Cell-to-Cell Signaling in *Xylella fastidiosa* Suppresses Movement and Xylem Vessel Colonization in Grape

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Cell-to-cell signaling mediated by a fatty acid diffusible signaling factor (DSF) is central to the regulation of the virulence of *Xylella fastidiosa*. DSF production by *X. fastidiosa* is dependent on *rpff* and, although required for insect colonization, appears to reduce its virulence to grape. To understand what aspects of colonization of grape are controlled by DSF in *X. fastidiosa* and, thus, those factors that contribute to virulence, we assessed the colonization of grape by a green fluorescent protein–marked *rpff*-deficient mutant. The *rpff*-deficient mutant was detected at a greater distance from the point of inoculation than the wild-type strain at a given sampling time, and also attained a population size that was up to 100-fold larger than that of the wild-type strain at a given distance from the point of inoculation. Confocal laser-scanning microscopy revealed that approximately 10-fold more vessels in petioles of symptomatic leaves harbored at least some cells of either the wild type or *rpff* mutant when compared with asymptomatic leaves and, thus, that disease symptoms were associated with the extent of vessel colonization. Importantly, the *rpff* mutant colonized approximately threefold more vessels than the wild-type strain. Although a wide range of colony sizes were observed in vessels colonized by both the wild type and *rpff* mutant, the proportion of colonized vessels harboring large numbers of cells was significantly higher in plants inoculated with the *rpff* mutant than with the wild-type strain. These studies indicated that the hyper-virulence phenotype of the *rpff* mutant is due to both a more extensive spread of the pathogen to xylem vessels and unrestrained multiplication within vessels leading to blockage. These results suggest that movement and multiplication of *X. fastidiosa* in plants are linked, perhaps because cell wall degradation products are a major source of nutrients. Thus, DSF-mediated cell-to-cell signaling, which restricts movement and colonization of *X. fastidiosa*, may be an adaptation to endophytic growth of the pathogen that prevents the excessive growth of cells in vessels.

Additional keywords: adhesins, biofilm, Pierce's disease, pit membrane.

Xylella fastidiosa is a xylem-limited bacterium transmitted by xylem sap-feeding insect vectors such as sharpshooter leafhoppers (Hemiptera, Cicadellidae) or spittlebugs (Purcell and Hopkins 1996). Although this pathogen can incite disease in many plant species, it also is widely distributed in asymptomatic plants. Pierce's disease (PD) of grape is one of the most economically important diseases caused by this pathogen. In

susceptible hosts such as grape, *X. fastidiosa* multiplies and spreads widely from the site of infection to colonize the xylem. Cells of *X. fastidiosa* attach to the vessel walls and multiply, forming biofilm-like colonies that can, when sufficiently large, completely occlude xylem vessels and block water transport (Purcell and Hopkins 1996; Tyson et al. 1985). The systemic spread of the pathogen is limited by the pit membranes that separate one xylem water conduit from its neighbors and, perhaps, by the production of tyloses and polysaccharide-rich gels by plants that block xylem cells following infection. The typical symptoms of PD, including leaf scorch and eventual death of the leaf and plant, are generally believed to be a result of progressive and increasingly limited water flow through the vine. Scorching symptoms are observed when high temperatures and the maximum water demand of a full canopy coincide in late summer, creating progressive water stresses to the plant (Hopkins and Purcell 2002; Purcell et al. 1979).

Considerable evidence indicates that disease symptoms associated with *X. fastidiosa* colonization of plants are related to the proportion of vessels that harbor large cell aggregates and not simply to the number of vessels that are colonized. The use of green fluorescent protein (gfp)-tagged cells of *X. fastidiosa* in conjunction with confocal microscopy has recently provided insight into the process of colonization of xylem vessels in grape. The colony size of *X. fastidiosa* in vessels in infected plants was highly variable, with vessels most commonly harboring colonies with relatively few cells (<100 cells/colony) (Newman et al. 2003). Although the number of vessels with small colonies was threefold higher in symptomatic leaves than asymptomatic leaves, the number with large, occluding colonies (>1,000 cells/colony) was 20-fold higher. This indicated that many cells in symptomatic leaves are in large colonies, whereas this is not true in asymptomatic leaves. This shift to larger colonies in symptomatic leaves also suggests that large colonies have a more deleterious effect on the host than do small colonies. Small colonies in asymptomatic leaves apparently grow into large colonies over time; however, cells must also be dispersing to new vessels because symptomatic leaves also had many more vessels colonized. Therefore, vessel blockage by bacterial colonization is apparently a critical variable in symptom expression.

