Pierce's disease system, because they also feed preferentially on that tissue. Spittlebugs were, as expected, shown to transmit *X. fastidiosa*. Their epidemiological relevance is still unknown, but it is likely small. Spittlebugs were also shown to transmit a strain of *X. fastidiosa* causing pecan leaf scorch in Louisiana in a pathosystem in which these insects may be responsible for significant pathogen spread (Sanderlin and Melanson, 2010). There are also reports showing that cicadas are vectors of *X. fastidiosa* in coffee in Brazil (Paião et al., 2002) and in grapevines in the United

States (Krell et al., 2007). However, both reports have small sample sizes, and *X. fastidiosa* transmission by cicadas needs to be studied in more detail. All these insects are xylem sap feeders, and probing into xylem tissue is a requirement for transmission (Houston et al., 1947). Taxa in other auchenorrhynchan families or Cicadellidae subfamilies have been tested for *X. fastidiosa* transmission, always with negative results (e.g., Purcell, 1981). This is in spite of the fact that sap-sucking insects belonging to other Cicadellidae subfamilies or Auchenorrhyncha families have been shown to occasionally feed on xylem sap. Therefore, occasional xylem sap ingestion is not sufficient for transmission. Future studies on why these insects are not *X. fastidiosa* vectors may yield interesting insights on transmission biology and the mechanics of leafhopper feeding.

Altogether, work on the identification of *X. fastidiosa* vectors led Frazier (1965) to propose that any species in the subfamily Cicadellinae (sharpshooter leafhoppers), the group primarily associated with plant diseases, should be considered vectors until proven otherwise. This hypothesis has, so far, been correct, as illustrated by the transmission of a South American isolate of *X. fastidiosa* by a North American vector species (Damsteegt et al., 2006). Only one report, to our knowledge, suggests that specificity may exist between vector species and *X. fastidiosa* strain (Lopes et al., 2009), but those observations need to be confirmed through the use of an artificial diet system (Killiny and Almeida, 2009b). In addition, a systematic analysis of vector species and *X. fastidiosa* subspecies in relation to their transmissibility must be performed, as suggested by Purcell (1989).

Retention Site

Early work on the characterization of *X. fastidiosa* transmission focused on the identification of vector species. In addition, Severin (1949) showed that transmission by adults was persistent and Freitag (1951) demonstrated lack of transovarial transmission. Persistence of infection in adults has been confirmed in other studies (Almeida and Purcell, 2003a; Hill and Purcell, 1995a), although transovarial transmission was not further tested. However, it is unlikely that vertical transmission of *X. fastidiosa* occurs. The lack of transstadial transmission and absence of a detectable latent period for acquisition or inoculation (Almeida and Purcell, 2003a; Purcell and Finlay, 1979) are strong indicators that this bacterium does not colonize the hemolymph or internal organs of vectors, as previously determined by microscopy (Purcell et al., 1979). In addition, evidence of vector foregut colonization by *X. fastidiosa* from microscopy studies (Brlansky et al., 1983; Purcell et al., 1979) (Fig. 12.1) support observations obtained through transmission experiments. The foregut cuticular lining is of ectodermic origin and is shed during each molt.

During acquisition from source plants, *X. fastidiosa* attaches to and multiplies in the foregut of vectors; multiplication was first shown by culturing (Hill and Purcell, 1995a) and more recently by quantitative PCR (Killiny and Almeida, 2009a) and inferred by microscopy at different time points after acquisition from plants (Almeida and Purcell, 2006). The foregut of vectors may house up to ~50,000–100,000 cells (based on quantitative PCR estimates) (R. P. P. Almeida, *unpublished*), although the number of cells should also be species dependent, with larger insects potentially harboring more cells. The generation time of *X. fastidiosa* cells within vectors, as estimated by quantitative PCR, is 7–8 h and remains constant for up to 4 days (Killiny and

Almeida, 2009a). In addition to an apparent constant multiplication rate over time during early stages of colonization, population growth rate is expected to slow down once space available on the cuticle is reduced; there is a physical barrier to the number of cells that can colonize vectors, since not every region of the precibarium and cibarium is colonized by *X. fastidiosa* (Almeida and Purcell, 2006; Purcell et al., 1979). However, it must be remembered that multiplication and biofilm (microbial colony attached to a solid surface embedded in an extracellular matrix) formation are not necessary for transmission, as demonstrated by the lack of a latent period (Purcell and Finlay, 1979). Nevertheless, it is possible that stage-specific events during biofilm maturation affect cell detachment rate, but this hypothesis has yet to be tested.

