Progress report for CDFA contract number 10-0276

Enhancing control of Pierce's Disease by augmenting pathogen signal molecules

Principal Investigator
Steven E. Lindow
Department of Plant and Microbial Biology
University of California, Berkeley 94720
icelab@berkeley.edu

Cooperators:
Michael Ionescu, Clelia Baccari, Rodrigo Almeida and Ellen Beaulieu
Department of Plant and Microbial Biology and Dept. ESPM
University of California, Berkeley 94720

Reporting period: Results reported here are from work conducted July 2010 to July, 2011.

Abstract:
X. fastidiosa produces an unsaturated fatty acid signal molecule called DSF that modulates gene expression in cells as they reach high numbers in plants. By increasing the expression of a variety of afimbrial adhesins while decreasing the expression of pili involved in twitching motility as well as extracellular enzymes involved in degrading pit membranes and hense movement between vessels, DSF accumulation suppresses virulence of *X. fastidiosa* in grape. We thus are exploring different ways to elevate DSF levels in plants to achieve disease control via "pathogen confusion". Plants expressing *rpfF* from *X. fastidiosa* produce low levels of DSF and are highly resistant to Pierce's disease. Chloroplast targeting of RpfF apparently substantially increases DSF production. *X. fastidiosa* moved much less rapidly in *rpfF*-transformed grape, colonized many fewer xylem vessels, and achieved a much lower population size indicating that elevated DSF levels suppressed movement within the plant. As exogenous sources of DSF applied in various ways to grape suppressed pathogen mobility and hence virulence we have further studied the chemical identity of DSF. Preliminary evidence suggests that DSF is comprised of three closely related fatty acid molecules. One component is 2-Z-tetradecenoic acid (hereafter called C14-cis) while a second compound termed C12-cis is apparently also produced. The chemical identity of a third component is as yet undetermined and is being investigated. We are currently determining the relative activity of these forms of DSF and if such molecules cooperate in regulating gene expression in *X. fastidious.* The various forms of DSF may preferentially affect different behaviors of *X. fastidiosa*. Since some reduction in disease severity was observed in grape scions grafted to DSF-producing rootstocks suggests that DSF produced by rootstocks can somewhat move to scions and confer disease control the control of disease, grafted plants are being made that have a relatively large rootstock to test the hypothesis that increased supply of DSF to the scion will be associated with a larger rootstock. Naturally-occurring endophytic bacteria within grape are being assessed for DSF production; only about 1% of the endophytic bacteria in grape produce DSF and these are being tested for their ability to move within plants after inoculation. As studies of pathogen confusion will be greatly facilitated by having an improved bioassay for the DSF produced by *X. fastidiosa*, we have been developing several immunological and biochemical means to assay for the presence of DSF using *X. fastidiosa* itself as a bioindicator. Bioassays based on immunological detection of the cell surface adhesin XadA and EPS as well as by quantifying mRNA associated with these genes in *X. fastidiosa* have been developed and more are in progress. Gene expression in *X. fastidiosa* exposed to various levels of DSF can also be directly assessed using *phoA* reporter gene fusions. RpfF- mutants of *X. fastidiosa* that do not produce DSF adhere much less tenaciously to grape vessels than do WT strains and we thus are developing assays to more rapidly screen transgenic plants for their resistance to Pierce's disease as well the efficacy of chemical analogs of DSF to induce resistance. The adherence of WT strains of *X. fastidiosa* to transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *X. fastidiosa* was much higher than non-transformed plants, indicating that DSF production in the plants has increased the adhesiveness of the pathogen, and thereby reduced it ability to move within the plant after inoculation.

Introduction:

We have found that the virulence of *Xf* is strongly regulated in a cell density-dependent fashion by accumulation of a signal molecule called DSF encoded by *rpfF* and involving signal transduction that requires other *rpf* genes. We now have shown that *Xf* makes at least one DSF molecule that is recognized by *Xanthomonas campestris pv. campestris (Xcc*) but slightly different than the DSF of *Xcc* (Figure 1). Our on-going work suggests that it also makes other, closely related signal molecules as well. In striking contrast to that of Xcc, *rpfF*- mutants of *Xf* blocked in production of DSF, exhibit dramatically <u>increased</u> virulence to plants, however, they are unable to be spread from plant to plant by their insect vectors since they do not form a biofilm within the insect. These observations of increased virulence of DFS-deficient mutants of *Xf* are consistent with the role of this density-dependent signaling system as <u>suppressing</u> virulence of *Xf* at high cell densities. Our observations of colonization of grapevines by *gfp*-tagged *Xf* are consistent with such a model. We found that *Xf* normally colonizes grapevine xylem extensively (many vessels colonized but with only a few cells in each vessel), and only a minority of vessels are blocked by *Xf.* Importantly, rpfF- mutants of *Xf* plug many more vessels than the wild-type strain. We thus believe that *Xf* has evolved as an endophyte that colonizes the xylem; blockage of xylem would reduce its ability to multiply since xylem sap flow would cease and thus the DSF-mediated virulence system in *Xf* <u>constrains</u> virulence. That is, *Xf* would benefit from extensive movement throughout the plant where it would partially colonize xylem vessels but would have evolved not to grow to excessively within a vessel, thereby plugging it and hence blocking the flow of necessary nutrients in the xylem sap. Given that the DSF signal molecule greatly influences the behavior of Xf we are investigating various ways by which this pathogen can be "confused" by altering the local concentration of the signal molecule in plants to disrupt disease and/or transmission. We thus are further exploring how DSF-mediated signaling occurs in the bacterium as well as ways to alter DSF levels in the plant. Our work has shown that the targets of Rpf regulation are

