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Gene regulation mediates host specificity of a bacterial pathogen

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Summary

Many bacterial plant pathogens have a gene-for-gene relationship that determines host specificity. However, there are pathogens such as the xylem-limited bacterium *Xylella fastidiosa* that do not carry genes considered essential for the gene-for-gene model, such as those coding for a type III secretion system and effector molecules. Nevertheless, *X. fastidiosa* subspecies are host specific. A comparison of symptom development and host colonization after infection of plants with several mutant strains in two hosts, grapevines and almonds, indicated that *X. fastidiosa* virulence mechanisms are similar in those plants. Thus, we tested if modification of gene regulation patterns, by affecting the production of a cell–cell signalling molecule (DSF), impacted host specificity in *X. fastidiosa*. Results show that disruption of the *rpfF* locus, required for DSF synthesis, in a strain incapable of causing disease in grapevines, leads to symptom development in that host. These data are indicative that the core machinery required for the colonization of grapevines is present in that strain, and that changes in gene regulation alone can lead *X. fastidiosa* to exploit a novel host. The study of the evolution and mechanisms of host specificity mediated by gene regulation at the genome level could lead to important insights on the emergence of new diseases.

Introduction

A wide range of mechanisms have been shown to lead to pathogen host specificity in animal hosts, including point mutations, duplications and horizontally acquired elements. Recently, for a mutualistic symbiosis, it was shown that host specificity was determined by the presence of a single regulatory gene (Mandel *et al.*, 2009). In that study, presence of one gene resulted in differential gene expression patterns, leading to bacterial colonization of a new host species. Therefore, gene regulation may also be responsible for host specificity of pathogens, potentially contributing to emerging diseases. This may be especially important as the evolutionary dynamics of disease systems differ from mutualistic symbioses and small changes to pathogen–host interactions may lead to changes in host range or disease severity.

Plant pathogens are ubiquitous in nature and may have great economic and social impact. To cause disease, pathogens need to overcome host plant defence lines. Initially, plant defences are mediated through recognition of general cell surface patterns, eliciting PAMP-triggered immunity (Bent and Mackey, 2007; Lindeberg *et al.*, 2009). A second level of defence is qualitative, in that host recognition of effectors, proteins secreted into host cells via type III secretion systems, leads to plant resistance to infection (i.e. effector-triggered immunity, ETI) (Bent and Mackey, 2007). Effector-coding genes are often in one-to-one relationships with plant resistance genes, such that secretion of an effector not recognized by its respective host resistance gene product leads to successful infection (Gabriel *et al.*, 1986; De Feyter *et al.*, 1993; Bent and Mackey, 2007). As such, host specificity is usually viewed under an arms-race framework where mutations to existing loci, or acquisition of new loci through lateral gene transfer, could increase the host range of a pathogen (Ma *et al.*, 2006). In addition, although host specificity at the plant species level is thought to occur via PAMP-triggered immunity mechanisms, deletion of effectors can lead pathogens to exploit new host plants (Wei *et al.*, 2007). Plant pathogenic fungi also have similar host–pathogen interactions; for example, breakage of plant resistance may be mediated by mutations in effectors (Van de Wouw *et al.*, 2010). Thus, plant pathogen host specificity is generally considered a qualitative rather than quantitative trait (Hajri *et al.*, 2009).

Xylella fastidiosa is different from other well-studied plant pathogens because its genome has no evidence of genes coding for effector proteins, nor does it have a type III secretion system (Van Sluys *et al.*, 2003). In the USA

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X. fastidiosa causes diseases of economic importance in grape and almond plants, among other hosts (Hopkins and Purcell, 2002). Two phenotypically and phylogenetically distinct subspecies, *fastidiosa* and *multiplex*, cause disease in grape and almond hosts respectively (Almeida and Purcell, 2003). Ssp. *multiplex* (i.e. almond strain) has never been isolated from grape plants, but occasionally ssp. *fastidiosa* (i.e. grape strain) has been isolated from almonds in California (Chen *et al*., 2005). Cross-inoculation studies in the laboratory showed that infection of grapevines with ssp. *multiplex* do not generate disease and lead to small pathogen populations within plants. On the other hand, ssp. *fastidiosa* causes disease in almond plants (Almeida and Purcell, 2003). These results suggest that the core machinery required for pathogenicity in almonds is present in both ssp. and that virulence factors required to cause disease in grape plants are missing in ssp. *multiplex*; however, genome comparisons among various *X. fastidiosa* strains have so far revealed no obvious candidates (Bhattacharyya *et al*., 2002; Van Sluys *et al*., 2003; Doddapaneni *et al*., 2006). Because ssp. *multiplex* is capable of minimal multiplication within grapevines under laboratory conditions, it is conceivable that genes required for pathogenicity are present in those genomes, but regulatory networks have changed enough after the lineages split so that cross-infection is no longer possible.