Two regions of the foregut have been implicated in *X. fastidiosa* transmission on the basis of spatial colonization patterns. Purcell et al. (1979) observed cells in the cibarium, the distal region of the precibarium, and the anterior region of the esophagus, while Brlansky et al. (1983) showed that the precibarium was also colonized by *X. fastidiosa*. However, those studies did not correlate bacterial visualization with vector transmission to plants. A more recent study showed an association between bacterial colonization of the precibarium with transmission to plants (Almeida and Purcell, 2006), suggesting that colonization of the esophagus and cibarium are not directly associated with inoculation events. These authors have mapped *X. fastidiosa* retention sites in the foregut of vectors, and the studies provide the backbone for morphology-based hypotheses on the

inoculation mechanism for this pathogen (see Almeida et al., 2005a). One hypothesis for *X. fastidiosa* inoculation events was based on the concept that cells would detach from the cuticle during feeding as a result of negative tension in xylem sap, but the infection of dormant plants with positive root pressure indicated that vector probing behaviors were responsible for bacterial inoculation (Almeida et al., 2005b). Studies linking specific vector probing behaviors with acquisition and inoculation events that lead to plant disease are necessary to dissect this process; characterization of probing behaviors by sharpshooter vectors is under way (Backus et al., 2005; Dugravot et al., 2008). However, vector probing behavior has yet to be directly linked to *X. fastidiosa* transmission. Factors that may affect probing behavior have been poorly explored. Vector sex and adult age have been analyzed in relation to *X. fastidiosa* transmission only once; in that study, neither factor impacted efficiency (Krugner et al., 2012). This is an interesting observation, suggesting that either probing behaviors by males and females of all ages are similar or that existing differences are not associated with behaviors connected with *X. fastidiosa* transmission.

Acquisition

Although different factors are expected to affect *X. fastidiosa* acquisition efficiency, so far only bacterial populations within plants have been shown to be of major importance. Transmission efficiency increases proportionally with increments in acquisition access period (Purcell and Finlay, 1979). Sharpshooters may have long-lasting individual xylem-sap-ingestion events and visit multiple vessels during the same probe (Almeida and Backus, 2004; Backus et al., 2005). Because *X. fastidiosa* is unevenly distributed within plants (Hopkins, 1981) and insects may move between probes, it is expected that longer plant access periods increase the opportunities for vector-pathogen encounters. Cross sections of infected plant tissue show that a relatively small proportion of vessels are colonized in individual cross sections, although higher proportions are observed in tissues with severe symptoms (e.g., Alves et al., 2004). In addition, a much higher percentage of individual vessels may be colonized if long segments of tissue are analyzed (Hopkins, 1981). It should be mentioned that although the probing behavior associated with *X. fastidiosa* acquisition is yet to be determined, it is presumed that ingestion from xylem vessels must occur.

The precibarium and cibarium of sharpshooter leafhoppers is far from a stress-free environment, and cell attachment in that environment is potentially a rare event. Although fluid-flow dynamics in this system have not been experimentally determined, xylem sap has been estimated to flow through the precibarium at average speeds of 8 cm/sec (Purcell et al., 1979). In addition, turbulence is likely present, since the muscle connected to the cibarium's diaphragm responsible for creating enough tension to pump sap from plants into the midgut is contracted and relaxed approximately once every second during ingestion (Dugravot et al., 2008). Thus, it is possible that very few colonization events occur given the number of cells, or cell aggregates, that may be ingested by a vector. That has been suggested by microscopy (Almeida and Purcell, 2006), but quantitative PCR data indicate that up to a few thousand cells can be detected in sharpshooters after feeding on infected plant material (Rashed et al., 2011a). These data suggest that many cells are acquired by vectors while ingesting sap from infected xylem vessels but very few are capable of colonizing vectors, indicating that initial



