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Genomic Insights into Xylella fastidiosa Interactions with Plant and Insect Hosts

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# Genomic Insights into *Xylella fastidiosa* Interactions with Plant and Insect Hosts

8

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## 8.1 Introduction

The utilization of genomic data and widespread availability of genomics tools is still incipient in plant pathology. The first genome of a plant pathogen, that of the bacterium *Xylella fastidiosa*, was only completed in 2000 (Simpson et al. 2000). Since then, many bacterial plant pathogens have been sequenced, but much of the scientific knowledge extracted from these data is still limited, especially when compared to human pathogens. Nevertheless, significant advances have been made during the last decade, when genomics became widely available to the plant pathology community. Our understanding of the functional role of genes and pathogen taxonomy

and evolution has improved significantly. The availability of genome sequences has served as the backbone for much of this work. In this chapter, we take a holistic approach and explore *X. fastidiosa* evolution, biology, and management based on information and insights that would not have been possible, or would have been technically challenging, during the pre-genomics period of plant pathology. Although *X. fastidiosa* is widely thought of as a plant pathogen, its biology is more complex and focus on its pathogenicity to crops clouds our broader understanding of its biology, ecology, and evolution. We predict that genomic data and further research on isolates that do not cause crop diseases will permit a more complete view of *X. fastidiosa*. This is especially important in the context of emerging diseases, as data suggest that novel *X. fastidiosa* isolates are particularly prone to emerge as crop pathogens of economic importance.

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In late 2013 *Xylella fastidiosa* was reported in southern Italy (Saponari et al. 2013). This report highlights the importance of *X. fastidiosa* as a quarantine pathogen, and the need to better understand its ecology and evolution.

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### 8.1.1 History

The first plant disease associated with the bacterium *X. fastidiosa* was described over one century ago (Pierce 1892). However, knowledge about this organism lags significantly behind other well-known plant pathogenic bacteria. Epidemics have been the major factor driving research on this pathogen. The first recorded epidemic caused by *X. fastidiosa* in grapevines in southern California in the late 1800s led to initial characterization of Pierce's disease of

grapevines (Pierce 1892). Later, parallel and inter-related epidemics of Pierce's disease and alfalfa dwarf in the Central Valley of California resulted in the identification of insect vectors, but also in the conclusion that the etiological agent of these diseases was a virus (Hewitt et al. 1949). Only in the mid-1970s, after it was shown that 'yellows' diseases of plants were of bacterial origin (phytoplasmas and spiroplasmas; Doi et al. 1967), were diseases caused by *X. fastidiosa* determined to also have bacterial etiology (Hopkins and Mollenhauer 1973). That breakthrough led to its axenic culture in 1978 (Davis et al. 1978); and the bacterium was named *X. fastidiosa* one decade later (Wells et al. 1987). Despite its importance, research on this pathogen remained limited until a new disease in citrus emerged in Brazil in 1987 and another re-emerged in California in the late 1990s (Hopkins and Purcell 2002). These diseases, principally in Brazil, led to efforts aimed at sequencing the genome of these pathogens, with the expectation that this novel information would assist research aimed at the development of better and new disease management practices. That culminated in the sequencing of the first bacterial plant pathogen, *X. fastidiosa*, in the year 2000 (Simpson et al. 2000). It is fair to say that, other than data available for PCR-based detection of *X. fastidiosa* (Minsavage et al. 1994), very limited information derived from molecular tools was available for this bacterium prior to 2000. We direct readers interested in a more complete perspective of the history of *X. fastidiosa* research to a recently published review by Purcell (2013).

### 8.1.2 Geographical Distribution

Initially thought to be limited to North America (Hewitt 1958), *X. fastidiosa* is now known to be present throughout the Americas and Taiwan. There is one report of *X. fastidiosa* in Kosovo, Europe (Berisha et al. 1998); however, the bacterium is considered to be absent from the continent. Taiwan represents an interesting case, where two distinct phylotypes of *X. fastidiosa* occur, causing disease in pear trees and grapevines,

respectively (Su et al. 2012, 2013). The grapevine disease is caused by isolates that are phylogenetically among those widely distributed in the United States, suggesting that it was recently introduced into the island, which is also evidenced by the lack of genetic diversity among Taiwanese isolates. The pear isolates, however, appear to have evolved in isolation, raising important questions regarding the worldwide distribution of *X. fastidiosa* and *X. fastidiosa*-like bacteria, which may have remained undetected due to significant biological, phenotypic, and genetic differences when compared to known taxa.

Within the Americas, *X. fastidiosa* occurs from the northeast region of the United States and Canada (Goodwin and Zhang 1997) to Argentina and southern Brazil (Hopkins and Purcell 2002). However, as discussed later, the distribution of individual subspecies is more limited, suggesting that geographical isolation has been important in the evolution of this bacterium. Furthermore, as with grapevine isolates in Taiwan, evidence indicates that at least some isolates causing disease in plum in Brazil originated from North America (Nunes et al. 2003) and that grapevine isolates in the United States originated from Central America (Nunney et al. 2010). Therefore, *X. fastidiosa* populations appeared to have remained largely isolated due to geographical barriers, but recent human activity has resulted in dispersal over continental distances.

### 8.1.3 Impact

*X. fastidiosa* causes disease in a wide range of host plants, from perennial fruit crops such as grapes and citrus to ornamental trees and shrubs. The economic impact of these diseases is poorly understood; to our knowledge, only Pierce's disease of grapevines in Northern California has been carefully studied in this context (Fuller 2012). The production of sweet orange in Brazil is severely impacted by this pathogen, which now infects up to half of plants in some regions, over one-third of citrus plants in São Paulo State are estimated to be infected with the pathogen. In rare instances, such as oleander leaf scorch in

southern California in the 1990s, most susceptible hosts were eliminated from the landscape due to *X. fastidiosa* infection. Control of *X. fastidiosa* diseases is dependent on host plant species, vector species, geographical location, and management practices, among other factors. We direct readers to other reviews that cover *X. fastidiosa* diseases, their impact, epidemiology, and management alternatives (Hopkins and Purcell 2002; Redak et al. 2004).

### 8.1.4 Quarantine Importance

This bacterium is of worldwide quarantine importance. Some countries where *X. fastidiosa* is established have enacted policies to avoid the introduction of new genotypes, while countries in which it is considered to be absent tend to prohibit the introduction of the species (i.e., Australia, New Zealand, and the European Union have strict regulations aimed at reducing the likelihood of *X. fastidiosa* introduction). It should be mentioned that these countries also list insect vectors of *X. fastidiosa* as quarantine organisms. The United States Department of Agriculture strictly regulates the introduction of foreign isolates and considered the genotype causing citrus variegated chlorosis (CVC), which is currently limited to South America, a bioterrorism agent until 2012 (Federal Register 2012).

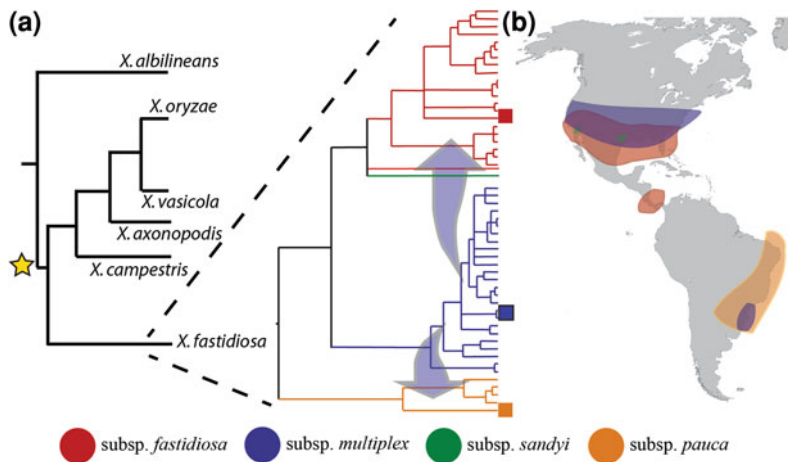
The European Union considers *X. fastidiosa* an EPPO A1 pathogen, which are quarantine pests absent from the region. Strict guidelines exist for positive diagnostic and reporting of *X. fastidiosa* in Europe (European and Mediterranean Plant Protection Organization 2004). European governments routinely survey for this bacterium using ELISA and PCR. In addition to regulations on pathogen introduction and field surveys, some countries have been more proactive, assuming that an eventual introduction will occur. Australia and New Zealand scientists have performed research in California, USA, to determine the host range of *X. fastidiosa* and one of its vectors using native plant species from those countries (Rathé et al. 2012a; Sandanayaka

and Backus 2008). Such efforts could have a significant impact on the eradication or management of *X. fastidiosa* introductions, as some biological knowledge would be already available to decision makers upon pathogen detection. Lastly, analysis of the potential threat of an introduction can provide important information to assess risk and develop science-based policies (e.g., Rathé et al. 2012b).

In addition to the above-mentioned risks, the introduction of *X. fastidiosa* into regions where the bacterium has already been detected increases chances of gene flow between endemic and invasive genotypes. The availability of novel loci and alleles can result in the emergence of epidemic isolates that have the potential to exploit host plants previously not susceptible to disease as a result of *X. fastidiosa* infection. Although not conclusively demonstrated, independent studies have suggested that *X. fastidiosa* diseases can emerge via gene exchange between isolates (Almeida et al. 2008; Nunes et al. 2003; Nunney et al. 2012). Therefore, increases in genetic diversity, driven by the introduction of foreign genotypes, carry risks that go beyond the potential host range of the newly arrived isolate.