Cell-to-cell signaling plays an important role in the colonization, pathogenesis, and biofilm formation of many plant-pathogenic bacteria, including *X. fastidiosa* (Kievit and Iglewski 2000; vonBodman et al. 2003). Bacteria can synthesize a variety of small signaling molecules which regulate different traits in a cell-density-dependent manner, a process referred to as quorum sensing in some species (Bassler 2002; Whitehead et al. 2001). *X. fastidiosa* harbors a cell-to-cell signaling system that requires a set of regulation of pathogenicity factor (*rpf*) genes, which is highly homologous to that in *Xanthomonas*

campestris pv. *campestris* (Barber et al. 1997; Chatterjee et al. 2008; Newman et al. 2004). The *rpff* gene of *Xylella fastidiosa* is required for the synthesis of a diffusible signaling factor (DSF) which is presumably similar to, but different than, a fatty acid produced by *Xanthomonas campestris* pv. *campestris* (Simionato et al. 2007; Newman et al. 2004). The DSF from *X. campestris* pv. *campestris* has been characterized as cis-11-methyl-2-dodecenoic acid (Wang et al. 2004). *rpff* mutants of *Xylella fastidiosa* are severely reduced in their ability to be transmitted by sharpshooters but, interestingly, are hypervirulent on grape (Chatterjee et al. 2008a; Newman et al. 2004).

DSF appears to regulate different virulence traits in *Xanthomonas* spp. and *X. fastidiosa*. In *Xanthomonas campestris* pv. *campestris*, DSF deficient mutants have lower expression of virulence-associated functions such as extracellular polysaccharide (EPS) and extracellular enzymes (Barber et al. 1997). However, in the closely related *X. oryzae* pv. *oryzae*, *rpff* mutants are reduced in both virulence and iron uptake but are proficient in extracellular enzyme and EPS production (Chatterjee and Sonti 2002). In contrast, in *Xylella fastidiosa*, the expression of genes encoding the production of the extracellular enzymes such as polygalacturonase and type IV pili are enhanced while those encoding a variety of adhesins are reduced in an *rpff* mutant (Chatterjee et al. 2008). Thus, whereas the enhanced virulence of DSF-deficient mutants of *X. fastidiosa* have been documented (Chatterjee et al. 2008a; Newman et al. 2004), it is unclear what aspects of the colonization of grape are altered in an *rpff* mutant. Because vessel blockage apparently is associated with symptom development, it is possible that the enhanced virulence of an *rpff* mutant is simply due to its enhanced growth in vessels that it colonizes. However, because movement of the pathogen through the plant is also apparently required for virulence, this process might also be regulated by DSF-based signaling. By examining colonization of plants with gfp-marked strains, we show here that not only do DSF-deficient mutants move more extensively than the wild type through the plant but they also colonize many more vessels in the process. Additionally, *rpff* mutants form larger colonies than do the wild type, apparently causing them to block a much higher proportion of the vessels in a plant, leading to enhanced symptom development. Thus, these results suggest that movement and multiplication of *X. fastidiosa* in plants is linked, perhaps because cell wall degradation products are a major source of nutrients. DSF-mediated cell-to-cell signaling, which restricts movement and colonization of *X. fastidiosa*, may be an adaptation to endophytic growth of the pathogen, preventing excessive growth of cells in vessels that may be detrimental to cell survival.

Fig. 1. Progression and severity of Pierce's disease of grape incited by a wild type (triangles) and an *rpff* mutant (squares) of *Xylella fastidiosa*. The vertical bars represent the standard error of the mean of number of infected leaves per vine determined from 15 replicate plants for each treatment. Similar results were obtained in three independent experiments (data not shown).

RESULTS

rpff mutants of *X. fastidiosa* move more extensively in grape than the parental strain.

The movement of *rpff* mutants and their ability to cause symptoms of PD were compared with the wild-type strain after mechanical inoculation into the stems of grape. Although disease symptoms first appeared at a similar time in plants inoculated with the wild type and the *rpff* mutant, at a given time subsequent to first appearance of disease the number of symptomatic leaves was always significantly higher in plants inoculated with the *rpff* mutant (Fig. 1). Disease symptoms first appeared on leaves near the point of inoculation, with more distal leaves subsequently becoming symptomatic with increasing time. At a given time, the severity of disease symptoms in plants inoculated with the *rpff* mutant was usually higher than that on plants inoculated with the wild-type strain. Thus, it appeared that cells of the *rpff* mutant were spreading more rapidly through the vine than the wild-type strain. The movement and multiplication of the *X. fastidiosa* strains in grape were determined by estimating the number of cells in leaf petioles at different distances away from the point of inoculation. At 12 weeks after inoculation, cells of the *rpff* mutants were detected as far as 115 cm from the point of inoculation whereas those of the wild-type strain could be detected only up to 90 cm (Fig. 2). The population size of the *rpff* mutant was always substantially higher than that of the wild-type strain at a given location away from the point of inoculation (Fig. 2). There were approximately fourfold more cells of the *rpff* strain compared with the wild type within approximately 40 cm of the point of inoculation whereas there were usually more than 80-fold more cells at greater distances (Fig. 2). These results indicate that *rpff* mutants of *X. fastidiosa* are apparently much more proficient in migration in both dimensions: longitudinally within xylem vessels, increasing their rate of spread along vines, and laterally to new vessels, increasing the number of vessels colonized and, hence, their population size.

rpff mutants colonize xylem vessels more frequently than do members of the wild-type strain.