Fig. 12.1. Top, leafhopper vector of *Xylella fastidiosa* and bottom, scanning electron microscopy image of the narrow canal in the foregut (precibarium) that is colonized by cells that form a “mat” of polarly attached bacteria. (Top, courtesy R. Krugner; bottom, courtesy R. P. P. Almeida—© APS)

adhesion is a rare event. This discrepancy highlights the fact that colonization of vectors is not a trivial process.

Several studies have reported differences in vector transmission efficiency that were host plant, vector species, or *X. fastidiosa* subspecies dependent. Although it is likely that various factors affect acquisition efficiency, *X. fastidiosa* populations (number of live cells per gram of plant tissue) within plants arose as a dominant hypothesis after Hill and Purcell (1997) demonstrated that vector acquisition efficiency was correlated with bacterial populations within grapevines. As discussed above, it is expected that higher pathogen populations yield higher acquisition efficiency, since the number of pathogen–vector encounters increases. Although this relationship was experimentally tested in only one other study (Daugherty et al., 2010), indirect evidence indicates that populations are a strong determinant of acquisition efficiency. Bacterial populations in *X. fastidiosa*-infected hosts vary by orders of magnitude. Citrus and almonds, for example, harbor low populations of *X. fastidiosa* compared with those in grapevines (Almeida and Purcell, 2003c; Almeida et al., 2001); transmission rates on the former are also lower (Almeida and Purcell, 2003a,b; Krugner et al., 1998; Marucci et al., 2008; Purcell, 1980). However, in one study in which vector transmission rates in various genotypes of the same species (grapevines) were compared, bacterial populations were not as variable and transmission rates were similar for all genotypes, suggesting that large, i.e., order(s) of magnitude, differences in populations are required to significantly impact transmission (Rashed et al., 2011b).

This relationship seems to hold as a general guideline, especially when one disease system is considered in relation to another. However, it does not necessarily explain the variability in transmission efficiency that has been observed when different vector species are tested on the same host plant. This was first observed in 1946, when it was found that the leafhopper *Graphocephala atropunctata* (Signoret) was a more efficient vector of *X. fastidiosa* from grape to grape than *Draeculacephala minerva* Ball, but the opposite occurred on alfalfa (Frazier and Freitag, 1946; Hewitt et al., 1946). A comparative study in which almond trees were used as hosts indicated vector variability in transmission efficiency (Purcell, 1980), as was also found with citrus (Krugner et al., 1998). Two factors appear to drive such differences. First, if *X. fastidiosa*'s heterogeneous distribution within plants (e.g., Krivanek and Walker, 2005) is coupled with vector within-plant feeding preference, these interactions may lead to major differences in transmission efficiency. This hypothesis was tested with the alfalfa–*G. atropunctata*–*D. minerva* system (Daugherty et al., 2010). Alfalfa harbors higher bacterial populations at the bases of stems than at the tips. In addition, given a choice, *G. atropunctata* feeds preferentially at the tops of plants, while *D. minerva* prefers the bases. When insects were confined, without choice, to the bases or tops of stems during pathogen acquisition, transmission efficiency was higher for both species when feeding at the bases. However, results also indicated that differences between species were independent of plant feeding tissue. This second source of variability is assumed to be a consequence of plant–vector interactions and deserves attention. For example, Rashed et al. (2011a) showed that two vector species preferred to alight on tissues that matched their forewing coloration, likely as a means to avoid visual notice by predators (background matching behavior) during long sap-ingestion events. These species also differ in transmission efficiency on grapevines (Daugherty and Almeida, 2009). Such

behavior could, for example, impact transmission efficiency caused by dissimilar vector probing behaviors on different plant tissues (Almeida and Backus, 2004), consequently affecting *X. fastidiosa* acquisition and inoculation efficiencies.