### 8.1.5 Taxonomy

*Xylella fastidiosa* is a gamma-proteobacterium in the order Xanthomonadales, family *Xanthomonadaceae* (Wells et al. 1987). This is the single species in the genus *Xylella*, which is monophyletic, and has *Xanthomonas* ssp. as a sister clade (Fig. 8.1). *X. fastidiosa* is currently subdivided into four subspecies, largely based on DNA–DNA hybridization and multi-locus sequence typing data (Sally et al. 2005; Schaad et al. 2004). Although these groupings are phylogenetically robust, they can be further subdivided into groups with well-supported genetic and biological distinctions (i.e., different host ranges) (e.g., Almeida and Purcell 2003b; Almeida et al. 2008; Nunney et al. 2010, 2013). The taxonomy of *X. fastidiosa* is discussed in detail later within an evolutionary context.



**Fig. 8.1** Evolutionary history and geographical distribution of *X. fastidiosa* subspecies in the Americas. **a** Relationship of *X. fastidiosa* to *Xanthomonas* species (after Rodriguez et al. 2012), rooted by *Stenotrophomonas* and *Pseudoxanthomonas*. The position of *X. albilineans* is ambiguous (star; see text). *X. fastidiosa* diversity (MLST) is shown in detail, with published

genome sequences identified with *squares*; the Taiwanese pear isolate is not included due to limited sequence data. Gene flow has been observed between subsp. *multiplex* and others (arrows). **b** Approximate spatial distribution of subspecies; it is assumed that the species extends throughout Central and South America

## 8.2 General Biology

The biology of *X. fastidiosa* is complex due to its requirement to successfully colonize two very distinct hosts, plants and insects. This bacterium emerged from plant-colonizing *Xanthomonas* spp., and its divergence is hypothesized to be driven by the novel utilization of insect vectors for dispersal (Killiny et al. 2010). Therefore, a discussion of the biology of *X. fastidiosa* must consider its plant and vector colonization, in addition to mechanisms necessary to switch from one host to another. This section provides an introduction to the general biology of plant and insect colonization by *X. fastidiosa*; genome-derived insights are addressed later in this chapter.

### 8.2.1 Colonization of Host Plants

Unlike many other bacterial plant pathogens, *X. fastidiosa* has a large host range, both as a species and for each individual subspecies. Hill and Purcell (1995) compiled published data and

concluded that plants in twenty-nine families were hosts of this bacterium. This bacterium is capable of multiplying in almost all tested plant species under greenhouse conditions when mechanically inoculated (Purcell and Saunders 1999). It can also be recovered from a wide range of plants in the field (Lopes et al. 2003), although *X. fastidiosa* does not cause disease in most of these species (Purcell and Saunders 1999). Thus, *X. fastidiosa* colonization of plants does not necessarily equal disease development. The list of host plants known to be susceptible to this pathogen has grown during the last decade, as has the even longer list of non-symptomatic host plants.

The details of pathogenicity mechanisms are yet to be fully understood, but disease appears to be a consequence of bacterial multiplication and movement within the xylem vessel network, leading to clogging of water flow through the plant (Chatterjee et al. 2008a; Newman et al. 2003). Although the bacterium is xylem limited, populations are not homogenous in the entire plant (Hopkins 1985). Bacterial accumulation at specific tissues within the plant are also host dependent; as an example, in Pierce's disease-

infected grapevines, *X. fastidiosa* is found at higher populations in symptomatic leaf veins and petioles (Baccari and Lindow 2011; Krivanek and Walker 2005). A positive correlation between symptom severity and pathogen populations within the plant has also been shown (Krivanek and Walker 2005). *X. fastidiosa* genomic analyses revealed a number of genes that encode plant cell wall-degrading enzymes; *X. fastidiosa* is strictly xylem limited, and these enzymes are predicted to solely function in pit membrane degradation. Using a GFP-tagged *X. fastidiosa* strain, symptom development was shown to be highly correlated with the number of vessels clogged (Newman et al. 2003). While structural differences in xylem vessels of susceptible versus nonsusceptible grape varieties did not correlate with probability of disease development (Chatelet et al. 2011), pit membrane degradation was more successful in susceptible versus resistant grapevine varieties (Sun et al. 2011). In addition, defensive responses of susceptible grapevines to *X. fastidiosa* colonization may further exacerbate restrictions on xylem fluid flow and symptom development (Sun et al. 2013).

### 8.2.2 Colonization of Insect Vectors

Severin (1949) showed that *X. fastidiosa* is persistently transmitted by adult sharpshooter leafhoppers (Hemiptera, Cicadellidae), 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 1995b); although transovarial transmission was not tested further, 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 circulate within vectors. In addition, microscopic studies on the colonization of the foregut of vectors (Almeida and Purcell 2006; Brlansky et al. 1983; Purcell et al. 1979)

supported the results obtained from transmission experiments.

After acquisition from source plants, *X. fastidiosa* attaches to and multiplies in the foregut of vectors; multiplication was first shown by culturing (Hill and Purcell 1995b) 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 generation time of *X. fastidiosa* cells within vectors as estimated by quantitative PCR was 7–8 h and remained constant for up to 4 days (Killiny and Almeida 2009a). We estimate that the foregut of vectors may house ~50,000 cells. Two regions of the foregut have been implicated in *X. fastidiosa* transmission based on 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; Brlansky et al. (1983) confirmed that the precibarium was colonized by *X. fastidiosa*. However, those studies did not correlate bacterial visualization with vector transmission to plants. A more recent microscopy-based study showed an association between bacterial colonization of the precibarium and transmission to plants (Almeida and Purcell 2006), suggesting that colonization of the esophagus and cibarium was not directly associated with inoculation events. However, the specific site(s) in the foregut as well as the vector probing behavior(s) associated with inoculation is yet to be determined.

The precibarium and cibarium of leafhoppers are highly turbulent environments, and cell attachment is not a trivial process. 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/s (Purcell et al. 1979). Turbulence is likely present as the muscle connected to the cibarium's diaphragm creates enough tension to pump sap from plants into the midgut by contracting and relaxing approximately every second during ingestion events (Dugravot et al. 2008). Thus, it is possible that very few colonization events of the precibarium

occur given the number of cells or cell aggregates that may be ingested by a vector through feeding on sap from an infected xylem vessel. While few colonization events have been suggested based on microscopy data (Almeida and Purcell 2006), quantitative PCR data indicate that approximately one to five thousand cells can be detected in sharpshooter heads after feeding on infected plant material (Rashed et al. 2011). Many of the ingested cells likely do not adhere to the insect cuticle and pass through the digestive tract, and therefore are considered to not be involved in vector colonization and *X. fastidiosa* transmission to plants. This discrepancy highlights the fact that successful attachment of *X. fastidiosa* to the foregut of vectors is probably a rare event requiring high-affinity ligand–receptor interactions.

### 8.2.3 Vector Transmission

Transmission experiments identified sharpshooter leafhoppers (Hemiptera, Cicadellidae, subfamily Cicadellinae) and spittlebugs (Hemiptera, Cercopoidea) as insect vectors of this pathogen (Severin 1949, 1950). A third group of xylem sap-sucking insects, cicadas (Hemiptera, Cicadidae), has been reported as a vector of *X. fastidiosa* (Paião et al. 2002; Krell et al. 2007), although more work is necessary to determine the contribution of this group to overall epidemiology and transmission dynamics. An important aspect associated with the transmission of *X. fastidiosa* is the lack of pathogen genotype and vector species specificity (Almeida et al. 2005). Frazier (1965) summarized the current state of knowledge at the time by concluding that all sharpshooter leafhoppers should be considered vectors of the Pierce's disease etiological agent until proven otherwise. The addition of various pathogen genotypes to this statement occurred later, after the advent of molecular tools that eventually allowed the split of the species into subspecies, when various studies showed that all tested vector species were capable of transmitting various *X. fastidiosa* genotypes (Almeida et al. 2005). The best

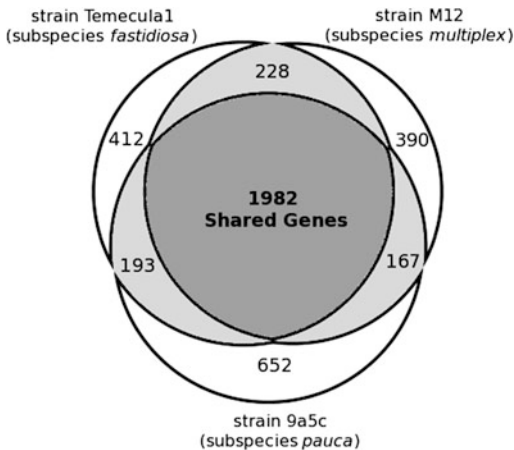
example was the demonstration that a North American vector species transmitted a South American isolate to plants (Damsteegt et al. 2006). Ultimately, more vector–pathogen combinations must be tested, but so far Frazier's 50-year-old statement remains true.