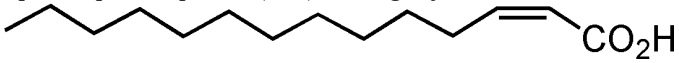


Figure 1

genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel as well as adhesins that modulate movement. Our earlier work revealed that several other bacterial species can both positively and negatively interact with the DSF-mediated cell-cell signaling in *Xf*. In this period we have extensively investigated both the role of DFS-production by *Xf* on its behavior within plants, the patterns of gene regulation mediated by DSF, the frequency with which other endophytes can produce signal molecules perceived by *Xf*, have further characvterized the behavior of *Xf* in grape genetically transformed to produce DSF, and explored other means to alter DSF abundance in plants to achieve PD control. We have particularly emphasized the development of various methods by which DSF abundance in plants can be assessed so that we can make more rapid progress in testing various ways to modulate DSF levels in plants, and have also developed more rapid means by which the behavior of *Xf* in plants can be assessed that does not require the multi-month Pierce's disease assay. Lastly, we have developed better methods to assess DSF-mediated changes in phenotypes in *Xf*.

Objectives:

1) Using novel, improved biosensors for the DSF produced by *Xf*, identify naturally-occurring endophytic bacteria which produce Xf DSF, and evaluate them for biological control of Pierce's disease after inoculation into plants in various ways.
2) Evaluate plants with enhanced production of DSF conferred by co-expressing RpfB, an ancillary protein to DSF biosynthesis, along with the DSF synthase RpfF for disease control as both scions and as rootstocks.
3) Optimize the ability of DSF-producing in rootstocks to confer resistance to Pierce's Disease in the scion.
4) Determine the movement and stability of synthetic DSF and chemical analogs of DSF applied to plants in various ways to improve disease control.

Results and Discussion:

Production of DSF in transgenic plants for disease control.

We have expressed the *rpfF* gene in several different plant species to investigate whether DSF excess can lead to reduced disease caused by *Xf*. Grape (Freedom) has been transformed at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis with a non-targeted *rpfF* construct. These plants produced only very low levels of DSF but are MUCH less susceptible to Pierce's disease (Fig. 2). While *Xf* spread throughout non-transformed plants causing disease on petioles located great distances from the point of inoculation, disease was observed only very close to the point of inoculation in *rpfF*-expressing plants. We measured the movement of *X. fastidiosa* in these plants by measuring both the population size of *X. fastidiosa* in stems and petioles at different distances from the point of inoculation, as well as to observe the fraction of vessels to which a gfp-marked strain of *X. fastidiosa* had moved using fluorescence microscopy (Figure 3). *X. fastidiosa* was greatly limited in its movement in plants producing DSF as evidenced by both a lower population size at sites distal to the point of inoculation and a lower incidence of vessel colonization at all points; both would contribute to low disease severity.

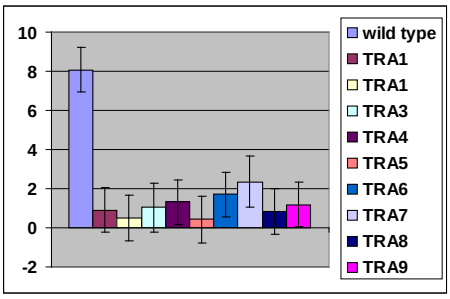


Figure 2. Disease severity (# symptomatic leaves/plant) on Freedom grape transformed with the r*pfF* gene encoding DSF production and inoculated with *Xf*.