To gain insight into the DSF-dependent processes contributing to disease, we compared the distribution of a GFP-marked *rpff* mutant with a GFP-marked wild-type *X. fastidiosa* strain in

Fig. 2. *rpff* mutants of *Xylella fastidiosa* are more proficient in movement and multiplication within grapevines. Bacterial population sizes from petioles of grape (Cabernet sauvignon) inoculated with wild-type *X. fastidiosa* strain Temecula (triangles) and an *rpff* mutant (squares) were determined in petioles harvested at the different heights above the point of inoculation (toward the shoot apex) shown on the abscissa. The vertical bars represent the standard error of the mean of log-transformed estimates of cell population sizes from six replicates for each treatment. Similar results were observed in two independent experiments (data not shown).

grape vessels. The presence of gfp-marked cells was visualized both in vessels in petioles of leaves exhibiting PD symptoms and in adjacent asymptomatic leaves by confocal laser-scanning microscopy (Fig. 3) (discussed below). A much higher proportion

of the vessels of symptomatic leaves were colonized by at least some cells of the wild-type strain (19%) than in the asymptomatic leaves (1.7%) (Fig. 4). The *rpfF* mutant was detected in a much larger proportion of vessels than the wild-type strain in

Fig. 3. Representative examples of images of cross sections of petioles on grape leaves symptomatic of Pierce's disease harboring green fluorescent protein (gfp) fluorescent cells of a wild-type strain of *Xylella fastidiosa* (left panels) and an *rpfF* mutant (right panels). Xylem vessels are visible due to their red autofluorescence. Bars: 10 μ M.

the petioles of both symptomatic and asymptomatic leaves. In symptomatic leaves, the *rpjF* mutant colonized threefold more xylem vessels (56%) than the wild-type strain (19%) (Figs. 3 and 4A). Similarly, in asymptomatic leaves, the *rpjF* mutant colonized approximately threefold more vessels (4.45%) than the wild-type strain (1.7%) (Fig. 4B).

rpjF mutants form larger colonies in vessels than do members of the wild-type strain.

Similar to observations in an earlier study (Newman et al. 2003), cells of the wild-type strain were observed in a wide

range of colony sizes when examined in xylem vessels in cross-sections of petioles (Fig. 3). Although most vessels harbored relatively few *gfp*-marked cells of the wild-type strain, some vessels were nearly filled with cells, and colony sizes as large as 1,000 cells or more were apparent (Fig. 3). Observation of over 3,000 vessels in a collection of different petioles enabled an estimate of the distribution of colony sizes of the wild-type strain to be described. Most colonies of the wild-type strain in vessels were relatively small (<100 cells/colony) and only approximately 18% of all of the colonies observed were large (>1,000 cells/colony) and apparently blocking vessels (Fig. 5). A range of colony sizes of the *rpjF* mutant was also observed; however, not only did the *rpjF* mutant colonize many more vessels than the wild-type strain, it more commonly formed large colonies in vessels (Fig. 3). Usually, more than twice as many vessels harbored colonies of the *rpjF* mutant of a given size than the wild-type strain (Fig. 5). Importantly, approximately 46% of the vessels harboring any cells of the *rpjF* mutant were colonized by large colonies (>1,000 cells) of this strain while the colonies in less than approximately 15% of the vessels colonized by the wild type were this large (Fig. 5). The proportion of colonized vessels harboring such large numbers of cells was significantly higher in plants inoculated with the *rpjF* mutant than with the wild-type strain ($\chi^2 = 4.96$; $P = 0.026$).

DISCUSSION

The results presented here on the patterns of vessel colonization by an *rpjF* mutant of *X. fastidiosa* reveal that DSF accumulation in this species controls at least two processes that contribute to the virulence of this pathogen. Not only did the *rpjF* mutant spread to many more xylem vessels after point inoculation into grape (Figs. 3 and 4) but it also multiplied much more within the vessels into which it moved (Fig. 5). It is the product of both of these phenomena that apparently accounts for the much larger population size that the *rpjF* mutant attains in plants compared with the wild-type strain (Chatterjee et al. 2008a) (Fig. 2). The results of this study also further support the conjecture that both the movement from vessel to vessel and the multiplication of cells of *X. fastidiosa* to form large colonies in vessels are important traits in the extensive colonization of *X. fastidiosa* (Newman et al. 2003, 2004). The higher ability of the *rpjF* mutant to move to new vessels after inoculation readily explains why the cells of this mutant were observed

Fig. 4. Incidence of colonization of xylem vessels in **A**, petioles of grape leaves exhibiting symptoms of Pierce's disease or **B**, adjacent asymptomatic leaves by at least one cell of the green fluorescent protein (*gfp*)-marked wild-type *Xylella fastidiosa* strain (solid bars) or a *gfp*-marked *rpjF* mutant (striped bars). The vertical bars represent the standard error of the mean frequency of colonization.