Various factors affect *X. fastidiosa* vector transmission efficiency. The length of time insects are allowed to acquire or inoculate *X. fastidiosa* into plants is proportional to overall transmission efficiency, up to approximately four days (Almeida and Purcell 2003a; Daugherty and Almeida 2009; Purcell and Finlay 1979). The most parsimonious interpretation of these results is that the probability of insects probing into xylem vessels with *X. fastidiosa* increases over time (Almeida and Backus 2004; Backus et al. 2005). In fact, the overall population of *X. fastidiosa* within host plant tissue has been the only parameter consistently correlated with transmission efficiency. Hill and Purcell (1997) were the first to demonstrate such relationship, which has been used to explain why specific vector–pathogen–plant combinations result in higher or lower transmission efficiency [e.g., Lopes et al. (2009)]. Furthermore, within-plant differences in pathogen population also affect transmission efficiency (Daugherty et al. 2010). Thus, host plants harboring larger *X. fastidiosa* populations generally result in higher transmission efficiency, and vice versa. Vector age and sex do not affect overall transmission efficiency (Krugner et al. 2012).

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## 8.3 Genome Structure

As of August 2013, five finished *X. fastidiosa* genome sequences have been published (PubMed Genomes Database; <http://www.ncbi.nlm.nih.gov/genome/genomes/173>), representing three of the four described subspecies (subspecies *sandyi* being the exception; Fig. 8.2) (Chen et al. 2010; Simpson et al. 2000; van Sluys et al. 2003). These genomes range from 2.5 to 2.7 megabase pairs (Mbp) and include a single chromosome along with zero to two plasmids (ranging from 1.3 to 51 kb). The nucleotide



**Fig. 8.2** Venn diagram for the number of predicted coding sequences that are shared and unique from representative genomes of subspecies *multiplex*, *fastidiosa*, and *pauca* calculated with EDGAR (Blom et al. 2009), using a protein–protein BLAST e-value cutoff of  $10^{-6}$  and 70 % amino acid identity

sequences are 51.8–52.6 % GC. Published annotations indicate that these genomes each contain from 2,066 to 2,294 protein-coding sequences, 2 ribosomal RNA operons, 49–50 tRNA genes (43 different anti-codons), and 1 tmRNA gene (van Sluys et al. 2003). Draft quality genomes are available for three other isolates, each appearing to be very similar to one of the finished genomes at the coarse scale (Bhattacharyya et al. 2002; Schreiber et al. 2010; Zhang et al. 2011), as are contigs from the shotgun sequencing of a mixed sample of subspecies *multiplex* and *sandyi* (Bhattacharyya et al. 2002; Nunney et al. 2012). The diversity of available sequences will increase rapidly in the near future, as several projects are underway that are sequencing tens of genomes from isolates collected around the world.

### 8.3.1 Core Genome

The location of the origin of replication can be confidently predicted from three different lines of genomic evidence: the location of the *dnaA* gene, a cluster of DnaA boxes, and inversion of the G/C and A/T skews (Mackiewicz et al. 2004; Simpson et al. 2000). The terminus can be identified by

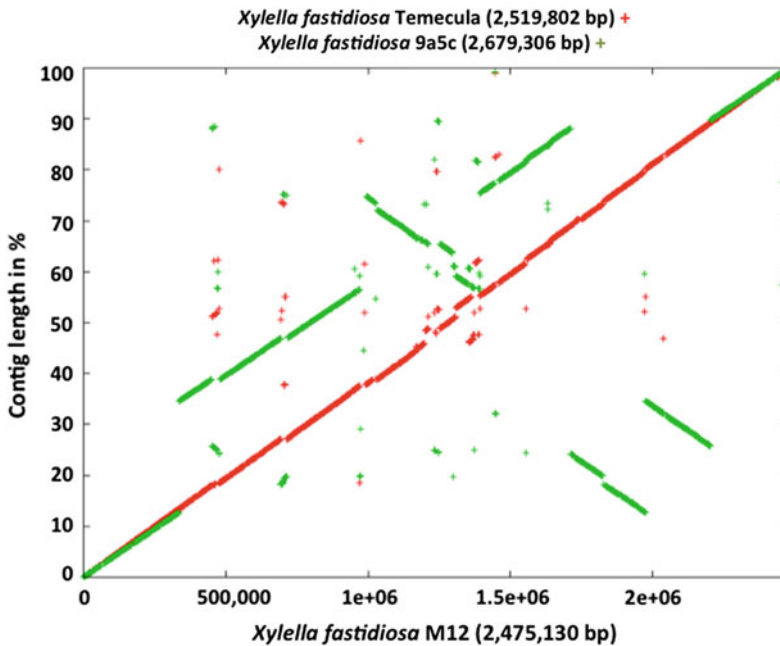
similar methods: Both the predicted *dif* sequence and oligonucleotide asymmetry analysis point to the same region of each sequenced chromosome (Yen et al. 2002; Kono et al. 2011). Based on the predicted origin and terminus of replication, we estimate that 59–60 % of genes are encoded on the leading replication strands (Rocha 2008). Notably, the chromosome arms of strain 9a5c are heavily imbalanced such that one replicore is approximately half the length of the other; in contrast, the origin and terminus of replication for the other complete *X. fastidiosa* chromosomes are approximately opposite from each other. Based on studies in *Escherichia coli*, this degree of replication imbalance is expected to be a substantial impediment to growth (Esnault et al. 2007).

We identified 1982 protein-encoding genes that are shared among subspecies *fastidiosa*, *multiplex*, and *pauca* (Fig. 8.2). This accounts for 71–86 % of the largest *X. fastidiosa* genome, 9a5c, depending on how conservative one is in predicting genes (e.g., Simpson et al. 2000; van Sluys et al. 2003). Only one study addressed this question at the subspecies level (subsp. *pauca*); it showed that the pan-genome of *X. fastidiosa* is potentially large and that differences among isolates were associated with genome island-like fragments (da Silva et al. 2007). However, representative genomes of different subspecies that have been fully sequenced are largely syntenic (Fig. 8.3). Several short sequence repeats (SSR) have been identified in the chromosome (Coletta-Filho et al. 2001). These loci tend to evolve quickly, and several have been confirmed as having variable number of tandem repeats (VNTR), making them useful for high-resolution studies of population structure (Coletta-Filho et al. 2011).

### 8.3.2 Mobile Elements

Several self-replicating genetic elements are present among *X. fastidiosa* genomes, including plasmids and prophages. These contribute to the gene diversity of *X. fastidiosa* and provide potential avenues for horizontal gene transfer (HGT) both within and between species. Due to the ability of HGT to confer new phenotypes on





**Fig. 8.3** Synteny map of representative genomes from each of the three sequenced subspecies, showing several rearrangements on the *X. fastidiosa* 9a5c (subsp. *pauca*)

chromosome compared to *X. fastidiosa* M12 (subsp. *multiplex*) and *X. fastidiosa* Temecula1 (subsp. *fastidiosa*)

bacteria and modify their ecological niche, there is intense interest in exploring the contribution of these elements to the distinct virulence traits of different isolates, including the ability to colonize novel host plants (Moreira et al. 2005; Nunes et al. 2003).

Despite the abundance of putative prophages embedded within the chromosome, the isolation of active phage particles has been difficult, with the first lysogen confirmed only in 2010 (Summer et al. 2010). The prevalence of prophages and phage remnants within *X. fastidiosa* genomes contrasts with the absence of identifiable transposons, which are abundant in *Xanthomonas* genomes (Monteiro-Vitorello et al. 2005). Prophages are also candidates for inducing rearrangements within the *X. fastidiosa* genome, due to the activity of their integrase genes (Moreira et al. 2005; Nunes et al. 2003).

Among subspecies, it is evident that *X. fastidiosa*'s flexible genome is primarily composed of laterally transferred phage-like elements, including prophages (Nunes et al. 2003; van Sluys et al. 2003; Varani et al. 2008).

Interestingly, transcription studies have suggested that this laterally transferred gene pool is differentially regulated in *X. fastidiosa* in comparison with genes in the core genome, suggesting that their regulation is yet to be tightly linked to the rest of the genome (Nunes et al. 2003). The finding that most obvious differences among *X. fastidiosa* genomes are connected to phage-like sequences has led several groups to propose that the divergence of lineages leading to host specificity is mediated by laterally transferred elements (e.g., van Sluys et al. 2003; Nunes et al. 2003; Varani et al. 2008). Thus, host adaptation in *X. fastidiosa* could be the result of one or few changes with large fitness benefits, despite the fact that overall pathogenicity mechanisms are similar regardless of host plant. This model is comparable to the effector–host resistance genes evolutionary model for most bacterial pathogens (Lindeberg et al. 2009; Ma et al. 2006). However, a competing hypothesis proposes that quantitative rather than qualitative differences are the major drivers of host specificity (Killiny and Almeida 2011).