Inoculation

Despite the fact that two essential questions regarding *X. fastidiosa* inoculation into plants remain unanswered, namely, the identification of specific probing behavior(s) associated with inoculation and the role of the biofilm stage on cell detachment, some factors that affect inoculation efficiency have been studied. Like acquisition, inoculation efficiency increases with longer inoculation access periods (Almeida and Purcell, 2003a; Purcell and Finlay, 1979). Longer plant access periods allow vectors to generate a larger number of inoculation events (i.e., infections) and probably deliver a greater number of cells into plants. Initially, the number of cells detected in the foregut (by use of bacterial culturing) and its relationship with vector inoculation of *X. fastidiosa* to plants was not clear but indicated that very few cells were enough to generate successful infections (Hill and Purcell, 1995a). This was expected because of the lack of a required latent period for transmission. Direct association of pathogen detection and quantification in vectors by PCR to transmission to plants showed this to be a complex relationship (Daugherty et al., 2009). The number of infected insects on plants, not the number of cells present in the heads of vectors, was correlated with transmission efficiency. This observation suggests that the overall number of inoculation events is more important in leading to successful transmission than the number of cells inoculated in an event. A similar relationship was observed by Jackson et al. (2008) using a protocol with chrysanthemum plant cuttings. However, the number of inoculated cells affects infection rate if plants are mechanically inoculated (Prado et al., 2008). The number of cells inoculated by vectors was estimated by Rashed et al. (2011a) using an artificial diet system. During a 3-h inoculation event, approximately 350 and 200 cells were inoculated into the diet solution by *Homalodisca vitripennis* and *G. atropunctata*, respectively. Although these estimates fall well within the expected number of cells inoculated by vectors into plants for this system, in situ studies quantifying the number of cells inoculated into plants during individual probes will be necessary to analyze these events in detail.

If the number of inoculation events were a strong determinant of efficiency, would one infective vector feeding on a plant for 4 days have the same likelihood of infecting that plant as four infective individuals feeding for 1 day? Purcell (1981) proposed a mathematical model in which these two parameters were equivalent. This hypothesis was experimentally tested, and the parameters were determined to be approximately equivalent (Daugherty and Almeida, 2009). Among the implications of these findings is the potential impact of multiple inoculation events on disease ecology. Because *X. fastidiosa* is a systemic pathogen colonizing the xylem network of hosts, movement within plants is a reasonably slow process (Hill and Purcell, 1995b). Furthermore, as vessel occlusion appears to be the major factor driving disease symptoms, it is possible that multiple infections at different sites result in faster colonization of plants and earlier symptom expression. Data from two studies, in which different *X. fastidiosa* subspecies and host plants were used, provide support for this hypothesis. Costa et al. (2000) showed that oleander plants inoculated by groups of three in-

sects died twice as often as those inoculated by individuals. Likewise, Daugherty and Almeida (2009) showed that disease symptoms in grapevines developed earlier in plants inoculated with multiple insects (for the same plant access period) or longer inoculation access periods (for the same number of insects). Faster symptom expression may result in bacterial populations within plants reaching higher levels earlier in the year, which could lead to more pathogen spread since the window for dispersal within a season increases (Hopkins and Purcell, 2002). A better understanding of the inoculation process and its consequences is necessary because it may have epidemiological consequences. This is especially true for *X. fastidiosa* because vectors discriminate against symptomatic plants (Daugherty et al., 2011; Marucci et al., 2005).