Fig. 5. Distribution of colony sizes of green fluorescent protein (*gfp*)-marked cells of the wild-type *Xylella fastidiosa* strain (black bars) and an *rpjF* mutant (striped bars) observed in petioles of grape leaves exhibiting symptoms of Pierce's disease 12 weeks after inoculation. Colony size was estimated based on number of cells visible in a cross-section of petiole within a 1- μ m depth of view. The vertical bars represent the standard errors of mean estimated frequency of occurrence of cell aggregates in each of four size classes.

to spread further distally along vines than the wild-type strain; not only could enhanced lateral vessel-to-vessel movement account for the higher proportion of vessels colonized on a given cross-section of stem (Fig. 4) but longitudinal movement also involves vessel-to-vessel movement that would also be enhanced in the *rpjF* mutant, as noted here (Figs. 1 and 2). An earlier study using a *gfp*-marked *X. fastidiosa* strain revealed that disease severity in grape was strongly associated with the frequency with which large colonies of *X. fastidiosa* were observed in the petioles of leaves (Newman et al. 2003). Similar results were observed here for both the wild-type strain and the *rpjF* mutant (Fig. 4). Presumably, water movement within a vessel is influenced directly by the presence of bacteria in the vessel only when relatively large cell aggregates form within the vessel. It is reasonable to expect that the severity of disease would thus increase with the increasing proportion of vessels that had become blocked by bacterial colonization because it would lead to a progressive decrease in water conduction capability as the number of alternative pathways for water flow in the plant is diminished (Tyree and Zimmermann 2002).

Recent reports of those traits in *X. fastidiosa* that are regulated by DSF accumulation provide some explanation for the observed differences in colonization of grape by the *rpjF* mutant. The expression of *pglA* as well as *pilY1* is higher in an *rpjF* mutant compared with the wild-type strain whereas that of *fimA*, *hxfA*, *hxfB*, and *gumJ* are lower (Chatterjee et al. 2008a; Wang et al. 2007). Cells of an *rpjF* mutant appear less “sticky” than that of a wild-type strain, being much less likely to be found in a biofilm on the sides of a culture flask than the wild-type strain (Chatterjee et al. 2008a). Thus, it would be expected that the *rpjF* mutant would be free to move within the plant than the wild-type strain. *X. fastidiosa* has been observed to traverse bordered pit membranes to access neighboring vessels through bordered pit membranes (Newman et al. 2003); therefore, it has been assumed that this is an active process requiring the activity of cell-wall-degrading enzymes. Support for this conjecture has been provided in a recent study that demonstrated that a *pglA* mutant of *X. fastidiosa* that is deficient in polygalacturonase production was unable to move beyond the point of inoculation and cause disease (Roper et al. 2007). By this logic, an *rpjF* mutant which overexpresses polygalacturonase would be expected to more readily breach pit membranes and spread to adjacent vessels. The observation that the *rpjF* mutant was present in threefold more vessels than the wild-type strain (Fig. 4) is consistent with this expectation and suggests that the expression of extracellular enzymes is an important virulence trait in the pathogen and that the levels of expression limit pathogen dispersal in plants and, thus, disease progression. Perhaps more unexpected was the fact that the colonies of the *rpjF* mutant of *X. fastidiosa* in vessels were larger than those of the wild-type strain (Fig. 5). Although the *rpjF* mutant formed colonies of a variety of sizes, like the wild-type strain, a significantly larger proportion of the colonies that it formed were very large (Fig. 5). We presume that the process of colony formation on the walls of the xylem vessels is a progressive one, with colonies initially being small and then enlarging due to growth in situ. Thus, it would seem that the elevated proportion of the colonies of the *rpjF* mutant that are very large reflects a change in the process by which the pathogen restricts the size of the colonies in a vessel. It has long been thought that *X. fastidiosa* grows in xylem vessels by consuming nutrients found at low concentrations in the xylem sap (Purcell and Hopkins 1996). It is not clear how the cells could regulate colony size in such a scenario, because the colony size would be expected to be limited by the abundance of nutrients available to it in the flowing liquid. The size of colonies, in turn, might be expected to be self-limiting in size due