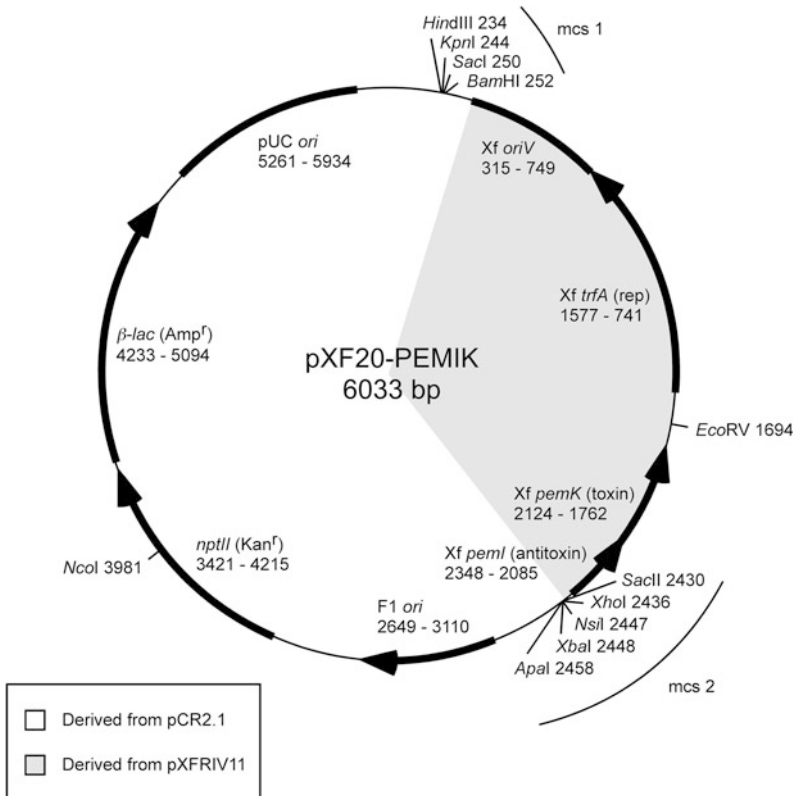
The existence of plasmids in *X. fastidiosa* cultures has been demonstrated in two manners: Some plasmids have been purified directly from *X. fastidiosa* cultures, while the existence of others have been inferred based on circular contigs generated during genome sequencing projects. These extra-chromosomal DNA molecules are commonly associated with bacteria, encoding accessory modules conferring selective advantage in specific environments (e.g., resistance to antibiotics or heavy metals, or an ability to degrade toxic organic compounds; Van der Auwera et al. 2009). Conjugative plasmids encode modules for DNA transfer, allowing for their own propagation independent of the host and potentially transferring chromosomal DNA. Complete nucleotide sequences are currently available for several plasmids of *X. fastidiosa*. Below, a brief summary of plasmids associated with *X. fastidiosa* is presented.

Known plasmids of *X. fastidiosa* represent a broad range of diversity. A 51 kilobase pair (kb) circular contig designated pXF51 was generated from genome sequencing of *X. fastidiosa* subspecies *pauca* strain 9a5c from citrus (Simpson et al. 2000; van Sluys et al. 2003). A smaller 6 kb plasmid (pXF5823) has been characterized from another citrus-infecting strain (Qin and Hartung 2001). Four *X. fastidiosa* strains isolated from mulberry in Southern California harbor closely related 25 kb plasmids (pXFRIV11, pXFRIV16, pXFRIV19, and pXFRIV25) that have been assigned to incompatibility group P1 (IncP1) (Stenger et al. 2010). A 38 kb plasmid (pXFRIV5) has been characterized from a subspecies *multiplex* strain (RIV5), isolated from ornamental plum in Southern California (Rogers and Stenger 2012; Chen et al. 2010). A nearly identical plasmid (pXFAS01), varying from pXFRIV5 at only six nucleotide positions, was discovered as a circular contig during genome sequencing of subspecies *fastidiosa* strain M23 (Chen et al. 2010) isolated from almond in California. Multiple strains of *X. fastidiosa* contain a small (1.3 kbp) plasmid that utilizes rolling circle replication (Guilhabert et al. 2006; Pooler et al. 1997). All of the plasmids mentioned above have been completely sequenced. Other, likely distinct,

plasmids are present in *X. fastidiosa* strains isolated from a variety of hosts and locations but so far have been characterized only for restriction endonuclease patterns (Hendson et al. 2001).

Beyond inferences from homology, there is limited information regarding function(s) of genes encoded by *X. fastidiosa* plasmids. These plasmids appear to lack modules that would provide selective advantages to their hosts. Interestingly, the only type IV secretion systems in *X. fastidiosa* are found on conjugative plasmids, where they presumably facilitate DNA transfer. Among the sequenced plasmids of *X. fastidiosa*, only those of the 38 kb class (pXFAS01 and pXFRIV5) encode what appear to be a complete type IV secretion system: *tra* and *trb* modules necessary for conjugative transfer and mating pair formation, respectively. Indeed, the occurrence of nearly identical 38 kb plasmids in subspecies *fastidiosa* (pXFAS01) and *multiplex* (pXFRIV5) may be due to recent conjugative transfer among strains representing different subspecies (Rogers and Stenger 2012).

Several genes of the 25 kb (Inc-P1) class of plasmids from mulberry-infecting strains of *X. fastidiosa* (Stenger et al. 2010) have been characterized for function. Sequence comparisons indicate that extensive regions (~75 %) the 25 kb IncP-1 plasmids of *X. fastidiosa* are most closely related to an IncP-1 plasmid (pVEIS01) from the earthworm symbiont *Verminephrobacter eiseniae* (Pinel et al. 2008). IncP-1 plasmids initiate DNA replication through a plasmid-encoded protein (TrfA), which binds to iterative elements of the vegetative origin of replication (*oriV*) (Mei et al. 1995). Phylogenetic analysis revealed that the TrfA homologues of *X. fastidiosa* and *V. eiseniae* plasmids represent a newly discovered and divergent lineage of IncP-1 plasmids (Stenger and Lee 2011). Replication modules (*trfA* and *oriV*), derived from IncP-1 plasmids of *X. fastidiosa* or *V. eiseniae*, placed into a standard *E. coli* cloning vector allowed plasmid replication in *X. fastidiosa* (Lee et al. 2010). Furthermore, hybrid IncP1 replication modules (e.g., heterologous combinations of *trfA* and *oriV* from *X. fastidiosa* and *V. eiseniae*) conferred the ability to replicate in *X. fastidiosa* (Stenger and Lee 2011).



**Fig. 8.4** Map of the *X. fastidiosa* shuttle vector pXF20-PEMIK. Colored arcs denote regions derived from *E. coli* plasmid pCR2.1 or *X. fastidiosa* plasmid pXFRIV11.

MCS1 and MCS2 denote locations of unique endonuclease restriction sites serving as multiple cloning sites

Plasmids bearing IncP-1 replication modules were stable in *X. fastidiosa* only when cultured under antibiotic selection. Growth of *X. fastidiosa* transformants in the absence of antibiotic selection resulted in extinction of plasmids bearing *trfA* and *oriV*. However, addition of the *pemI/pemK* toxin–antitoxin (TA) system homologue encoded by pXFRIV11 conferred stable inheritance of plasmids bearing the *X. fastidiosa* IncP-1 replication module in the absence of antibiotic selection (Lee et al. 2010). Functional analysis of the *X. fastidiosa pemI/pemK* TA system indicated that PemK toxin is an endoribonuclease and that PemI is the cognate antitoxin that blocks PemK ribonuclease activity via direct and reversible binding (Lee et al. 2012). These results, and the effects of unbound PemK toxin on cell growth, indicate that the *X. fastidiosa pemI/pemK* TA system is a classic plasmid

addiction system (stable toxin, labile antitoxin) in which daughter cells not containing plasmid are killed by residual toxin. The construct pXF20-PEMIK (Fig. 8.4) bearing the *X. fastidiosa* IncP-1 replication module and *pemI/pemK* TA system represents a stable shuttle vector able to replicate in both *E. coli* and *X. fastidiosa*.

Of what benefit are plasmids to *X. fastidiosa* fitness as a plant pathogen? At present, the answer to this question is not known. Currently, no pathogenicity or virulence factors of *X. fastidiosa* are known to be plasmid-encoded. In some cases (e.g., the 1.3 kb rolling circle replicon), plasmids simply may be acting as selfish DNA. In another case (e.g., the 38 kb plasmids), encoding a functional type IV secretion system may allow for (or has facilitated) acquisition of new traits, potentially including pathogenicity islands or other genetic modules of benefit to *X. fastidiosa*.

### 8.3.3 Comparative Sequence Analysis

Like other xanthomonads, *X. fastidiosa* has a circular chromosome and a variety of plasmids, none of which are present among all isolates. A notable difference between *Xanthomonas* and *Xylella* genomes is size; *X. fastidiosa* shares ~ 74 % of its genome with *Xanthomonas* strains (Moreira et al. 2004), but *X. fastidiosa* genomes range from 2.5 to 2.7 megabase pairs (Mb), the smallest *Xanthomonas* genome (*X. albilineans*) consists of 3.7 Mb and other genomes range from 4.5 to 5.5 Mb. Unlike *X. fastidiosa*, *Xanthomonas* ssp. genomes are highly plastic and there is very high diversity within the genus.