X. fastidiosa–Vector Interactions

Knowledge of the molecular interactions between plant-pathogenic bacteria and their respective insect vectors significantly behind that of plant viruses. Most of these bacteria are either challenging to manipulate *in vitro* (e.g., *Spiroplasma* spp. and *X. fastidiosa*) or are so far unculturable (e.g., *Candidatus Phytoplasma* spp. and *Candidatus Liberibacter* spp.). Protocols to transform *X. fastidiosa* have been available since 2001 (Guilhabert et al., 2001; Monteiro et al., 2001). The availability of gene-specific mutants has opened a new frontier for *X. fastidiosa* transmission research, but these efforts have also encountered a major stumbling block. Most mutants tested so far have important phenotypes when inoculated into plants, for example, causing more (Guilhabert and Kirkpatrick, 2005; Newman et al., 2004) or less (Reddy et al., 2007; Roper et al., 2007) symptom development in plants than the wild-type controls, which is a direct consequence of bacterial multiplication and movement rates. Because transmission efficiency is affected by *X. fastidiosa* populations within plants, comparing mutants in relation to the wild type becomes a difficult and laborious process, because appropriate experimental controls that address multiple alternative explanations of the outcomes of transmission assays must be performed (e.g., Newman et al., 2004). The development of an *in vitro* protocol to deliver transmissible cells to vectors addresses this problem and represents an important development in the field (Killiny and Almeida, 2009b).

Because *X. fastidiosa* is transmitted without specificity, interactions between vector and bacterium were initially expected to be straightforward. The first evidence contrary to that assumption came from experiments with a cell–cell signaling mutant (Newman et al., 2004). *X. fastidiosa* gene expression is dependent on, among many factors, cell density. As in many other bacteria, a regulatory system exists that responds to signal molecules produced by individual cells in a population. These diffusible molecules accumulate in the environment and trigger population-wide changes in gene expression when a threshold is reached. The regulation of pathogenicity factor (*rpf*) operon is responsible for synthesizing and responding to the diffusible signaling factor (DSF) molecule, a short-chain fatty acid that is the cell–cell signaling molecule in *X. fastidiosa* (Chatterjee et al., 2008a). Disruption of DSF production results in hypervirulence within plants, probably resulting from upregulation of plant-colonization-related genes and downregulation of adhesins (Newman et al., 2004). However, these cells are not capable of colonizing the precibarium of vectors and are very poorly transmitted to plants. The limitation of this study in identify-

ing specific interactions associated with the lack of transmission resides in the fact that a cascade of genes is regulated by DSF, and identification of individual targets is not possible. In a later study (Chatterjee et al., 2008b), a DSF-blind mutant was found to be hyperadhesive *in vitro* and was vector transmitted, yet with efficiency lower than that of the wild type. The authors interpreted those results to be a consequence of reduced cell detachment from vectors resulting from its adhesive phenotype. Similarly, however, the identification of specific proteins involved in vector colonization was not possible.

The chemical composition of the external layer of an insect's exoskeleton (the epicuticle) is not well understood for several insect groups. In the case of insect vectors of *X. fastidiosa*, to our knowledge, there is no information on its composition, especially of the foregut. The epicuticle of insects is composed of several layers, the inner and outer epicuticle, above which is a wax layer, and in some insects a cement layer exists. The thickest layer, the inner epicuticle, is 0.5–2 μm thick (Chapman, 1998). The cement layer is a thin layer composed of mucopolysaccharides associated with lipids. The wax layer is composed largely of lipids with embedded proteins and serves as a water-proofing element for the cuticle. In addition, proteins and other potential molecules are present in the cuticle. Research on the structure and chemical composition of the cuticle of leafhoppers (and other plant-pathogen vectors) is necessary and represents an important gap in our understanding of how insects transmit pathogens that bind to or colonize the foregut of vectors. Although the chemical composition of the cuticular surface of arthropods is generally not well understood, interactions between bacteria and the exoskeleton of such animals has been studied in other systems in which chitin was used as a proxy with success (e.g., Meibom et al., 2004).