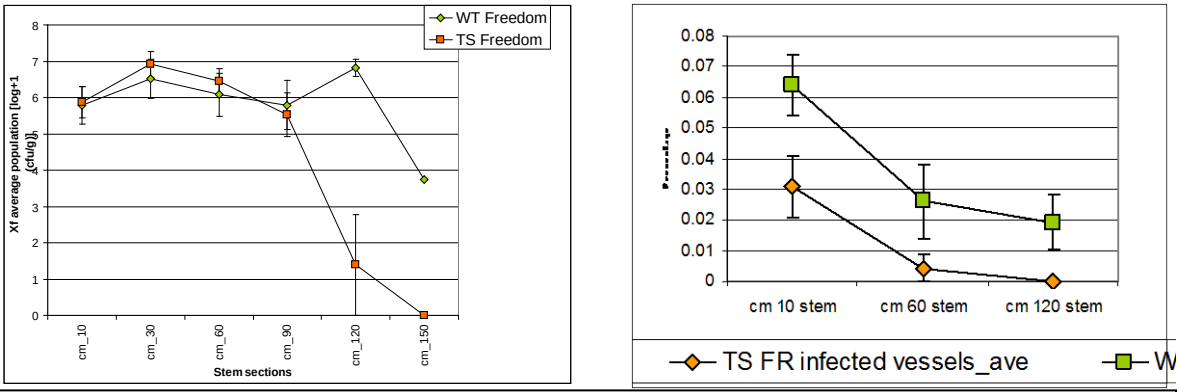


Figure 3. (Left) Population size of *X. fastidiosa* in 1 cm stem segments at different distances from the point of inoculation on non-transformed Freedom grape (green) and in *rpfF*-transformed Freedom (red). The vertical bars represent the standard error of mean log population size. (Right). Proportion of xylem vessels in stems of transformed Freedom grape (green) and in *rpfF*-transformed Freedom (orange) colonized with a gfp-marked strain of *X. fastidiosa* at different distances from the point of inoculation.

We have recently transformed grape (Thompson seedless) with an *rpfF* gene that has been modified to direct the protein product to the chloroplast where fatty acid synthesis (and DSF synthesis) should be much enhanced compared to its production in the cytosol, the presumed location of RpfF in the current transformed plants. Our preliminary results with an improved DSF bioindicator (described

below) revealed that DSF could be detected in several of the transgenic targeted RpfF lines (Figure 4) – this is in contrast to the non-targeted plants were levels of DSF are apparently too low to detect with this biosensor.

We also have inoculated the chloroplast-targeted rpfF plants with a gfp-marked strain of *X. fastidiosa* to assess differences in its movement within the DSF-producing and normal Thompson seedless grape using fluorescence microscopy. Ten Transgenic Chloroplast-targeted rpfF Thompson seedless plants, (which was a mix of several trnasformed lines) and ten non-transformed Thompson seedless were stem inoculate with a gfp-marked wild type *X. fastidiosa*. At eight weeks after inoculation one cm stem segments were sampled at 10,60,120, cm distal from the point of inoculation, and *X. fastidiosa* populations were determined by culturing and CFU/gr populations were estimated via dilution plating.

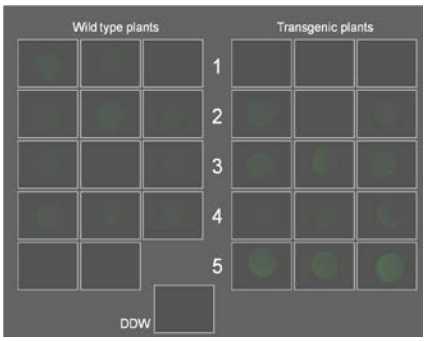


Figure 4. Detection of DSF, visualized as green gfp fluorescence from macerates of leaves from wild-type Thompson seedless (left panel) or different *rpfF*-transformed plants (right panel). In each panel the left-most column is from samples taken near the point of inoculation while the center and right column are from stem segments taken 60 cm and 120 cm from the point of inoculation, respectively.

While the population size of *X. fastidiosa* in the rpfF-transformed lines were similar to that in untransformed lines near the point of inoculation, population sizes were about 10-fold lower in the rpfF-transformed lines at more distal sites on the vine such as 120 cm from the point of inoculation (Figure 5).

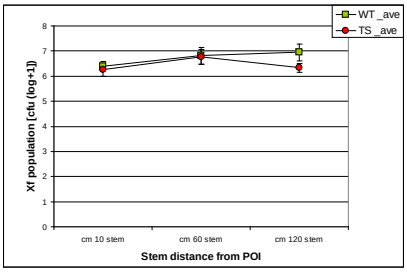


Figure 5. Population size of *X. fastidiosa* in wild type Thompson seedless grape (squares) or transgenic rpfF-expressing grape (circles) at different distances from the point of inoculation.