*X. fastidiosa* appears to be derived from a form of *Xanthomonas* (Fig. 8.1); the relationship between *X. fastidiosa*, *Xanthomonas albilineans*, and the remainder of *Xanthomonas* species is unclear, but the most comprehensive published analysis identifies *X. albilineans* as an outgroup with low confidence (Rodriguez et al. 2012). Our own analysis supports this relationship, but *Xylella* has also been inferred to be the sister clade to *Xanthomonas albilineans* (Pieretti et al. 2009). The family *Xanthomonadaceae* is very diverse, but there is no indication that plant association is common outside of *Xanthomonas* and *Xylella*. The family (and order) itself has a complicated evolutionary history; while it is nominally within the gamma-proteobacteria, its relationship to that group is not straightforward. Phylogenies generally depict the lineage as splitting from the rest of the gamma-proteobacteria, and a gene-by-gene analysis has revealed that many genes are more similar to genes of the alpha or beta proteobacteria, implying that much of the genome was acquired from these other lineages and the placement within gamma-proteobacteria may not be reliable (Comas et al. 2007).

Like *X. fastidiosa*, *X. albilineans* is considered to have a reduced form of the *Xanthomonas* genome. However, gene loss occurred subsequent to their divergence from each other, indicating that the lineage leading to *X. fastidiosa*

likely underwent the loss of 2 Mb of genome content independent of any known lineage (Pieretti et al. 2009). This reduction in genome size is consistent with the stereotypical genome degradation process that has been described for assorted host-dependent bacteria (Moran and Plague 2004). The genome erosion syndrome is also revealed in the absence of detectable selection on codon usage and the slow growth rate of *X. fastidiosa* (Sharp et al. 2005; Rocha 2004), relative to *Xanthomonas* species. Two explanations are available for this evolutionary pattern. The ecological explanation posits that bacteria growing in a stable, nutrient-limited environment do not benefit from a diverse gene repertoire or from the ability to rapidly produce proteins (Vieira-Silva and Rocha 2010). Alternatively, the genetic explanation appeals to a reduction in selective efficacy due to reduction in effective population size that results from frequent population bottlenecks associated with transmission between hosts (Sharp et al. 2010). Combined with the deletion-biased mutation processes that are typical of bacterial genomes, this results in a reduction in genome size as genes that provide little fitness advantage are lost.

Only a small number of genes distinguish *X. fastidiosa* from *Xanthomonas* species. Due to evolutionary association of such genes with the defining ecological traits of *X. fastidiosa* (particularly insect transmission), they are candidates for participation in these functions. The pear leaf scorch pathogen from Taiwan appears to represent a lineage of *X. fastidiosa* that branched from the American lineages prior to their diversification. If it is confirmed to exhibit the same basic ecological and physiological traits of *X. fastidiosa*, then its genome sequence will help us to narrow in on the genomic changes responsible for the evolution of those traits.

Although four subspecies are commonly recognized within *X. fastidiosa*, there are reports of variants that may belong to two other subspecies, though these have yet to be well described (Su et al. 2012; Randall et al. 2009). Although various approaches have been used to

study *X. fastidiosa* genetic diversity, multi-locus sequence typing (MLST) has provided the most valuable insights into the evolutionary history of this pathogen (Yuan et al. 2010). MLST is based on the sequencing of seven housekeeping genes showing neutral variation and are distributed throughout the chromosome of *X. fastidiosa* such that they are unlinked during recombination. This approach has been used to characterize the genetic diversity of *X. fastidiosa* subspecies, providing insights into the history of pathogen dispersal and genetic recombination between subspecies, as described below.

Subspecies *fastidiosa* and *sandyi* in the United States are composed of isolates with very little diversity when typed using MLST (Yuan et al. 2010), but subsp. *fastidiosa* isolates from Costa Rica colonizing various plant species have more allelic diversity than those causing disease in grapevines in the United States (Nunney et al. 2010). These results suggest that Pierce's disease in the United States was introduced from Central America. The genetic structure of subsp. *multiplex* is somewhat distinct in that there are multiple clusters of isolates, with indications of host-range differences among clusters (Nunney et al. 2013; Scally et al. 2005; Almeida and Purcell 2003b). Lastly, subsp. *pauca* is limited to South America; while most studied isolates colonize either citrus or coffee sympatrically, there is no overlap in host range and the groups are phylogenetically distinct (Almeida et al. 2008). Interestingly, isolates recovered from plum in Brazil fall within subsp. *multiplex*, which is otherwise limited to North America, or fall between subsp. *multiplex* and *pauca* (Nunes et al. 2003), suggesting it was likely introduced from North America via contaminated plant material.

Horizontal gene transfer between subspecies is a recurring theme in *X. fastidiosa* evolution, as shown by both MLST and whole-genome data, along with distribution of plasmids as described above. Both the coffee and citrus isolates of subspecies *pauca* appear to have acquired alleles

from subspecies *multiplex* (Nunney et al. 2012). Likewise, subspecies *multiplex* has participated in recombination with subspecies *fastidiosa* (Nunney et al. 2010; Kung et al. 2013). This process of allele conversion has even gone as far as to produce new lineages with roughly equal contributions from subsp. *multiplex* and *fastidiosa*, as found in the strains isolated from mulberry (Nunney et al. 2014). Overall MLST has proven to be a robust tool for reconstructing the relationship between *X. fastidiosa* isolates despite gene flow between subspecies and has therefore become the paradigm for *X. fastidiosa* taxonomy (Fig. 8.1).

In contrast to effector-mediated host/microbe interactions shown for *Xanthomonas* ssp. and most other bacterial plant pathogens, host specificity in *X. fastidiosa* violates the dominant paradigm of host–pathogen specificity among bacterial plant pathogens, as this bacterium does not have effector-encoding genes or a type III secretion system (Simpson et al. 2000). *X. fastidiosa* is further distinguished from *Xanthomonas* ssp. In that genome comparisons do not reveal gene content differences that could be responsible for the observed host specificity (van Sluys et al. 2003).

All *Xanthomonas* have six secretion systems (type I–VI) to export proteins. Within individual *Xanthomonas* ssp., pathovars show specific host associations, largely driven by secreted effectors (White et al., 2009), of which each strain secretes a unique blend of 20–40 directly into the host cytoplasm. While the exact function of many effectors in host cells is just beginning to be understood, many interfere or suppress induced plant defenses. *X. albilineans* is the only sequenced *Xanthomonas* ssp. without an Hrp-T3SS, but contains an SP1-T3SS. Mutations at the T3SS locus eliminate virulence among *Xanthomonas* isolates; therefore, the absence of a T3SS in *X. fastidiosa* indicates that its pathogenicity mechanisms are distinct and not yet fully described compared to most other bacterial pathogens (Simpson et al. 2000; Chatterjee et al. 2008a).

## 8.4 Applications from Genomic Data

### 8.4.1 Genetic Tractability

Determination of the complete genome sequence of *X. fastidiosa* (Simpson et al. 2000) raised questions about the putative function of several genes in plant pathogenicity and insect transmission, among other topics. Genetic analysis of these genes typically requires the ability to transform bacteria with appropriate plasmids carrying complementary wild-type genes. The first description of a system for genetic analysis was the transformation of a construct carrying ORF1 of *X. fastidiosa* along with its origin of replication, originally present in the sequence of one of its plasmids (Qin and Hartung 2001). Several studies described different systems used to transform *X. fastidiosa* (e.g., Silva Neto et al. 2002) or extracted from broad-host-range plasmids (Guilhabert and Kirkpatrick 2003). However, many of these early constructs were not stable once introduced in *X. fastidiosa* chromosome without antibiotic selection, and genetic analysis of knockouts within plants or insects was impossible. Development of tools allowing the stable transformation of *X. fastidiosa* by homologous recombination through the utilization of plasmids carrying its own chromosomal replication origin (*oriC*) circumvented this limitation (Monteiro et al. 2001; Newman et al. 2003, 2004). More recently, the pAX1 plasmid series carrying a *colE1*-like (pMB1) replicon associated with four different antibiotic selection markers allowed the recovery of double recombinants (Matsumoto et al. 2009). The pXF20-PEMIK plasmid with an addiction system, described above, may be successful in allowing for complementation of mutants, which has been a major limiting factor in *X. fastidiosa* research. To our knowledge, only Chatterjee et al. (2010) performed studies with complemented knockouts in both plants and insects using the broad-host-range plasmid pBRR-5 (Kovach et al. 1995), which was previously used by Reddy et al. (2007).

*Xylella fastidiosa* is naturally competent and able to transform and recombine linear and circular DNA during its growth phase (Kung and Almeida 2011). Transformation and recombination efficiencies increase with increments in the length of homologous flanking regions of inserts, up to 1 kb in size. In addition, efficiency reduces as the size of the non-homologous insert increases, with recombination not detected once the region was 6 kb in length (Kung et al. 2013). Competency, as in other bacteria, appears to be mediated by a type IV pilus-like apparatus, with associated *com* genes that transport DNA fragments through the cell membrane. The ease of transformation with this protocol, in addition to its high efficiency, should facilitate the genetic tractability of *X. fastidiosa*.