The first step in determining the nature of *X. fastidiosa*–vector interactions was to learn whether cell surface proteins are involved in adhesion to vectors. Killiny and Almeida (2009a) demonstrated that cells bind to carbohydrates and that treating intact cells with proteases reduces adhesion to compounds such as chitin. Thus, surface proteins are involved in cell adhesion to carbohydrates; however, *X. fastidiosa* has variable affinity to different molecules. For example, in competition assays, *N*-acetylglucosamine (a monomer of chitin) acted as a strong competitor in binding assays in which vector foregut extracts were used as a substrate, reducing cell adhesion. On the other hand, mannose and galactose did not affect binding. When mutants of fimbrial types I and IV pili and afimbrial (hemagglutinin-like proteins) surface adhesins were tested *in vitro* for their binding to foregut extracts, in addition to other mutants, only hemagglutinin-like proteins and cell-signaling mutants (discussed above) were affected in adhesion. However, this approach assumed that extracts are good proxies for the intact surface of the foregut colonized by *X. fastidiosa*. Potentially better surrogates are the hind wings of sharpshooter leafhoppers, which are easy to collect and represent surfaces of the exoskeleton that may be chemically and structurally more similar to intact foreguts. Specificity and competition assays showed that to be the case (Killiny and Almeida, 2009a). Altogether, these biochemical and other biological assays indicated that initial cell adhesion to vectors is mediated by carbohydrate–lectin interactions and that specific surface proteins can be identified *in vitro* as potential candidates for more comprehensive studies.

The delivery of *X. fastidiosa* cells to vectors from growth media has been tried since the bacterium was first cultured in the

laboratory (Davis et al., 1978). It was tested on and off for three decades, until it was discovered that plant structural polysaccharides, pectin and glucan, induce phenotypic changes in *X. fastidiosa* that induce its transmissibility by leafhoppers (Killiny and Almeida, 2009b). There are conceptual similarities in this case with vector-borne nonpersistent and semipersistent viruses that do not use the capsid strategy for transmission, since purified virions are not retained in the mouthparts of vectors but are viable if mechanically inoculated into plants (Froissart et al., 2002). Furthermore, it illustrates the need for in situ microscale analysis and the context dependence of the transmission process, which in fact needs to be addressed for all vector-borne plant pathogens. The relevance of such detailed analyses has

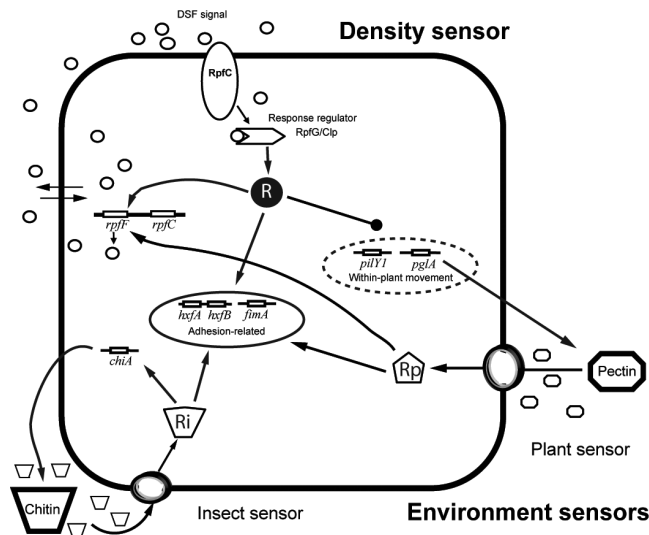


Fig. 12.2. Simplistic model integrating the cell density signal (diffusible signaling factor, DSF) and environmental cues, focusing on adhesion and within-plant movement genes. At high cell densities, the presence of high levels of the DSF signaling molecule induces the expression of adhesion-related genes and suppresses within-plant movement. In the presence of plant polysaccharides, the DSF-induced response is upregulated through an unknown regulatory mechanism, linking plant signals to the density sensor. Within insects, chitin induces an adhesive state but does not up-regulate the expression of *rpfF*; thus, this environmental cue may regulate genes in parallel rather than under the density sensor. Downstream regulators: R = cell density, Rp = plant signal, and Ri = insect signal. (Developed by N. Killiny, S. E. Lindow, and R. P. P. Almeida—© APS)