to the decreased flow of nutrient-laden xylem sap that would pass by growing colonies as they progressively blocked the flow due to their growing size. It is tempting to speculate that *X. fastidiosa* gains substantial nutrition from the consumption of cell wall or pit membrane components released by the action of polygalacturonase. Not only would the apparent derepression of *pglA* in the *rpjF* mutant enable it to breach more pit membranes by enzymatic digestion but the resulting products of digestion also may be a major source of carbon to the cells. The genome sequence of *X. fastidiosa* reveals that it encodes putative sugar transport systems (Bhattacharyya et al. 2002; Meidanis et al. 2002; Van Sluys et al. 2003) which further support the concept that cell-wall-derived sugars are consumed by this pathogen. The apparent repression of *pglA* upon the accumulation of DSF in vessels as the numbers of pathogen cells become large also can be rationalized as a means for it to avoid excessive multiplication in vessels. The finding that most vessels harbor only relatively small colonies of wild-type *X. fastidiosa* cells has been suggested to be the signature of an endophytic bacterium with the goal of achieving large population sizes in plants. By colonizing a large number of xylem vessels to only a modest degree without impeding the flow of xylem sap needed to provide needed resources such as oxygen or other limiting resources, *X. fastidiosa* could optimize its ability to exploit the plant (Chatterjee et al. 2008b; Newman et al. 2003). Presumably, the formation of large colonies would be avoided because they would result in vessel blockage and, thus, lead to cell stress or death (Chatterjee et al. 2008b; Newman et al. 2003). Therefore, the *rpjF* mutants could not repress *pglA* expression even in heavily colonized vessels, likely resulting in the excessive growth of the pathogen. The severity of PD is clearly directly related to the number of vessels that harbor high numbers of *X. fastidiosa* (Frittschi et al. 2007; Newman et al. 2003; Tyson et al. 1985) (Fig. 4); therefore, the elevated PD disease incidence and severity incited by the *rpjF* mutant (Fig. 1) (Chatterjee et al. 2008a; Newman et al. 2004) is likely due to the higher number of vessels that are blocked by this mutant. It had been previously proposed that *X. fastidiosa* *rpjF* mutants exhibit more disease due to their inability to escape from vessels, thereby leading to their occlusion while limiting the number of vessels colonized (Newman et al. 2004). This proposal was based on the knowledge that, in bacteria such as *Xanthomonas campestris* pv. *campestris*, DSF-deficient mutants were deficient in synthesis of exoenzymes, which are required for hydrolysis of plant cell wall and dispersal of bacterial cells from the biofilm state to planktonic lifestyle and, hence, deficient in spread from vessel to vessel (Barber et al. 1997; Dow et al. 2003). The subsequent finding that *pglA* expression is oppositely regulated by DSF in *Xylella fastidiosa* and *Xanthomonas campestris* pv. *campestris* (Chatterjee et al. 2008a, Wang et al. 2007) readily explains both why many more vessels are colonized by the *rpjF* mutant (Fig. 4) and why they might form larger colonies in the vessels into which they move (Fig. 5).

The DSF-dependent expression of a variety of adhesins in *Xylella fastidiosa* can also account for the enhanced movement of *rpjF* mutants within grape tissues. Although exoenzymes are likely required to open portals for movement between vessels, cells must also be free to move and adhesiveness of cells would appear to suppress movement. Previously, it has been reported that mutants of *X. fastidiosa* which are deficient in the adhesins HxfA and HxfB migrated more rapidly and reached a higher population density in grape (Guilhabert et al. 2005). These adhesins as well as FimA are also required for biofilm formation and cell aggregation in vitro, suggesting that they are involved in both cell-surface and cell-to-cell association, leading to colony formation within xylem vessels (Feil et al.

2007; Guilhabert et al. 2005; Meng et al. 2005). Direct evidence for the lower ability of *rpff* mutants to form a biofilm in vitro was recently reported (Chatterjee et al. 2008a). Because the expression of these genes are all lower in an *rpff* mutant than in the wild-type strain, we speculate that the mutant cells are less restricted in their ability to move within the plant upon the breaching of pit membranes.

The DSF-dependent repression of active motility of *X. fastidiosa* also apparently contributed to the enhanced movement of the *rpff* mutants. It has been reported that *X. fastidiosa* uses twitching motility to spread in the xylem vessel of plants. A type IV pilus mutant of *X. fastidiosa* was deficient in twitching motility and spread more slowly in grape (Meng et al. 2005). The expression of pilY1 is expressed at a higher level in the *rpff* mutant than the wild-type strain (Wang et al. 2007) and preliminary results of a twitching motility assay indicated that *rpff* mutants were more motile than the wild-type strain (S. Chatterjee and S. E Lindow, *unpublished results*). It is probably not coincidental that the downregulation of twitching motility and upregulation of adhesins is affected by DSF accumulation because this would appear to be a coordinated set of behaviors that would enable the cells to move before DSF has accumulated. By this logic, it would appear that *X. fastidiosa* restricts not only its ability to move upon reaching high cell densities (and, thus, higher DSF levels) in xylem vessels by increasing adhesiveness and lowering its ability for active movement but also that its ability to multiply within vessels would slow in such vessels upon decreasing its production of extracellular enzymes such as polygalacturonase.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