### 8.4.2 Genomics Opens New Research Venues

While there has been much less study of the virulence mechanisms utilized by *X. fastidiosa* compared to its closest relatives in the genus *Xanthomonas*, the availability of genome sequences has enabled several putative virulence factors to be investigated. Both the lack of an apparent type III secretion system, commonly used in other plant pathogens to suppress plant host defense responses, and the fact that this pathogen probably seldom encounters living plant cells while colonizing the xylem vessels suggests that *X. fastidiosa* differs in the factors that contribute to its virulence compared to other plant pathogens. Most attention has been directed toward the study of the role of other secretion systems in its virulence, and it appears that *X. fastidiosa* is capable of type I secretion. Analysis of the genome of *X. fastidiosa* reveals the presence of at least 23 systems comprising 46 proteins that belong to the ABC (ATP-binding cassette) superfamily of proteins (Meidanis et al. 2002). Type I secretion systems are often used in tolerance of toxic compounds and encode efflux pumps for small molecules as well as enabling the secretion of extracellular

proteins such as hemolysins. A central component of many bacterial type I secretion systems is a protein similar to TolC that spans the inner and outer membranes of gram-negative species. Importantly, a *tolC* knockout mutant of *X. fastidiosa* exhibited nearly complete loss of virulence to grape (Reddy et al. 2007). Perhaps more importantly, no viable cells of the *tolC* mutant could be recovered after inoculation into grape. This latter observation suggests strongly that an efflux pump in which TolC participated was required for survival in plants after inoculation, perhaps by enabling export of toxic compounds found in the xylem of the plant. Support for this conjecture was provided by the observation that the *tolC* mutant was more sensitive to toxic phytochemicals such as berberine, certain detergents, as well as the variety of compounds found in crude plant homogenates than the wild-type strain (Reddy et al. 2007). While such efflux systems can also export toxins and proteins that might elicit a host plant response, no studies have appeared to suggest that *X. fastidiosa* uses such a process to interact with living plant cells. Clearly, more work is needed to understand to which extent *X. fastidiosa* interacts with living plant tissues. The fact that tyloses that invade the xylem vessel are induced in plants infected with this pathogen (Hopkins and Purcell 2002) suggests that some communication with living plant tissue does occur. Furthermore, others have posited that many of the water stress symptoms associated with *X. fastidiosa* infection are not due solely to plugging of xylem vessels by cells of the pathogen, but instead are a result of excessive self-induced blockage of vessels by tyloses that are induced by the presence of the pathogen (Fry and Milholland 1990; Hopkins 1989; Hopkins and Purcell 2002; Purcell and Hopkins 1996).

The type II secretion system appears to be particularly important for the virulence of *X. fastidiosa* in the absence of a type III secretion system. The type II secretory system is primarily involved in the export of extracellular enzymes, frequently for the hydrolysis of various plant structural features. The type II secretion system, encoded by a collection of genes commonly

known as Xps (Xanthomonas protein secretion), has been widely studied in other *Xanthomonas* species. Not only does *X. fastidiosa* harbor a complete set of Xps homologues, but it also contains genes capable of encoding several different extracellular enzymes such as several  $\beta$ -1,4 endoglucanases, xylanases, xylosidases, and a polygalacturonase (Simpson et al. 2000; van Sluys et al. 2003). Only the role of the polygalacturonase, encoded by *pglA* on the virulence of *X. fastidiosa*, has been addressed experimentally. Importantly, a *pglA* mutant exhibited greatly reduced ability to colonize grape and therefore incited very little symptom development (Roper et al. 2007). While the mutant could be re-isolated from near the point of inoculation, it was severely restricted in its long-distance movement along the grape xylem vessels (Roper et al. 2007). It seems likely that its greatly reduced motility is attributable to its inability to degrade pit membranes that serve to restrict both lateral and longitudinal movement of the pathogen between xylem vessels. As both the growth and movement of the *pglA* mutant was suppressed compared to the parental strain, it seems possible that pectin may serve not only as a barrier to intercellular movement, but might also be a nutrient source for *X. fastidiosa* although pectin does not appear to affect bacterial growth in vitro (Killiny and Almeida 2009b). In fact, the differential polysaccharide composition of pit membranes has been suggested to account for the relative differences in susceptibility of different grape varieties to invasion by *X. fastidiosa*, with homogalacturans and xyloglucans playing an important role in the susceptibility of various varieties to infection (Sun et al. 2011). Further evidence for the role of pectin as a constituent of a physical barrier for intercellular movement of *X. fastidiosa* is provided by the observation that grape expressing a polygalacturonase-inhibiting protein from pear exhibited higher resistance to symptom development after inoculation with this pathogen than the parental line (Agüero et al. 2005). It is also important to note that *rpfF* mutants of *X. fastidiosa* that do not produce diffusible signaling factor (DSF) express *pglA* and genes encoding

certain other extracellular enzymes at a higher level than that of the wild-type strain (Chatterjee et al. 2008b; Wang et al. 2012). Given that *rpfF* mutants are hyper-virulent to grape (Newman, et al. 2004), it is reasonable to assume that the elevated expression of these cell wall-degrading enzymes in this mutant background could have contributed to at least some of the enhanced virulence that they exhibit.

Active motility appears to be an important factor contributing to the virulence of *X. fastidiosa*. While *X. fastidiosa* is a non-flagellating bacterium, it harbors several genes that encode proteins involved in production and function of type IV pili (Simpson et al. 2000; van Sluys et al. 2003). These long, polar located pili have been shown to be involved in twitching motility in *X. fastidiosa* (Meng et al. 2005). Intriguingly, such twitching motility was demonstrated to enable the movement of *X. fastidiosa* not only along abiotic surfaces, but also along xylem vessels. Importantly, *X. fastidiosa* exhibits the ability to actively move against the flow of xylem fluids in vessels by twitching motility (Meng et al. 2005). The apparently high efficiency with which cells of *X. fastidiosa* move through the orifices of pits (Newman et al. 2004), which are apparently enlarged due to the action of extracellular enzymes secreted by this pathogen (Perez-Donoso et al. 2010), may be due at least in part to its ability to move along surfaces by retraction of the type IV pili. *X. fastidiosa* also produces relatively short type I pili, encoded by *fimA*, that apparently act to restrict motility (De La Fuente et al. 2007). *fimA* mutants exhibited a higher rate of twitching motility than that of the wild-type strain, suggesting that it may serve as an “anchor,” being involved in attachment but serving to repress motility (De La Fuente et al. 2007). Perhaps not surprisingly, the expression of the genes for type IV pili and that of *fimA* tend to be oppositely regulated by the accumulation of DSF signal molecule (Chatterjee et al. 2008b; Wang et al. 2012). It therefore would seem prudent for *X. fastidiosa* to balance the abundance of these two pilus types depending on those stages of its colonization to which they might primarily

contribute. That is, the initial attachment of *X. fastidiosa* to surfaces such as insect vectors may be facilitated by *fimA* (Killiny and Almeida 2009b), but its presence would tend to suppress active movement by twitching within the plant after inoculation. Curiously, a *tonB* homologue was found to be required for twitching motility and appropriate biofilm formation and virulence of *X. fastidiosa*, phenotypes unexpected from its role in transport of non-permeable molecules across the outer membrane and other bacteria (Cursino et al. 2009). Further evidence of the need for complex regulation of type IV pili function in *X. fastidiosa* is the observation that a chemosensory system is operative. An operon named Pil-Chp containing genes homologous to those found in chemotaxis systems of other bacteria were found to be required for proper pilus function but not biogenesis in *X. fastidiosa* (Cursino et al. 2011). Such regulatory mutants were also deficient in colonization of plants, suggesting that complex regulation of pilus function may also occur in plants (Cursino et al. 2011). While such regulators are apparently required for motility, it remains unclear whether *X. fastidiosa* exhibits chemotactic movement toward or away from particular compounds. It is intriguing to consider that the host range or virulence of *X. fastidiosa* might be modulated by the differential presence of plant compounds that might anticipate in regulation of type IV pilus function.

The role of extracellular polysaccharide (EPS) production in the behaviors of *X. fastidiosa* is not completely understood. EPS production is an important virulence factor in many *Xanthomonas* species. *X. fastidiosa* contains homologues of many but not all of the so-called *gum* genes necessary for EPS production in *Xanthomonas* species (Simpson et al. 2000; van Sluys et al. 2003). It therefore has been suggested that *X. fastidiosa* makes an EPS molecule similar to that of xanthan gum made by *Xanthomonas campestris*, but one which is lacking terminal mannosyl residues (Silva et al. 2001). Antibodies that could recognize the EPS produced by *X. fastidiosa* were recently used to illustrate its production both in culture and *in*



*planta* (Roper et al. 2007). Generation of *gum* mutants (Killiny et al. 2013) recently helped to better characterize the role of EPS in *X. fastidiosa* virulence. In addition to their reduced capacity to form biofilms in culture, *gum* mutants, once mechanically introduced in plants, were avirulent and did not result in the development of any symptoms in grapevines (Killiny et al. 2013). These traits were also associated with an apparent lack of plant colonization and an altered motility compared to that of the wild-type cells. In addition, EPS was also shown having an important role in vector transmission, probably due to its implication in biofilm formation, essential step for a successful insect colonization.