Although the *X. fastidiosa* genome is free of typical virulence-associated genes such as effectors, several pathogenicity factors hypothesized to be important in plant colonization due to their inferred function have, in fact, been demonstrated to affect pathogenicity. Like many other pathogens, *X. fastidiosa* pathogenicity factors are expressed in a context-dependent manner, which is under control of a cell–cell signalling system that is not based on acyl-homoserine lactones, but a short chain fatty acid named diffusible signalling factor (DSF) (Chatterjee *et al*., 2008). This system is also present in the sister genus *Xanthomonas*; however, in *Xanthomonas* plant pathogenicity genes are upregulated at high cell density, while the opposite occurs in *X. fastidiosa*. Accordingly, a *X. fastidiosa* mutant of *rpfF* (ssp. *fastidiosa*), which encodes an enoyl-CoA hydratase responsible for DSF synthesis, causes more disease in grapevines than its parental wild-type strain but is deficient in vector transmission between plants (Newman *et al*., 2004). Thus, DSF functions as a disease suppressor in *X. fastidiosa*, suggesting that regulation of pathogenicity factors mediates host colonization.

To test the hypothesis that gene regulation is responsible for the host specificity of *X. fastidiosa* ssp. *multiplex*, which does not cause disease in grapes, one could disrupt *rpfF* and challenge grapevines with that strain. Such mutant would be qualitatively similar to the wild type, but with quantitative differences in the expression of pathogenicity genes. A requirement would be for RpfF to have a similar role in ssp. *multiplex* as it does in *fastidiosa*; we show that to be the case here. Furthermore, infection of grapevines with the ssp. *multiplex rpfF* mutant leading to disease would support our proposition, while absence of disease symptoms would indicate that host-specific pathogenicity factors are responsible for the biological observations made in cross-inoculation and field studies. Performed experiments indicate that changes in gene regulation alone are sufficient for the ssp. *multiplex rpfF* mutant to exploit a new host.

Results and discussion

Single-gene mutants behave similarly in grape and almond hosts

In order to test the hypothesis that *X. fastidiosa* uses the same mechanisms to cause disease in different hosts, we inoculated almond and grape plants with the ssp. *fastidiosa* wild type (Temecula strain) and a number of mutant strains in which single genes were disrupted (Table S1). Although most of these mutants have been tested in grapevines, none has ever been tested in almond plants. Results obtained with individual mutants in relation to the wild type were similar to previously published results by other groups, but this comparative approach allowed for relevant insights on their relative role in disease development (Fig. 1). The short pilus (type I, *fimA*) mutant was the most virulent strain in both almonds and grapevines, followed by the DSF-deficient *rpfF*. In grapevines, absence of the afimbrial adhesins HxfA and HxfB equally impacted symptom development, both resulting in significantly more disease than the wild type. However, in almond plants the *hxfA* mutant was statistically equivalent to the wild type in disease severity, suggesting that HxfA has little impact on the ability of ssp. *fastidiosa* wild type to colonize almonds. HxfA is an afimbrial adhesin involved in cell adhesion to surfaces, including plant and insect vectors (Guilhabert and Kirkpatrick, 2005; Killiny and Almeida, 2009a). We speculate that HxfA binds to grapevine structural polysaccharides that are absent from the surface of almond xylem vessels, so that inoculation of almonds with the *hxfA* mutant did not impact symptom development. Two groups of tested mutants were less virulent than the wild type, including the exopolysaccharide-deficient strains *gumD* and *gumH* and those deficient in within-plant movement (*pilB*), pectin degradation (*pglA*) and intracellular signalling (*cgsA*, cyclic di-GMP mutant). Thus, except for one afimbrial adhesin (HxfA) that appears to not have a phenotype in almonds, symptom development in both hosts was equivalent for all strains tested.