X. fastidiosa Temecula (ATCC 700964) strains were grown in liquid (PW) and solid PWG media as previously described (Hill and Purcell 1995; Newman et al. 2003) with supplementation of either kanamycin at 30 µg/ml or spectinomycin at 50 µg/ml. *Escherichia coli* strains were grown in Luria-Bertani media with kanamycin at 50 µg/ml and spectinomycin at 80 µg/ml.

Construction of gfp-marked *X. fastidiosa* strains.

All standard molecular biology methods were done as previously described (Sambrook et al. 1989). To obtain the gfp-marked *rpff* mutant, wild-type *X. fastidiosa* harboring a constitutively expressed gfp marker gene (KLN59.3) (Table 1) (Newman et al. 2003) was used as the parental strain. Plasmid pKLN121 containing the *X. fastidiosa* *rpff* gene with an Ω

fragment inserted into an internal deleted region (Table 1) was obtained as follows. The Ω fragment (conferring spectinomycin resistance) was isolated from pUPC1318 Ω (Miller et al. 2000) by digestion with *SalI*. The *rpff* deletion-containing plasmid KNL61 having a kanamycin resistance marker (Table 1) (Newman et al. 2004) was then digested with *XhoI* and treated with alkaline phosphatase before ligation of the isolated Ω fragment to yield pKLN121. DNA sequences of the insert and junctions were determined to confirm each cloning step. pKLN121 was then electroporated into competent gfp-marked *X. fastidiosa* strain KLN59.3 as described earlier (Newman et al. 2003). Electroporated cells were plated on PWG containing kanamycin and spectinomycin. Double recombinants containing the marked deletion was confirmed by polymerase chain reaction and sequencing (data not shown).

Pathogenicity and migration assays.

Greenhouse-grown *Vitis vinifera* 'Cabernet sauvignon' grapevines that were approximately 50 cm tall were mechanically inoculated by needle puncture at a distance of approximately 20 cm from the soil surface using standard procedures (Hill and Purcell 1995; Newman et al. 2003). The number of symptomatic leaves distal to a point two nodes from the inoculation site was noted weekly after inoculation. Symptomatic leaves were scored for the presence of typical PD symptoms (loss of chlorophyll and drying of the leaf margins). For cell migration assays, petioles of leaves showing symptoms were harvested at different distances above the point of inoculation at 12 weeks after inoculation. The petioles were surface sterilized with 3% NaOCl and 50% ethanol and cut into 1-cm pieces which were then ground in 1 ml of distilled water using a mortar and pestle, serially diluted, and plated on PWG containing kanamycin to determine the number of CFU.

Microscopy.

Leaves were removed from the plants immediately prior to sectioning as described previously (Newman et al. 2003). Briefly, for each leaf, the petiole was hand sectioned with a thin razor, mounted on a 4% gelatin pad, overlaid with 25% glycerol, and immediately examined under the microscope. Images were captured using a Zeiss 510 confocal laser-scanning microscope at the University of California, College of Natural Resources Biological Imaging Facility. GFP was excited using the 488-nm wavelength of an argon ion laser. GFP fluorescence was captured using a 505- to 550-nm band-pass emission filter placed in front of the detector. Auto fluorescence was collected using a 385- to 470-nm BP filter from the sample to see plant features and was excited using 385- to 470-nm light from a coherent UV laser.

Vessel colonization analysis.

The extent of vessel colonization by gfp-marked strains was assessed similarly as described before (Newman et al. 2003). For both symptomatic and asymptomatic plants, four petioles from each of four different plants were analyzed as follows. The total number of vascular bundles from a single hand section of the basal end of the petiole was recorded. Each bundle in that cross-section was examined at both ×40 and ×60 magnification using confocal laser-scanning microscopy to determine whether any colonization was present. To ensure an accurate count of cells present in small colonies, every vessel was systematically observed at the higher magnification that allowed resolution of individual cells. For each section, the total number of vessels was counted. The number of colonized vessels was then enumerated, and the colony size, as visible in one 0.8- to 1.0-µm focal plane, was determined by enumerating all cells when less than 100 cells were present and by estimation when