*Xylella fastidiosa* appears to have a surprisingly large number of adhesin and hemagglutinin-encoding genes compared to other bacteria (Simpson et al. 2000; van Sluys et al. 2003), and such adhesins appear to play a central role in its complex lifestyle. Initial results of studies of virulence factors in *X. fastidiosa* involving the screening of random insertional mutants revealed a surprising role for adhesins. Specifically, *hxfA* and *hxfB* mutants that no longer expressed these two related hemagglutinin-like proteins exhibited a hyper-virulent phenotype in grape (Guilhabert and Kirkpatrick 2005). Furthermore, these mutants exhibited reduced cell–cell aggregation and moved further in grape xylem vessels in the wild-type strain after inoculation (Guilhabert and Kirkpatrick 2005). Because such proteins would be expected to facilitate both cell–cell aggregation as well as cell surface attachment, it would be expected that their presence would tend to reduce the virulence of *X. fastidiosa* by impeding its movement along xylem vessels. Further evidence for the negative effects of a fimbrial adhesins on the virulence of *X. fastidiosa* has come from studies of various mutants altered in DSF-mediated cell–cell signaling. For example, *rpfF* mutants, which are blocked in the accumulation of DSF, express a variety of adhesins such as HxfA, HxfB, FimA, and XadA at a much lower level than that of the wild-type strain (Chatterjee et al. 2008b; Wang et al. 2012). The hyper-virulence of the *rpfF* mutant could be

conferred by the lower level of these adhesins, accounting for the ability of such mutant cells to move more extensively within plants (Chatterjee et al. 2008c). Further support for the role of adhesins as anti-virulence factors comes from studies of *rpfC* mutants which accumulate excessive amounts of DSF and *cgxA* mutants of *X. fastidiosa* which are predicted to have relatively low concentrations of the intracellular signaling molecule cyclic di-GMP (Chatterjee et al. 2008b, 2010). In both mutant backgrounds, the expression of various fimbrial and afimbrial adhesins is higher than in a wild-type strain, accounting for their relatively low ability of these mutants to move throughout plants and therefore to cause disease symptoms (Chatterjee et al. 2010). In addition to the hemagglutinin-like proteins and other fimbrial and afimbrial adhesins that have been noted to affect the adhesiveness and cell–cell aggregation capabilities of *X. fastidiosa*, LPS also may play a similar role. Disruption of PD0914, which encodes a Wzy polymerase involved in biosynthesis of a high molecular weight O-antigen in the LPS of *X. fastidiosa*, led to measurable differences in the structure of biofilms formed by such a mutant as well as reducing its virulence (Clifford et al. 2013). It is also fascinating to find that several afimbrial adhesins produced by *X. fastidiosa* can be found in the extracellular milieu of this pathogen. For example, the adhesin XadA1 was found associated not only with the surface of *X. fastidiosa* cells, but was also often found in intercellular spaces of bacterial aggregates (Caserta et al. 2010). In contrast, the related molecule XadA2 was found associated only with intact cells (Caserta et al. 2010). Curiously, a portion of the population of the adhesins HxfA and HxfB produced by *X. fastidiosa* in culture were found in culture supernatants, presumably associated with membranous vesicles (Voegel et al. 2010). The role of such extracellular forms of these adhesins is unknown, as is the identity of any factors that may control the apparent release of these molecules from the producing cell.

It is probably quite significant that several different regulatory systems have been found to coordinate expression of various fimbrial and

afimbrial adhesins in *X. fastidiosa*. Given that adhesins almost certainly play a strong but contextual role in the biology of *X. fastidiosa*, their proper temporal and spatial expression would be essential for this pathogen to maintain optimal fitness in its plant and insect niches. The response regulator XhpT, composed of a receiver domain and a histidine phosphotransferase output domain, was found to control surface attachment, cell–cell aggregation, and EPS production as well as virulence of *X. fastidiosa* (Voegel et al. 2013). While any signal that this response regulator might perceive remains unknown, it is intriguing to consider that it might be involved in habitat-specific expression of those genes such as *hxfA*, *hxfB*, and *tonB* which, as discussed above, play central roles in the behavior of *X. fastidiosa* in different settings. The global regulator GacA was also found to positively regulate several virulence factors in *X. fastidiosa* including the adhesins XadA and Hsf (Shi et al. 2009). GacA has been found to control a variety of physiological processes as well as pathogenicity factors in many other gram-negative bacteria (Heeb and Haas 2001). As such, it is thought to play an important coordinating role in context-dependent expression of virulence factors. The suppression of adhesins in *X. fastidiosa* provides further evidence that the proper temporal and spatial expression of these molecules is important in the context-dependent behavior of this pathogen.

#### 8.4.2.1 Vector Colonization

The chemical composition of the external layer of an insect's exoskeleton (the epicuticle) is not well understood for several insect groups; and to our knowledge, there is no information on its composition for *X. fastidiosa* insect vectors. The epicuticle of insects is composed of several layers: the inner and outer epicuticle covered by a wax layer; and in some insects an additional cement layer also exists above this wax layer. The thickest layer, the inner epicuticle, is 0.5–2  $\mu\text{m}$  thick (Chapman 1998). The cement layer is a thin layer composed of mucopolysaccharides associated with lipids. The wax layer is

largely composed of lipids with embedded proteins, and serves as a waterproofing element for the cuticle. In addition, proteinaceous molecules would also be present in the cuticle, along with other potential molecules. Although the chemical composition of the cuticular surface of arthropods is generally not well understood, interactions between bacteria and the exoskeletons have been successfully studied in other systems using chitin as a proxy, as this system mimics bacterial behavior on the surface of actual hosts (Tarsi and Pruzzo 1999).

The first step in determining the nature of *X. fastidiosa*-vector interactions was to learn whether *X. fastidiosa* surface proteins were involved in cell adhesion to vectors. Killiny and Almeida (2009a) demonstrated that *X. fastidiosa* cells bind to carbohydrates, and that treating intact cells with proteases reduced adhesion to compounds such as chitin. Thus, surface proteins were involved in cell adhesion to carbohydrates; however, *X. fastidiosa* had variable affinity to different molecules. For example, competition assays showed that *N*-acetylglucosamine (GlcNAc, the monomer of chitin) acted as a strong competitor in binding assays where vector foregut extracts were used as a substrate, reducing cell adhesion. On the other hand, mannose and galactose did not affect binding. In addition, to look for specific *X. fastidiosa* proteins involved in adhesion, several mutants were tested in vitro for binding to foregut extracts (Killiny and Almeida 2009a). Only hemagglutinin and cell–cell signaling mutants were affected in adhesion. Altogether, these biochemical and other biological assays indicated that initial cell adhesion to vectors is mediated by carbohydrate–lectin interactions and that specific *X. fastidiosa* surface proteins can be identified in vitro as potential candidates for more comprehensive studies.

Since it was first cultured in the laboratory, attempts to deliver *X. fastidiosa* cells to vectors from growth media have been unsuccessful (Davis et al. 1978), until it was discovered that plant structural polysaccharides result in phenotypic changes in *X. fastidiosa*, inducing its transmissibility by leafhoppers (Killiny and

Almeida 2009b). Plant polysaccharides induce phenotypic changes leading to higher degrees of adhesiveness and, consequently, attachment to vectors after acquisition from plants (Killiny and Almeida 2009b). The pattern of gene expression of *X. fastidiosa* exposed to pectin-supplemented media is also similar to that of *X. fastidiosa* cells occurring at high cell density (Newman et al. 2004; Chatterjee et al. 2008b).

The gene expression profile is also modified when media is supplemented with chitin, the main component of the insect foregut surface (Killiny et al. 2010). Adhesins involved in *X. fastidiosa* initial adhesion to insect cells are upregulated, and biofilm formation is also enhanced on chitinous surfaces. In addition, a functional chitinase (ChiA; Killiny et al. 2010) was recently discovered, as part of the machinery used by *X. fastidiosa* to degrade and assimilate chitin as its sole carbon source. Molecular mechanisms involved in chitin utilization are not completely understood. Until now, no known chitin-binding domains have been found in that enzyme, and the implication of other *X. fastidiosa* chitin-binding proteins are likely to be involved in the process (Labroussaa and Almeida, unpublished).

#### 8.4.2.2 Host Switching and Cell-Cell Signaling

One intriguing aspect of *X. fastidiosa*'s biology unique among xanthomonads is that it is restricted to colonizing two very different, yet highly specialized environments—plants and insect vectors. Water conducting xylem vessels of plants and the foregut of vectors are extremely different environments. Cells of *X. fastidiosa* are attached to surfaces in the insect foregut and experience very rapid fluid flow (estimated  $> 5$  cm/s, Purcell et al. 1979) caused by a powerful pumping system used by insects to suck sap under negative tension; turbulence is created in the mouthparts once every second when leafhoppers pull sap from plants and push it into the gut (Dugravot et al. 2008; Purcell and Finlay 1979). Sap flow conditions are not nearly as extreme in xylem vessels, where flow was

calculated to achieve ca.  $1 \times 10^{-2}$  to  $1 \times 10^{-4}$  cm/s inside grapes growing in the field (Andersen and Brodbeck 1989; Greenspan et al. 1996). The dramatic differences in the flow speed of different environments (100 to 10,000 times faster inside insect foregut compared to xylem in plants) will have an impact on bacterial cell attachment to surfaces and to each other, since they need to overcome the external shear force stress. In plants, *X. fastidiosa* multiplies within individual vessels and moves actively to adjacent vessels in the xylem network by producing enzymes that degrade pit membranes separating individual xylem vessels (Perez-Donoso et al. 2010). In plant xylem vessels, *X. fastidiosa* also has a larger surface area to colonize compared to the foregut of leafhoppers (Newman et al. 2003).