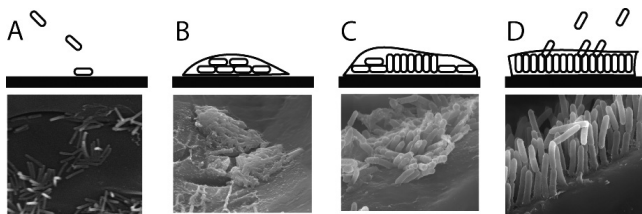


Fig. 12.3. Hypothetical model of *Xylella fastidiosa* colonization of leafhopper vectors. **A**, Cells initially attach laterally to the cuticle of insects, a process mediated by HxfA and HxfB and possibly other carbohydrate-binding proteins. **B**, Microcolonies establish and **C**, change in morphology, with cells in the center becoming polarly attached to increase exposed surface area; type I pili may be important for polar attachment. **D**, Mature biofilm forms; newly divided daughter cells not attached to the leafhopper cuticle are subject to detachment from the biofilm. (Reproduced, by permission of the publisher, from Killiny and Almeida, 2009a)

been highlighted by work on the aphid transmission of *Cauliflower mosaic virus* (Martiniere et al., 2009; Uzeit et al., 2007).

In the case of *X. fastidiosa*, plant polysaccharides induce regulons that result in drastic phenotypic changes leading to higher degrees of adhesiveness and, consequently, transmission by vectors (Killiny and Almeida, 2009b). Although the transcriptional profile induced by pectin, for example, is significantly different from that of the control conditions (same medium without pectin), the general pattern of gene expression is similar to that of *X. fastidiosa* cells occurring at high densities (Chatterjee et al., 2008b; Newman et al., 2004). Although a complex matrix of mutants and media for cell growth must be analyzed, it appears that the induction of a vector-transmissible state occurs as a result of overexpression (up or down) of genes under control of the DSF system. As such, afimbrial adhesins are upregulated, while genes associated with plant host colonization are downregulated (see Chatterjee et al., 2008a, for discussion). Importantly, the hemagglutinin-like proteins previously identified as important for initial colonization of vectors were present at several orders of magnitude more than in the absence of pectin (Killiny and Almeida, 2009b). D-Galacturonic acid, one of the subunits of pectin, and not the polysaccharide itself, induced changes in gene expression. Altogether, these experiments showed that colonization of insect vectors requires carbohydrate-binding proteins, which likely act as adhesins during the first contact between insect and microbe. However, these studies have not addressed other stages of vector colonization by *X. fastidiosa*.

One of the open questions about the biology of *X. fastidiosa* is related to its nutritional physiology. The major carbon source used by this bacterium while colonizing plants is still not clear. The fact that it has enzymes capable of degrading plant polysaccharides does not mean it uses them as nutrient sources. On the other hand, the xylem sap of plants is usually nutrient dilute and by itself may not sustain cell growth at the rates observed in biological studies. Similarly, within the foregut of vectors, it has been assumed that *X. fastidiosa* uses ingested xylem sap as a carbon source. Although that is a possibility, research has shown that cells can use chitin and do not depend on other carbon sources (Killiny et al., 2010). In situ evidence of *X. fastidiosa* utilization of the foregut cuticle of insect vectors as a source of nutrients is lacking, but cell growth on leafhopper hind wings indicates the bacterium can use cuticle as a nutrient source. In addition, data on gene expression and phenotypic changes show that chitin is used, probably in the form of N-acetylglucosamine monomers, as a carbon source and maybe as a signaling molecule. The role of chitin on *X. fastidiosa* gene expression and phenotype needs to be studied in more detail since it is an essential component in our understanding of how *X. fastidiosa* colonizes vectors.

A complex picture of *X. fastidiosa* gene regulation in relation to vector transmission is emerging on the basis of this research. Cells respond to at least two distinct groups of signals, one based on cell density and the other on environmental cues (Fig. 12.2). In addition, at least one intracellular signaling molecule, cyclic di-GMP, is involved in gene regulation and transmission (Chatterjee et al., 2010) but is ignored here for simplicity. Under high cell densities within plants, DSF accumulates in the environment and cells switch from a plant-colonization to an adhesive state that is required for insect colonization. Although this regulatory switch occurs in the absence of plant cues such as pectin (i.e., polygalacturonate), the presence of plant carbohydrates results