Microscopy was also carrying out at the same sampling sites to assess the frequency with which xylem vessels were colonized by *X. fastidiosa*. We recorded as positive any vessel harboring *X. fastidiosa* irrespective of whether they harbored few cells or many cells. An average of five stem cross sections were examined for each sampling distance from the point of inoculation on each plant (Figure 6). The proportion of vessels of the *rpfF*-transformed grape that were colonized by *X. fastidiosa* was only about 50% that in non-transgenic lines, suggesting that the movement of the pathogen through the plant was inhibited by expression of *rpfF* and thus production of DSF in the plant. It was also noteworthy that the incidence of vessel colonization varied greatly between transgenic lines evaluated, with some lines having a similar incidence of colonization as the wild-type line while others having very little colonization.

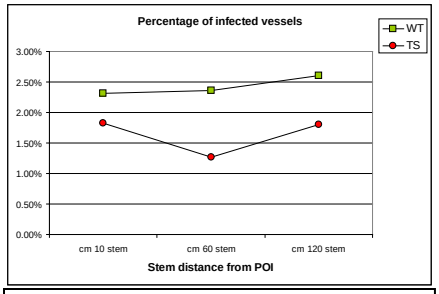


Figure 6: Average of *X. fastidiosa*-infected vessels per grape stem cross section in transgenic rpfF-transformed Thompson grape (red circle) and in non-transformed Thompson seedless in (green squares).

Further support for the possibility that more than one fatty acid signal molecule is made by RpfF was obtained by the use of a Thin Layer Chromatography (TLC) method to assess the fatty acids produced by *X. fastidiosa*. In this method, acidified ethyl acetate extracts of culture supernatants of a wild-type *X. fastidiosa* strain and an RpfF- mutant and a RpfB mutant were subjected to TLC and fatty acids visualized by iodine vapors. Interestingly, three different fatty acids were visualized in the wild type strain, while these were largely missing in an RpfF- mutant, with only very small amounts of two other putative fatty acids present (Figure 7). It also was of interest to see that the RpfB mutant produced an altered pattern of putative fatty acids, with the major chemical species produced by the WT strain missing, and much larger amounts of one of the other species produced. The top-most spot observed in extracts of a WT strain of *Xf* co-migrates with C14-cis, a chemical form of DSF that we have previously characterized. Interestingly, the middle band found in the WT strain, which migrated similarly to the topmost, and most abundant band seen in the rpfB mutant, co-migrates with C12-cis. It is noteworthy, that a re-examination of the DSF species produced by *Xanthomonas campestris* pv. campestris using different methods have revealed that this species also produces C12-cis. The most prominent, lower, band seen in the WT strain is not observed in the *rpfB* mutant. This supports the model that RpfB, a putative long-chain fatty acyl CoA ligase, serves to produce suitable substrates for RpfF, the DFS synthase. Although RpfB is not required for DSF synthesis in *Xf*, it presumably aids in DSF synthesis by encoding long chain fatty acyl CoA ligase which might increase availably of the appropriate substrates for DSF synthesis by RpfF. It is interesting to note that *rpfB* mutants have an altered behavior compared to *rpfF* mutants and WT strains of *Xf*. While rpfF mutants are hypervirulent in grape, *rpfB* mutants were nearly as virulent as WT strain. In contrast, while rpfF mutants are non-transmittiable by sharpshooters, the rpfB mutants exhibit only a slight decrease in their transmission (Figure 8) suggesting that they are retained by sharpshooters more efficiently than the rpfF mutant. Given that the *rpfB* mutant

Figure 7. Fatty acids resolved by TLC from a RpfF mutant of *X. fastidiosa* (left lane) a RpfB mutant (center lane) and a wild type strain of *X. fastidiosa* (right lane). Fatty acids were visualized after exposure to iodine vapor.

appears to make C12-cis but not C14-cis, this suggests that C12-cis is sufficient to enable signaling that leads to insect transmission but does not greatly affect virulence. We expect that co-expression of RpfB and RpfF in the chloroplast will further enhance the DSF levels in plants. We have produced transgenic *Arabidopsis* plants with such a construct and find evidence of high levels of DSF production. Pathogenicity assays with the *rpfF* mutant of *Xcc* indicated that the transgenic plants expressing both *rpfB* and *rpfF* transgenic plants can better complement the virulence of the non pathogenic *rpfF* mutant of *Xcc*. Based on this and other data, we are expressing both RpfF and RpfB simultaneously in transgenic for optimum production of suitable DSF molecules. We thus are preparing genetic constructs to transform grape with these two genes to further enhance DSF production.