In addition to responding to host or environment specific cues, many bacteria also utilize highly specific quorum-sensing signals that induce gene expression above a certain concentration threshold. By using density-dependent signaling, populations of bacteria can quickly coordinate the expression of metabolically costly traits in response to environmental cues that would be ineffective if expressed by low populations of cells. The role of cell-cell signaling toward host colonization is often not well documented (Bassler and Losick 2006) but is implicated in expression of colonization and virulence traits for an increasing number of plant and animal pathogenic bacteria (Ham 2013; Ryan and Dow 2008).

Like many other bacteria, *X. fastidiosa* has a regulatory system that responds to signal molecules produced by individual cells in a population; these diffusible molecules accumulate in the environment and trigger population-wide phenotypic changes, likely associated with global changes in gene expression when the signal threshold is reached (Wang et al. 2012). Many traits implicated in insect and host plant colonization are also under control of the regulation of pathogenicity factor (*rpf*) operon. In *Xanthomonas* ssp. and *X. fastidiosa*, *rpf* controls synthesis and detection of DSF, a medium-chain fatty acid that functions as a signaling molecule (Colnaghi Simionato et al. 2007; Dow et al.

2003). The *rpf* cluster in *Xanthomonas* ssp. contains 12 genes, 9 of which are conserved in *X. fastidiosa*. In both genera, *rpfF* produces DSF, which diffuses freely through cell membranes and is sensed by a two-component transmembrane receptor RpfC and RpfG (Barber et al. 1997; Newman et al. 2004), but many of the genes in this operon remain functionally uncharacterized.

The *Xanthomonas campestris* pv. *campestris* DSF molecule was described as cis-11-methyl-2-dodecanoic acid (Barber et al. 1997; Wang et al. 2004); while *X. fastidiosa* can respond to *Xanthomonas* ssp. DSF, the weaker response (Newman et al. 2004) suggested that *X. fastidiosa* synthesizes and responds to a distinct DSF molecule. Recently, the structure of an *X. fastidiosa* DSF was identified as 2(Z)-tetradecanoic acid and was shown to control DSF-dependent traits in *X. fastidiosa*, including biofilm formation and attachment to surfaces (Beaulieu et al. 2013).

Experiments with DSF deficient and blind mutants have shown that DSF signals are important in regulating *X. fastidiosa* phenotypes in plant and insect hosts. Disruption of DSF production (in an *rpfF* knockout mutant) results in hyper-virulence within plants, possibly due to up-regulation of plant colonization-related genes and down-regulation of adhesins (Newman et al. 2004; Wang et al. 2012) that decrease rates of both self-aggregation and attachment to xylem vessels. However, the *rpfF* mutant is not capable of colonizing the precibarium of vectors and is very poorly transmitted to plants. It appears that the induction of a vector-transmissible state occurs due to up-regulation of genes under control of the *rpf* system.

In contrast, DSF-blind *rpfC* mutants over-produce *hxfA* and *hxfB*. This ‘stickier’ phenotype is less virulent than wild type in planta due to reduced movement through and colonization of xylem. While *rpfC* is not impaired in attachment to vector foreguts, transmission is reduced due to increased adhesin expression and lower rates of cell detachment (Chatterjee et al. 2008b). In *X. campestris*, DSF regulates the production of an enzyme that controls cell

dispersal from a biofilm (Dow et al. 2003). The inability of *rpfC* to detach from the biofilm in the insect foregut implicates DSF signaling as an important but uncharacterized regulator of expression controlling cell detachment from biofilm bound cells in the insect foregut. Consequently, afimbrial adhesins are over-expressed, while genes associated with plant host colonization are down-regulated (see Chatterjee et al. 2008a for discussion). A complex picture of *X. fastidiosa* gene regulation in relation to vector transmission is emerging based on this research.

Although adhesion is essential for retention in insects, it limits colonization of plants compared to the *rpfF* mutant, highlighting the distinct requirements for life in such different hosts (Newman et al. 2004; Guilhabert and Kirkpatrick 2005; Chatterjee et al. 2008c). Thus, *X. fastidiosa*’s conflicting life history is framed by the contrasting requirement to move within plants to increase its population size and thus its chances of being acquired by insects, but at the same time, it must increase its adhesiveness so it can attach to insects, which consequently reduces within-plant movement.

Recent characterization of *rpfB* in both the rice blight pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (He et al. 2010) and *X. fastidiosa* (Almeida et al. 2012) have demonstrated that both species produce at least three distinct DSF signals, that they accumulate at different cell densities, and that *rpfB* has a role in DSF processing (Almeida et al. 2012). In *X. fastidiosa* and *Xoo*, an *rpfB* mutant only produced one of these signals, indicating a regulatory role in DSF processing for RpfB (Almeida et al. 2012). *rpfB* is spatially separated from the *rpf* operon in the *X. fastidiosa* genome compared to *Xanthomonas* ssp., suggesting a more complex role for *rpfB* in *X. fastidiosa*, which is vector transmitted compared to *Xanthomonas* ssp., which are not. The production of multiple DSF signals in wild-type cells and the ability of *rpfB* mutants, which produce only one of these signals, to adhere to but not colonize insect suggests the intriguing but unexplored possibility that host cues influence the production of different ratios of DSF to

modulate complex host colonization behaviors in alternating plant and insect hosts (Almeida et al. 2012).

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## 8.5 Disease Management Strategies

The availability of a collection of approaches to control *X. fastidiosa* diseases is highly desirable, as integration of various strategies will likely be necessary to sustainably control this pathogen. Unfortunately, disease spread in these systems is controlled primarily through the extensive use of pesticides, which often have negative short- and long-term consequences to integrated disease and pest management. In addition, this approach has obvious negative impacts on the environment and communities that rely on agricultural activities. Alternative, efficient, pathogen-specific, environmentally friendly, and safe approaches to control these diseases would lead to long-term sustainability of crop systems. Strategies to control *X. fastidiosa* currently in development, based on genomics-derived knowledge and the production of transgenic plants, focus on either the pathogen (e.g., Chatterjee et al. 2008c; Dandekar et al. 2012) or the vector (such as RNAi to impact insect development, not discussed here; Rosa et al. 2012), or on both partners and their interactions during insect transmission (Killiny et al. 2012). We limit our discussion to these approaches, although it should be mentioned that genomic data has provided great insights into the biology, ecology, evolution, and taxonomy of *X. fastidiosa*, much of which has been useful to devise other disease management strategies and for detection and quarantine purposes.

First, the DSF signaling system of *X. fastidiosa* has been used as the basis to develop a confusion strategy, where presence of DSF in the environment (i.e., xylem stream) at all times should limit bacterial movement and plant colonization. Chatterjee et al. (2008c) showed that the *rpfC* mutant strain is indeed limited within

plant movement and multiplication, suggesting that the presence of DSF functions as a suppressor of population growth. If constitutively expressed in transgenic plants, DSF molecules should lead to adherent *X. fastidiosa* populations with limited movement within plants and, as a result, the expression of disease symptoms should be reduced.

Another strategy concerns the utilization of cecropin A or B as antibacterial peptides for limiting the growth of *X. fastidiosa* (Ishida et al. 2004). Recently, the introduction of a construct carrying such a peptide into transgenic grapevines, allowing its specific expression into the xylem of plants, was efficient protecting grapes against the development of Pierce's disease (Dandekar et al. 2012). Other concepts leading to the constitutive expression of exogenous proteins in transgenic plants include the expression of polygalacturonase-inhibiting proteins (PGIPs) that inhibit *X. fastidiosa* polygalacturonase (*pglA*) responsible for the systemic movement of *X. fastidiosa* in plants (Agüero et al. 2005). Strategies may also emerge from research on the identification of xylem compounds produced during plant exposure to low temperatures (Wilhelm et al. 2011; Meyer and Kirkpatrick 2011). Grapevines with *X. fastidiosa* and expressing symptoms of Pierce's disease are cured of infections when subject to cold winters (Purcell 1977, 1980), via a yet to be determined mechanism.

Lastly, a strategy following the hypothesis that blocking interactions between both partners occurring during initial adhesion could lead to the impossibility for the bacteria to successfully colonize its vectors is also being pursued (Killiny et al. 2012). *X. fastidiosa* proteins identified as able to bind to insect receptors will be used as transmission-blocking molecules. Once expressed in grapevines, those molecules will compete for insect receptors with *X. fastidiosa* preventing the attachment of the bacteria on insect cells, essential step for its transmission and consequently, disrupting bacteria transmission from plant to plant.

## 8.6 Conclusion

The availability of *X. fastidiosa* genome sequences have allowed research on this fastidious organism to move significantly faster than in the past. In addition to improving diagnostic tools and promoting research on the functional role of genes, which has led to the incipient technologies briefly discussed here, it has provided insights into its evolution that would not have been possible otherwise. Because advances in technology will continue to make sequence data available at a larger scale with diminishing costs, we foresee that it will become an integral part of *X. fastidiosa* research. The complete integration of this tool with others now routinely used in plant pathology is yet to be realized, but efforts to use genome sequences have already generated exciting findings and will continue to do so.

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