in overexpression of the DSF-mediated phenotype, resulting in cells with a phenotype capable of colonizing insects (Killiny and Almeida, 2009b). Carbohydrate-binding afimbrial adhesins involved in transmission are then upregulated by several orders of magnitude and mediate the initial stages of cell adhesion to vectors. The host transition, from plants to insects, is thus dependent on cell density and plant structural polysaccharides. Once in the insect, chitin (or its subunits) or structurally similar carbohydrates induce another regulon, with a gene expression profile that also matches the general trends observed with cells in high cell density (Killiny et al., 2010). However, even at low densities, during the early stages of vector colonization *X. fastidiosa* is in an adhesive state, as cells are adhered to the cuticle of vectors. Thus, carbohydrates or other environmental cues are probably involved in gene regulation of cells colonizing vectors at low cell density, since *X. fastidiosa*'s phenotype at that stage is incongruent with that observed within plants. It should be noted that cell aggregates with fewer than 100 cells might be enough to reach a quorum, inducing density-dependent genes, in natural environments (Dulla and Lindow, 2008).

In summary, *X. fastidiosa* colonization of vectors is a complex process under the control of multiple regulatory systems. A conceptual model has been proposed describing the essential steps of colonization (Fig. 12.3) (Killiny and Almeida, 2009a). First, cells switch from a plant-colonization state to an immobile state at high cell densities, which is necessary for the plant-to-insect transition. Once acquired by insects, adhesive cells bind laterally to the cuticle through carbohydrate-binding afimbrial adhesins. Low cell density biofilms grow, becoming surrounded by an extracellular matrix, potentially under low DSF concentrations. As the biofilm matures, cells at the center of colonies become polarly attached via type I pili. Why biofilms develop from cells attached sideways in a polar fashion is unknown, although the arrangement increases surface area and the number of individuals per unit area. In *G. atropunctata*, a mature biofilm is composed of a monolayer of cells (Almeida and Purcell, 2006), with dividing cells on top of the biofilm detached from the insect surface. This mechanistic hypothesis for *X. fastidiosa* colonization of vectors is an exciting development in the field, because a framework now exists that can guide future studies.

Future Directions

Like research on many other vector-borne plant pathogens, that on *X. fastidiosa* has benefited from sporadic epidemics, which resulted in significant advances in our understanding of its transmission biology. However, this is an unfortunate trend that impacts the long-term pursuit of answers to important questions. Regardless, work since 2000 has opened several new and exciting research venues that should be pursued in the future. Among these, four topics have emerged:

***X. fastidiosa*-vector specificity.** *X. fastidiosa* isolates can now be easily and robustly assigned to different subspecies, and a plant-independent protocol is available for delivery of transmissible cells to vectors. These advances permit testing for the existence of vector-pathogen specificity, a hypothesis that has never been systematically addressed with appropriate tools.

Vector behavior. Vector tissue preference and within-plant distribution can affect transmission efficiency, because *X. fastidiosa* is heterogeneously distributed within plants. Vector discrimination against symptomatic plants is also relevant and should be studied in more detail. Studies analyzing the role of

vector behavior in relation to transmission are expected to yield results that will assist in our understanding of the ecology of *X. fastidiosa* diseases.

Quantitative analysis of transmission. Tools such as quantitative PCR and approaches like mathematical modeling are slowly being incorporated into studies addressing different aspects of *X. fastidiosa* transmission. Quantitative approaches may explain many phenomena that are currently interpreted through alternative, potentially incorrect, perspectives.

***X. fastidiosa*-vector interactions.** The availability of site-specific mutants and a protocol to deliver transmissible cells to vectors, among other technological advances, will allow researchers to ask elaborate questions on how *X. fastidiosa* colonizes sharpshooters. This area holds promise, since this bacterium is as much an insect as a plant inhabitant and its life history within insects has been largely neglected.

Acknowledgments

I acknowledge and thank Sandy Purcell, João Lopes, Matt Daugherty, Nabil Killiny, Arash Rashed, and Stephanie Kung for comments on the manuscript; omissions and mistakes are entirely mine. I also thank Sandy Purcell, João Lopes, and many other colleagues I have worked with for insightful discussions on this topic over the years.

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