Studies of movement of *X. fastidiosa* in plants

Our studies have suggested strongly that adhesion of Xf to plant tissues inhibits movement of the pathogen through the plant, and hence tends to reduce the virulence of the pathogen. RpfF- mutants of *Xf* that do not produce DSF adhere to glass surfaces and to each other much less effectively than WT strain that produce DSF. This is consistent with the apparent rpfF-regulation of adhesins such as HxfA, HxfB and XadA etc. To better correlate levels of DSF in the plant and the stickiness of the *X. fastidiosa* cells we have developed a practical assay to measure and compare stickiness of *X. fastidiosa* cells in grapes infected with *X. fastidiosa gfp-Wt* and *X. fastidiosa* mutants. In this assay, the release of cells of *Xf* from stems and petioles tissue from grape infected with *X. fastidiosa* wild type Tem and gfp-rpfF mutant were compared. Tissues from infected Thompson seedless grapes were surface sterilized with 70% alcohol, and 30 % bleach. From the sterile tissues, 5mm stem or petiole segments were cut and placed individually in sterile buffer and shaken gently for 20 minute. After 20 minutes the number of cells released from the cut end of the segment were estimated by dilution plating on PWG. To determine the total number of cells in a given sample (the number of cells that potentially could have been released by washing) the washed segment was macerated and *X. fastidiosa* populations again evaluated by dilution plating. Total cell populations were calculated by summing the cells removed by washing and those retained in the segment. The ratio of easily released cells to the total cells recovered in the samples was termed the release efficiency. In both stems and petioles the release efficiency of the *rpfF* mutant was much higher than that of the WT strain (Figure 8). This very striking difference in the adhesiveness of the *Xf* cells experiencing different levels of DSF in the plant suggest that this release efficiency assay will be valuable for rapidly assessing the susceptibility of grapes treated in various ways. For example, the adhesion of cells could be measured within a couple of weeks after inoculation of WT *Xf* cells into transgenic plants harboring various constructs designed to confer DSF production in plants, or in plants treated with DSF producing bacteria or topical application of chemicals with DSF-like activity. Such an assay would be far quicker than assays in which disease symptoms must be scored after several months of incubation, and could be employed during those times of the year such as the fall and winter when disease symptoms are difficult to produce in the greenhouse.

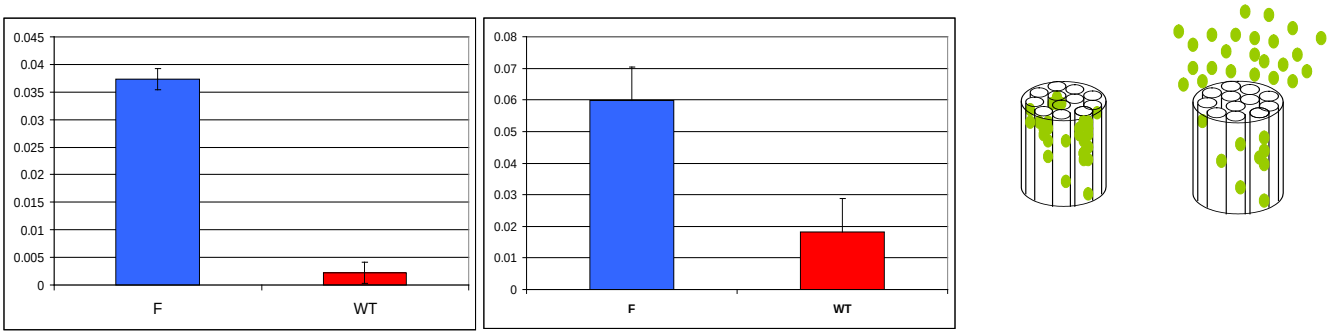


Figure 8. Proportion of total cells of a gfp-marked WT strain of *X. fastidiosa* (red) and a gfp-marked rpfF mutant of *X. fastidiosa* (blue) in petioles (left) or stems of Thompson seedless grape (right) that were released during gentle washing of the segments in buffer. Stems were excised and surface sterilized before a 5 mm segment is cut. Each segment was then shaken in 2 ml of buffer for 20 minutes and the number of cells released from the cut end are determined by dilution plating. The segment is then macerated and the number of cells within the segment determined by dilution plating of the segment macerate. The total *Xf* population was taken as the sum of the cells released by washing and the number remaining within the segment. The released population is the number washed from the segment normalized for the total population size of *Xf*. The vertical bars represent the standard error of estimates of the proportion of released cells for a given treatment.