Table 1. Strains and plasmids used

Strain or plasmid	Description ^a	Source or reference
<i>Xylella fastidiosa</i>		
Temecula	Wild type, Pierce's disease strain	ATCC 700964
KLN61	<i>rpff</i> deletion strain, Km ^r	Newman et al. 2004
KLN59.3	Gfp ⁺ , Km ^r	Newman et al. 2003
KLN121	Gfp ⁺ , Km ^r , <i>rpff</i> deletion strain containing Sp ^r Ω cassette	This study
Plasmids		
pKLN61	<i>X. fastidiosa</i> Δ <i>rpff</i> -Km ^r in pGEM-SZf(+), Ap ^r , Km ^r	Newman et al. 2004
pKLN121	pKLN61 containing Ω cassette insertion in the <i>kan</i> gene, <i>kan</i> ::Ω, Ap ^r , Sp ^r	This study

^a Km^r, Sp^r, and Ap^r = resistant to kanamycin, spectinomycin, and ampicillin; Gfp = green fluorescent protein.

more than 100 cells were present. It was also determined whether the vessel was completely occluded by the colony or not. Because the cut surface of the sample was potentially subject to superficial spreading of cells of *X. fastidiosa* during the cutting process, images were captured from the deepest optical section that still afforded adequate resolution of colony size (8 to 12 µm below the cut surface) to ensure that only undisturbed cells were examined. In all, 3,024 vessels in symptomatic and 1,788 vessels in asymptomatic leaf petioles infected by the wild-type strain were examined, while 3,178 and 1,445 vessels were examined in rpf-infected leaves, respectively.