Fifteen weeks after inoculation the size of live *X. fastidiosa* populations was estimated from the inoculation site and 50 cm above it for grapevines and 25 cm for almonds

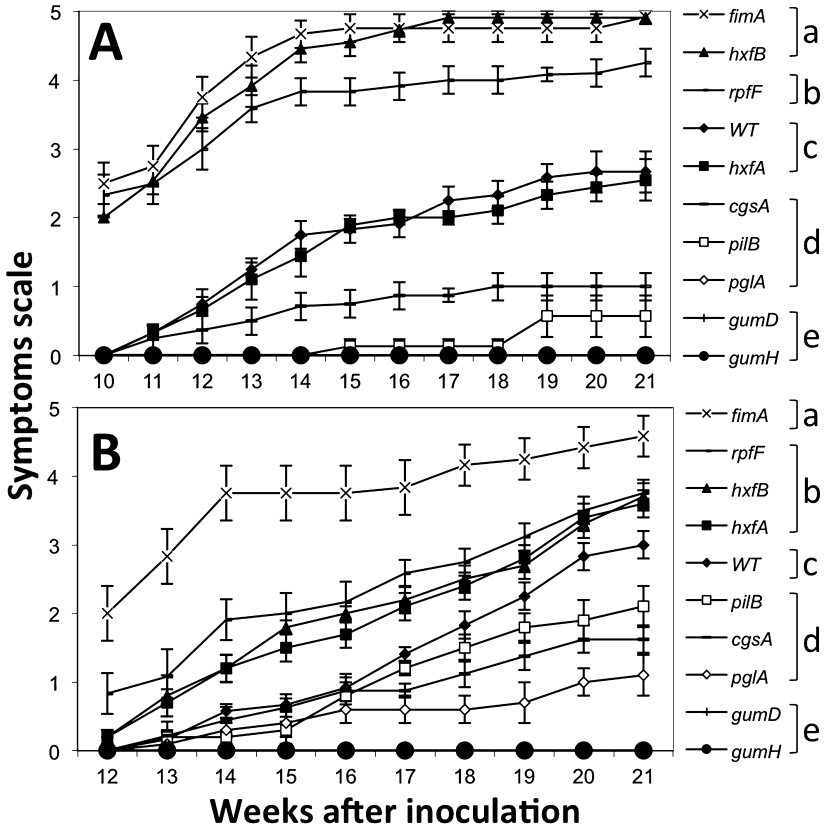

Fig. 1. Symptom development in almond (A) and grapevine (B) plants after inoculation with *X. fastidiosa* single-gene mutant strains and the wild-type Temecula. Letters cluster strains into groups according to statistically significant differences among treatments ($P < 0.05$).

using a culture-based protocol. The goal was to study the capacity of these mutants to multiply and colonize these hosts, which are essential processes leading to *X. fastidiosa*-induced disease. A general correlation between symptom development and bacterial populations within plants was observed (Figs 1 and S1), although not all groups were statistically different from each other as in the symptom development analysis. The larger within-treatment variability observed at the site far from the inoculation point was expected given that only one sample was taken from plants and *X. fastidiosa* colonization of hosts is heterogeneous (Hopkins and Thompson, 1984). All strains were recovered from both host plants at similar rates (Table S2), with the exception of the *pglA* mutant, which was recovered twice as often from grapevines but yet the recovery rate was not statistically different ($P = 0.08$, Fisher exact test). Altogether, *X. fastidiosa* pathogenicity mechanisms appear to be similar in grape and almond hosts. Van Sluys and colleagues (2003) had previously reached this conclusion through a comparative analysis of genome sequences.