To further address the usefulness of the cell release assay to assess treatments designed to limit the movement of X. fastidiosa in plants to achieve disease control, we tested the adherence of WT strains of *X. fastidiosa* to transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *X. fastidiosa* compared with that to non-transformed plants. Plants were inoculated with a gfp-marked wild type strain of *X. fastidiosa* and petioles were removed from plants at a distance of about 20 cm from the point of inoculation, and the percent of the cells removed during a brief washing step measured as above. The adherence of WT strains of *X. fastidiosa* to transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *X. fastidiosa* was much greater than that of cells in the non-transformed plants, indicating that DSF production in the plants has increased the adhesiveness of the pathogen, and thereby reduced it ability to move within the plant after inoculation. That is, the percentage of cells that was released from *rpfF*-expressing plants was from 2 to 3-fold less than that of control plants (Figure 9). As seen before, cells of an *rpfF* mutant that does not produce DSF exhibited about 3-fold higher percentage of cells released from a normal Thompson grape compared to the WT strain in the non-transgenic grape (Figure 9).

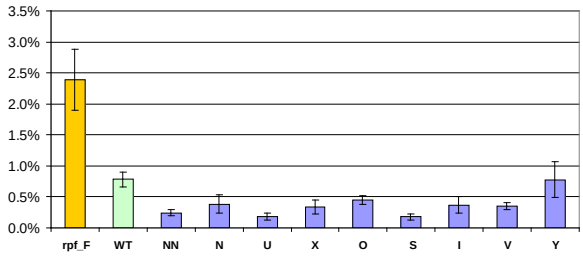


Figure 9. Percentage of total cells of a gfp-marked WT strain of *X. fastidiosa* (blue and green) and a gfp-marked rpfF mutant of *X. fastidiosa* (orange) in petioles of non-transformed Thompson (Orange and Green) or of transgenic Thompson seedless expressing a chloroplast-targeted *rpfF* gene from *X. fastidiosa* (blue) that were released during gentle washing of the segments in buffer. The vertical bars represent the standard error of estimates of the proportion of released cells for a given treatment.

To further test the utility of the cell release assay to relate the virulence of *X. fastidiosa* to their adhesive properties, the retention of a large number of different mutants of *X. fastidiosa* are being assessed with this method. Plants have been inoculated with 11 different mutants altered in cell-cell signaling as well as other genes contributing to virulence and the retention of the cells will be related to disease symptoms expressed in plants inoculated with these strains. As the adhesiveness of cells of X. fastidiosa and their ability to cause disease in grape are expected to be inversely correlated, we examined the disease severity of the normal and rpfF-expressing Thompson seedless grape that were inoculated with either the WT *X. fastidiosa* strain or an RpfF mutant. Although these studies were done during winter months when disease symptoms are hard to distinguish from other stress symptoms on grape, substantial differences in putative disease severity were observed between the rpfF-expressing grape and the non-transformed Thomopson seedless; most of the transgenic lines expressed less than half as many symptoms. In general, there was a direct relationship between the release efficiency of the cells of *X. fastidiosa* and the severity of disease in the same plants (Figure 10). These results suggest strongly that DSF production presumably has occurred in the *rpfF*-expressing plants, and that the enhanced adhesiveness of these cells is associated with their reduced ability to spread through the plant and cause disease. These results suggest that the release efficiency assay should be a useful tool to rapidly assess treatments designed to control Pierce's disease.

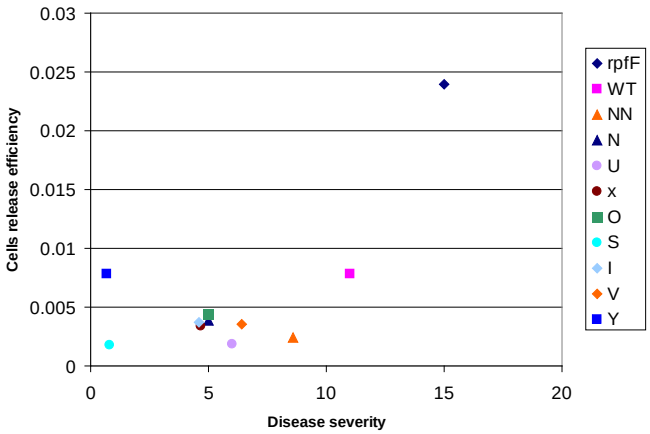


Figure 10. Relationship between release efficiency of cells of *X. fastidiosa* in normal Thompson seedless grape (pink squares) and that in *rpfF*-expressing Thompson seedless grape (all other symbols except purple diamonds) and the severity of Pierce's disease in those vines assessed as the number of symptomatic leaves per vine (abscissa).