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LITERATURE CITED

- Barber, C. E., Tang, J. L., Feng, J. X., Pan, M. Q., Wilson, T. J. G., Slater, H., Dow, J. M., Williams, P., and Daniels, M. J. 1997. A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol. Microbiol.* 24:555-66.
- Bassler, B. L. 2002. Small talk: Cell-to-cell communication in bacteria. *Cell* 109:421-424.
- Bhattacharyya, A., Stilwagen, S., Reznik, G., Feil, H., Feil, W. S., Anderson, I., Bernal, A., D'Souza, M., Ivanova, N., Kapatral, V., Larsen, N., Los, T., Lykidis, A., Selkov, E., Walunas, T., Purcell, A., Edwards, R. A., Hawkins, T., Haselkorn, R., Overbeek, R., Kyrpides, N. C., and Predki, P. F. 2002. Draft sequencing and comparative genomics of *Xylella fastidiosa* strains reveal novel biological insights. *Genome Res.* 12:1556-1563.
- Chatterjee, S., and Sonti, R. V. 2002. *rpfF* mutants of *Xanthomonas oryzae* pv. *oryzae* are deficient for virulence and growth under low iron conditions. *Mol. Plant-Microbe Interact.* 15:463-71.
- Chatterjee, S., Wistrom, C., and Lindow, S. E. 2008a. A cell-cell signaling sensor is required for virulence and insect transmission of *Xylella fastidiosa*. *Proc. Natl. Acad. Sci. U. S. A.* 105:2670-2675.
- Chatterjee, S., Almeida, R. P. P., and Lindow, S. E. 2008b. Living in two worlds: The plant and insect lifestyles of *Xylella fastidiosa*. *Annu. Rev. Phytopathol.* 46:243-271.
- Dow, J. M., Crossman, L., Findlay, K., He, Y. Q., Feng, J. X., and Tang, J. L. 2003. Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. *Proc. Natl. Acad. Sci. U. S. A.* 100:10995-11000.
- Feil, H., Feil, W. S., and Lindow, S. E. 2007. Contribution of fimbrial and afimbrial adhesins of *Xylella fastidiosa* to attachment to surfaces and virulence to grape. *Phytopathology* 97:318-324.
- Fritsch, F. B., Lin, H., and Walker, M. A. 2007. *Xylella fastidiosa* population dynamics in grapevine genotypes differing in susceptibility to Pierce's disease. *Am. J. Enol. Viticult.* 58:326-332.
- Guilhabert, M. R., and Kirkpatrick, B. C. 2005. Identification of *Xylella fastidiosa* antivirulence genes: Hemagglutinin adhesins contribute to *X. fastidiosa* biofilm maturation and colonization and attenuate virulence. *Mol. Plant-Microbe Interact.* 18:856-868.
- Hill, B. L., and Purcell, A. H. 1995. Multiplication and movement of *Xylella fastidiosa* within grapevine and four other plants. *Phytopathology* 85:1368-1372.
- Hopkins, D. L., and Purcell, A. H. 2002. *Xylella fastidiosa*: Cause of Pierce's disease of grapevine and other emergent diseases. *Plant Dis.* 86:1056-1066.
- Kievit, T. R., and Iglewski, B. H. Bacterial quorum sensing in pathogenic relationship. 2000. *Infect. Immun.* 68:4839-4849.
- Meidanis, J., Braga, M. D. V., and Almeida, S. V. 2002. Whole genome analysis of transporters in the plant pathogen *Xylella fastidiosa*. *Microbiol. Mol. Biol. Rev.* 66:272-299.
- Meng, Y., Li, Y., Galvani, C. D., Hao, G., Turner, J. N., Burr, T. J., and Hoch, H. C. 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. *J. Bacteriol.* 187:5560-5567.
- Miller, W. G., Leveau, J. H. J., and Lindow, S. E. 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. *Mol. Plant-Microbe Interact.* 13:1243-1250.
- Newman, K. L., Almeida, R. P. P., Purcell, A. H., and Lindow, S. E. 2003. Use of a green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. *Appl. Environ. Microbiol.* 69:7319-7327.
- Newman, K. L., Almeida, R. P. P., Purcell, A. H., and Lindow, S. E. 2004. Cell-cell signaling controls *Xylella fastidiosa* interactions with both insects and plants. *Proc. Natl. Acad. Sci. U. S. A.* 101:1737-1742.
- Purcell, A. H., and Hopkins, D. L. 1996. Fastidious xylem-limited bacterial plant pathogens. *Annu. Rev. Phytopathol.* 34:131-151.
- Purcell, A. H., Finlay, A. H., and McLean, D. L. 1979. Pierce's disease bacterium: Mechanism of transmission by leafhopper vectors. *Science* 206:839-841.
- Roper, M. C., Greve, L. C., Warren, J. G., Labavitch, J. M., and Kirkpatrick, B. C. 2007. *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in *Vitis vinifera* grapevines. *Mol. Plant-Microbe Interact.* 20:411-419.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Simionato, A. V. C., da Silva, D. S., Lambais, M. R., and Carrilho, E. 2007. Characterization of a putative *Xylella fastidiosa* diffusible signal factor by HRGC-ESI-MS. *J. Mass Spectrom.* 42:490-496.
- Tyree, M. T., and Zimmermann, M. H. 2002. *Xylem Structure and the Ascent of Sap*. Springer-Verlag, New York.
- Tyson, G. E., Stojanovic, B. J., Kuklinski, R. F., Divittorio, T. J., and Sullivan, M. L. 1985. Scanning electron microscopy of Pierce's disease bacterium in petiolar xylem of grape leaves. *Phytopathology* 75:264-269.
- Van Sluys, M. A., de Oliveira, M. C., Monteiro-Vitorello, C. B., Miyaki, C. Y., Furlan, L. R., Camargo, L. E. A., da Silva, A. C. R., Moon, D. H., Takita, M. A., Lemos, E. G. M., Machado, M. A., Ferro, M. I. T., da Silva, F. R., Goldman, M. H. S., Goldman, G. H., Lemos, M. V. F., El-Dorry, H., Tsai, S. M., Carrer, H., Carraro, D. M., de Oliveira, R. C., Nunes, L. R., Siqueira, W. J., Coutinho, L. L., Kimura, E. T., Ferro, E. S., Harakava, R., Kuramae, E. E., Marino, C. L., Gigliotti, E., Abreu, I. L., Alves, L. M. C., do Amaral, A. M., Baia, G. S., Blanco, S. R., Brito, M. S., Cannavan F. S., Celestino, A. V., da Cunha, A. F., Fenille, R. C., Ferro, J. A., Formighieri, E. F., Kishi, L. T., Leoni, S. G., Oliveira, A. R., Rosa, V. E. Jr., Sasaki, F. T., Sena J. A. D., de Souza, A. A., Truffi, D., Tsukumo, F., Yanai, G. M., Zaros, L. G., Civerolo, E. L., Simpson, A. J. G., Almeida, N. F., Setubal, J. C. Jr., and Kitajima, J. P. 2003. Comparative analysis of the complete genome sequences of Pierces disease and citrus variegated chlorosis strain of *Xylella fastidiosa*. *J. Bacteriol.* 185:1018-1026.
- von Bodman, S. B., Bauer, W. D., and Coplin, D. L. 2003. Quorum sensing in plant-pathogenic bacteria. *Annu. Rev. Phytopathol.* 41:455-482.
- Wang, L. H., He, Y., Gao, Y., Wu, J. E., Dong, Y. H., He, C., Wang, S. X., Weng, L. X., Xu, J. L., Tay, L., Fang, R. X., and Zhang, L. H. 2004. A bacterial cell-cell communication signal with cross-kingdom structural analogues. *Mol. Microbiol.* 51:903-912.
- Wang, N., Feil, W., and Lindow, S. E. 2007. Identification of traits of *Xylella fastidiosa* conferring virulence to grape and insect transmission by analysis of global gene expression using DNA microarrays. (Abstr.) *Phytopathology* 97:S120.
- Whitehead, N. A., Barnard, A. M. L., Slater, H., Simpson, N. J. L., and Salmond, G. P. C. 2001. Quorum-sensing in gram-negative bacteria. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev.* 25:365-404.