RpfF is responsible for DSF production in ssp. multiplex *strain*

Because a *X. fastidiosa* ssp. *fastidiosa rpfF* mutant behaved similarly in grapevines and almonds, in that it was hypervirulent in relation to its wild type in both hosts, modifying *X. fastidiosa* gene regulation could lead a non-pathogenic strain to cause disease in a novel host. To test this possibility a ssp. *multiplex* DSF-deficient mutant (NK22, Dixon as the parental strain) had to be generated; Dixon is a strain that does not cause disease in grapevines (Almeida and Purcell, 2003). As expected, neither strain NK22 nor KLN61 (Temecula strain *rpfF* mutant) induced GFP expression in a *Xanthomonas campestris* pv. *campestris* DSF reporter strain (Fig. S2A), which is biological evidence that *rpfF* was disrupted. The genotype of strains used was confirmed by PCR, as was the disruption of the *rpfF* locus (Fig. S2B). Interesting, but not a focus of this work, was the observation that the reporter strain did not grow near or around the disk with extracted DSF from the wild-type strains, as was observed with both *rpfF* mutants.

Dixon rpfF *mutant causes disease in grapevines*

Mechanical inoculation of wild type and *rpfF* mutants of ssp. *fastidiosa* and *multiplex* strains into both almonds and grapevines was performed to determine if NK22 could cause disease in a novel host. In both hosts, infection with KLN61 resulted in symptom development faster than any other strain (Fig. 2A). Strain Dixon did not cause disease in grape, but its *rpfF* mutant (NK22) behaved