While the movement of *X. fastidiosa* has been recognized as an important trait necessary for disease, the process is still poorly understood. Other studies we have performed in our lab are strongly supportive of a model of progressive and sequential colonization of a large number of xylem vessels by *X. fastidiosa* after inoculation of a single vessel. Furthermore, we believe that the process of movement of *X. fastidiosa* through plants is a stochastic one which is characterized by growth in a given xylem vessel into which it is introduced followed by "active escape" of at most a few cells into adjacent uncolonized vessels, and then further multiplication of the cells which starts the process anew. We thus are exploiting the use of mixtures of phenotypically identical strains of *X. fastidiosa* differing by only one or two genes to better understand the process of progressive movement of *X. fastidiosa* through plants. We hypothesize that anatomical features of plants (nature of pit membranes and other barriers to vessel to vessel movement in the stem) limit the number of *X. fastidiosa* cells that can transit from one vessel to another and are major factors conferring resistance in plants. It would be expected that the stochastic (random) processes that would tend to segregate cells of one strain from another in the process of progressive movement would increase the degree of segregation with distance from the point of inoculation (with increasing numbers of vessels the cells had to traverse to get from one part of the stem to another given that each vessel in grape is an average of only about 10 cm long). That is, *X. fastidiosa* must move from one xylem vessel to another dozens or hundreds of times to be able to move longitudinally down a vine as well as laterally across the vine to achieve the extensive colonization of the stem that

are typical of diseased vines. If, at each step in this movement process only a few cells are transferred then, by chance, xylem cells distal to the inoculation point will receive by chance only one of the two genotypes of the pathogen. Thus for a given plant inoculated with a mixture of cells, the proportion of one strain compared to the other would either increase or decrease along a predictable trajectory given the stringency of the "bottleneck" that it faced while moving from one vessel to another. Our efforts to test this model of movement of *X. fastidiosa* through grape tissue has been hindered by the fact that isogenic strains of this pathogen that differ even slightly in virulence (ability to move within the plant) result in one strain predominating at distal parts of the plant, presumably due to a slightly higher likelihood of successful movement to adjacent xylem vessels at each step in the colonization process. For example, WT cells of *X. fastidiosa* always moved further and at higher frequency than *rpfB* mutants. Likewise, random Tn5-mutants of *X. fastidiosa* generated by the Kirkpatrick lab also were inferior to the WT strain and were not recovered when mixtures were inoculated into grape. We now have identified *phoA* mutants as having identical behavior in grape as the WT strain, enabling us to examine the process of spatial segregation of cells of *X. fastidiosa* during colonization of grape. The population size of the WT strain of *X. fastidiosa* was similar to that of the *phoA* mutant whether inoculated singly or in a mixture (Figure 11). More importantly, the proportion of cells of the two strains that were recovered from different locations within a given plant differed greatly between plants (Figure 12). For example, 8 plants were inoculated with an equal mixture of the WT and *phoA* mutant, yet in some plants all of the cells recovered from locations either 10 cm or 120 cm form the point of inoculation were either one strain or the other; seldom was a mixture of both strain found, and a similar fraction of the plants harbored one strain or the other, suggesting that the two strains had an equal likelihood to move within the plant, but that stochastic processes determined the movement. We hypothesize that resistant grape varieties harbor anatomical differences from susceptible varieties that limit the movement of *X. fastidiosa* from vessel to vessel. Such plant would thus present a more extreme "bottleneck" to *X. fastidiosa* at each movement event and hence we would expect a more rapid segregation of mixtures of *X. fastidiosa* at a given point away from inoculation. We thus are currently further exploring the spatial dependence of this segregation process in different grape varieties that differ in resistance to Pierce's disease. Plants have been inoculated and assessment of the ratios of cells of the two strains in the mixture are currently being made. Not only should this provide considerable insight into the process of movement which, while central to the disease process, remains very poorly understood, but it should also provide new tools for screening grape germplasm for resistance to *X. fastidiosa*.

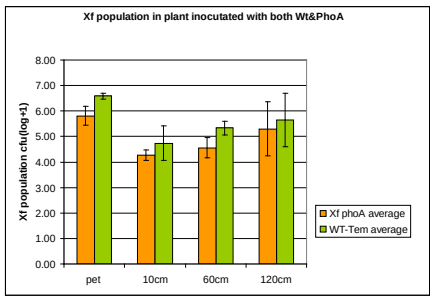
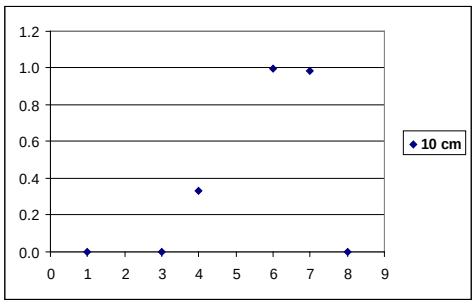


Figure 11. Population size of a WT strain of *Xf* (green) or of a PhoA mutant (orange) at various distance from the point of inoculation when inoculated single (left panel) or in equal mixtures (right panel).