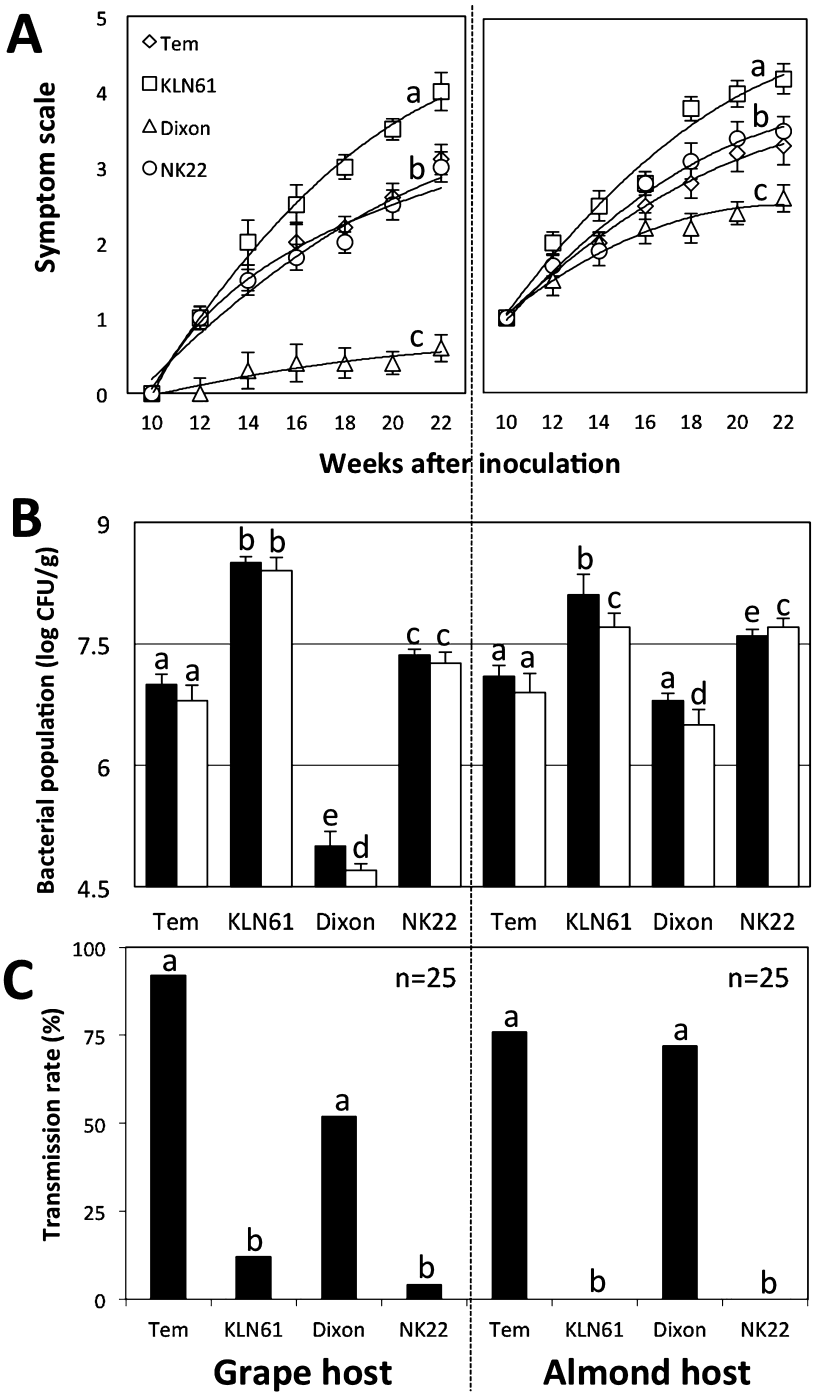

Fig. 2. *Xylella fastidiosa* ssp. *multiplex* strain with *rpfF* locus disrupted is biologically similar to ssp. *fastidiosa* wild type in plants, but as the ssp. fastidiosa mutant it is also deficient in vector transmission. The left part of figure shows results using grapevines while the right side represents results with almond plants; treatments are the wild types Temecula and Dixon and their respective *rpfF* mutants.
A. Symptom development from week 10 after inoculation.
B. Bacterial populations at the point of inoculation and 25 or 50 cm above that site 5 months after inoculation.
C. Insect transmission efficiency using an artificial diet system for acquisition and grape or almond plants for inoculation. Letters indicate statistically significant differences among treatments ($P < 0.05$) for each host plant.

similarly to the ssp. fastidiosa wild type. A similar trend was observed in almonds where Dixon caused disease, although symptoms did not develop as fast as with the other strains. Comparisons of bacterial populations in both hosts show that strains with higher numbers of live cells within plant tissue caused more severe disease symptoms (Fig. 2B). Furthermore, results show that although strain Dixon was capable of multiplying and moving within grapevines, its populations were orders of magnitude lower than other strains in that host. Temecula and KLN61 were both recovered from all grape and almond plants inoculated ($n = 12$). Dixon, however, was recovered from 11 almonds and 5 grapevines, and its *rpfF* mutant NK22 from 12 and 11 plants respectively. In summary, the Dixon *rpfF* mutant was as pathogenic to grapevines as the Temecula wild type, indicating that upregulation of genes associated with plant movement, among others, was sufficient to overcome physical and/or

chemical barriers needed for effective exploitation of a novel host.

RpfF mutants are defective in vector transmission

We also conducted tests to determine if the phenotype of Dixon and NK22 were similar to Temecula and KLN61 in relation to vector transmission. Both KLN61 and NK22 were transmitted at very low rates to grapevines relative to their respective parental wild-type strains, while no transmission was obtained when almond plants were used (Fig. 2C). Wild-type strains were transmitted at high efficiency to both hosts, suggesting little if any differences among strains in this regard. It was already known that KLN61 was deficient in vector transmission (Newman *et al*., 2004). We note that pathogen detection at the site of vector feeding during inoculation, and not disease, was used as a measurement of vector transmission. This approach allowed us to show that ssp. *multiplex* was transmitted to both grape and almond hosts, despite the fact that it does not cause disease in grapevines. Therefore, based on the experimental assays performed RpfF has similar roles in the biology of *X. fastidiosa* strains belonging to different subspecies.

Summary of findings

We showed that host specificity of *X. fastidiosa* was mediated by gene regulation and not specific loci associated with pathogenicity factors. With only one exception, nine *X. fastidiosa* mutants tested in relation to their pathogenicity in grape and almond hosts behaved similarly in relation to the wild-type controls. RpfF appears to be functionally identical in ssp. *fastidiosa* and *multiplex*, both in its DSF production and phenotype in plants and insects. Although ssp. *multiplex* does not cause disease in grapevines, the Dixon *rpfF* mutant caused symptoms similarly to the ssp. *fastidiosa* wild type in that host. Altogether, these results indicate that the ssp. *multiplex* requires no additional genes to colonize grapevines, and that gene regulation is at least one factor precluding this from occurring in nature.

Results indicate that host specificity in *X. fastidiosa*, at least between the two strains tested, is not mediated by a single or few loci. The fact *X. fastidiosa* does not have a type III secretion system and has no obvious evidence of other mechanisms implicated in host specificity is suggestive that this is not a trait acquired or modified in short periods of time. This would be different from many bacterial plant pathogens, such as *Pseudomonas syringae* (Ma *et al*., 2006; Bent and Mackey, 2007). These results disagree with proposals suggesting that determinants of *X. fastidiosa* host specificity are based on laterally acquired elements, primarily phage-related genes (Nunes *et al*., 2003; Van Sluys *et al*., 2003; Varani *et al*., 2008).

Experimental procedures

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table S1. *X. fastidiosa* strains were grown at 28°C for 7 days on modified periwinkle wilt solid medium (PWG) with or without kanamycin at 5 μg ml^{-1} (Hill and Purcell, 1995). No differences in growth rate were observed *in vitro* for the strains used. For the transmission tests, *X. fastidiosa* strains were grown in XFM-pectin with or without kanamycin (Killiny and Almeida, 2009b). The DSF reporter, *Xanthomonas campestris* pv. *campestris* (*Xcc*) strain 8523 (Newman *et al*., 2004), was grown on King's B agar with 100 μg ml^{-1} rifampicin, 100 μg ml^{-1} spectinomycin and 50 μg ml^{-1} streptomycin for 24 h. *Escherichia coli* DH5α transformed with pKLN61 (Newman *et al*., 2004) was grown in Luria–Bertani liquid medium with 5 μg ml^{-1} of kanamycin for 24 h.

Dixon rpfF *mutant*

The *rpfF* gene encodes an enoyl-CoA hydratase that is implicated in DSF production. The protein in strain Dixon (ssp. *multiplex*) has 97% (283/290) sequence identity to the ssp. *fastidiosa* strain Temecula. We used the allelic exchange pKLN61 constructed by Newman *et al*. (Newman *et al*., 2004) to generate an *rpfF* mutant for Temecula (*rpfF* KLN61). The plasmid was extracted from *E. coli* cells using QIAprep Spin Miniprep Kit (Qiagen) following manufacturer's instruction. The plasmid was electroporated into Dixon, and transformants were selected on PWG with kanamycin. Disruption of the *rpfF* locus was confirmed by PCR. To confirm the inability of the mutant to produce DSF, cells were grown on PWG for 10 days, harvested and subject to a water-saturated ethyl acetate extraction; extracts were then tested using a DSF reporter strain [*Xcc* strain 8523, pKLN55; (Newman *et al*., 2004; 2008)]. Additionally, we used primers 'rpfFKOFNotI' and 'rpfFRBglII' according to Newman and colleagues (2004) to confirm that the locus was disrupted. Furthermore, we differentiated between ssp. *fastidiosa* and *multiplex* using PCR primer sets RST31-33 followed by restriction of the PCR product with RsaI, as previously described (Hendson *et al*., 2001).

Movement and pathogenicity of single-gene mutants within grape and almond plants

Xylella fastidiosa inocula were prepared by suspending strains in 1 ml of succinate-citrate buffer (Hopkins and Thompson, 1984) after growth on PWG solid medium for 10 days. Suspensions were adjusted to $OD_{600} = 0.25$ reflecting to cell concentrations of 10^8 to 10^9 cfu ml^{-1}. A 20 μl drop of the suspension was placed on a young stem of the test plant, and the tissue was pricked through the drop five times with a No. 00 entomological pin (Hill and Purcell, 1995). One site per plant was inoculated; each mutant was inoculated into 12 almond plants (grafted Sonora/Hansen) and 12 grape plants (Cabernet Sauvignon cuttings). Negative control plants were inoculated with buffer alone, positive controls with Temecula and Dixon respectively. We kept one branch per plant to

observe symptom development. Ten weeks after inoculation for almonds and 12 weeks for the grapevines, we started to quantify symptom on a weekly basis development until week 21. We used the symptom scale described by Guilhabert and Kirkpatrick (2005) for the grapevines, and designed a similar scale for almonds. Briefly, scores ranged from 0 to 5, with 0 = no symptoms, 1 = one or two leaves with scorching, 2 = two to three leaves with scorching, 3 = all the leaves with mild scorching, 4 = all the leaves with sever scorching, and 5 = leaves only at the end of cane and numerous matchsticks (Fig. S3). To estimate *X. fastidiosa* populations and its movement within the plants, we cultured *X. fastidiosa* from leaves immediately above inoculation site. We also cultivated 50 and 25 cm above the inoculation site for grape and almond plants respectively. All plants were sampled 15–16 weeks after inoculation. The same protocol was used to compare strains Temecula, Dixon and their respective rpfF mutants, with evaluations starting 10 weeks after inoculation.

Vector transmission assays

Transmission tests were performed as described by Killiny and Almeida (2009b). Briefly, cells (Temecula, Dixon and their respective *rpfF* mutants) were grown on XFM-pectin medium, suspended on a diet solution and made available to blue-green sharpshooters (*Graphocephala atropunctata*, Hemiptera, Cicadellidae) within a parafilm sachet. After acquisition, insects were transferred and caged individually to single grapevine leaves for an inoculation access period of 12 h. Three weeks later, bacterial cells were recovered from petioles of the same leaves by culturing Hill and Purcell (1995). This approach would reduce potential problems associated with the limited movement of certain *X. fastidiosa* mutants within plants, although we did not expect this to be an issue as the Temecula *rpfF* mutant was previously shown to be hypervirulent in grapevines. However, ssp. *multiplex* strains do not cause disease in grapevines (Almeida and Purcell, 2003).

Statistical analyses

To compare the bacterial populations within plants when comparing a set of mutants or the *rpfF* mutants in relation to wild-type strains, one-way ANOVA followed by the Tukey test as a *post hoc* test was performed on log-transformed estimates of cell numbers. Ordinal multinomial logistic regression analyses were used to compare the rate of symptom development among strains. To study the populations within plants of various mutant strains we used 10 treatments (mutants) and a reference treatment (Temecula wild type); to compare the biology of *rpfF* mutants we used three treatments (two mutants and Dixon wild type) and reference treatment (Temecula wild type). For the transmission tests we used a two by four contingency table to analyse data obtained, followed by pairwise comparisons using Fisher's exact test (∞ 0.05).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Bacterial populations of *X. fastidiosa* mutant strains and wild-type Temecula within almonds (A) and grapevines (B), as determined by culturing. Populations were estimated from the point of inoculation (grey bars) and 25 and 50 cm (white bars) above the point of inoculation for almond and grape plants respectively (asterisks represent strains with populations statistically different at these sampled sites; $P < 0.05$). Letters on bars indicate statistically significant differences among treatments ($P < 0.05$).
Fig. S2. *In vitro* characterization of Dixon *rpfF* mutant (NK22, ssp. *multiplex*). (A) Detection of the DSF using a DSF bioreporter strain. The wild-type strains Temecula and Dixon are presented with their respective *rpfF* mutants. The photos show growth of bioreporter under light (top) and epifluorescence (bottom) microscopes; GFP expression is an indication that DSF is present in the medium. (B) PCR products confirming construction of NK22; (a) identification of grape and almond genotypes using RsaI restriction digestion fragments of the PCR amplified fragment (RST31/RST33) for the strains Temecula, KLN61 (Temecula *rpfF*), Dixon and NK22; (b) PCR product fragment (rpfFKOFNotI/rpfFRBglII) showing disruption of *rpfF* locus – lane 1 – Dixon, 2 – NK22, 3 – pKLN61 plasmid, 4 – Temecula, and 5 – KLN61.
Fig. S3. Almond leaf scorch disease symptoms scale used in this study. Healthy control (0), images from 1 to 5 almond were plants infected with *X. fastidiosa* ssp. fastidiosa strain Temecula.
Table S1. Bacterial strains and plasmid used in this study. Strains description and phenotype are based on previously published work.
Table S2. Recovery of *Xylella fastidiosa* strains from grape and almond plants mechanically inoculated with the wild-type Temecula strain (Tem) or several mutants (ssp. *fastidiosa*). Twelve individual plants were inoculated per treatment, the number of positive plants is shown; results are based on cell culturing on PWG medium.

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