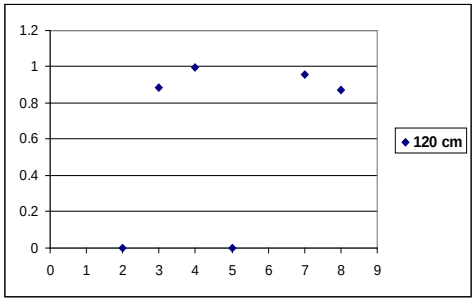
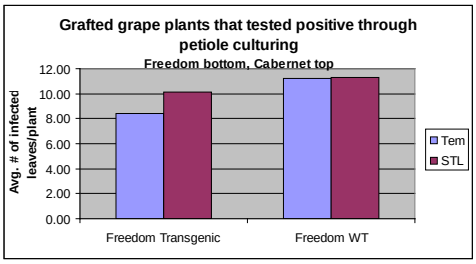


Figure 12. Proportion of cells recovered at 10 cm (left panel) or 120 cm (right panel) from the point of inoculation from individual plants that were inoculated with an equal mixture of a WT strain of *Xf* and a PhoA mutant (abscissa) that were the WT strain.

Graft transmissibility of DSF. To test whether DSF is mobile within the plant we are performing grafting experiments in which DSF-producing Freedom grape transformed with the rpfF of *Xf* are used as rootstocks to which normal Cabernet Sauvignon grape were green-grafted as a scion. As a control, normal Freedom was also used as a rootstock. These plants were inoculated with *Xf* to test whether normal scions on DSF-producing rootstocks have a lower susceptibility to *Xf* colonization as a rootstock than as a scion. Initial estimates of disease severity indicate that there were about 30% less symptomatic leaves of the normal Cabernet scion when grafted onto a *rpfF*-expressing rootstock compared with plants on a normal Freedom rootstock (Figure 13). Thus, like in the studies of the *rpf*F-expressing tobacco, it appears that DSF production in the scion is more efficacious for disease control than is the expression of *rpfF* in the rootstock. We are repeating these experiments and will be inoculating the plants in a variety of ways to determine the efficiency of disease control from rootstock modification. We are repeating these grafting experiment both with the non-targeted *rpfF* Freedom as a rootstock as well as the chloroplast-targeted *rpfF* Thomson seedless as a rootstock. We are testing the hypothesis that increasing the size of the rootstock will increase its potential to distribute DSF to the scion. We thus are producing while types ions grafted to rootstocks of differing sizes. This is proving difficult because her normal process of green grafting roots a small root stock at the same time that a scientist grafted onto the top. Establishing a large rootstock before grafting has made establishment of the grafted scion more difficult. Some success has now been achieved in these plants will soon be inoculated and their disease susceptibility related to the size of the rootstock.



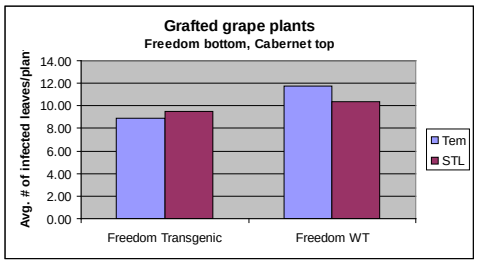


Figure 13. Severity of Pierce's disease (left box) or number of leaves per vine infested with *X. fastidiosa* (right box) of Freedom grape having a Cabernet sauvignon grape scion grafted onto the rootstock noted on the abscissa. Plants were inoculated with either *X. fastidiosa* strain Temecula (blue) or strain STL.

Disease control with endophytic bacteria**.** The severity of Pierce's disease is reduced when DSF-producing bacteria such as rpfF-expressing *E. coli* and *E. herbicola* and certain *Xanthomonas* strains are co-inoculated with Xf into grape. Importantly, the control of disease by DSF-producing bacteria was associated with their ability to produce DSF since strains that were blocked in ability to produce DSF were greatly reduced in their ability to suppress disease, indicating that elevated DSF in the plants was the cause of disease suppression. We have been successful in producing large quantities of DSF in endophytes such as *Erwinia herbicola* and also in lab strains of *E. coli*. Unfortunately, these bacterial strains do not move within grape after inoculation, thereby restricting their ability to interact with *X. fastidiosa* except at sires of co-inoculation. Presumably to achieve control of Pierce's disease by endophytic bacteria where *X. fastidiosa* might be inoculated at any point in the plant by insect vectors it will be important to utilize endophytic bacteria that can colonize much of the plant in order that DSF be present at all locations within the plant. Naturally-occurring endophytic bacteria that produced the DSF sensed by *X. fastiosa* and which might move extensively within the plant would presumably be particularly effective as such biological control agents, but until now it was impossible to identify such strains. We recently were able to transform a putative efficient endophyte of plants, *Rizobium etili* G12 with both the *Xcc* and *X. fastidosa rpfF* (DSF biosynthetic gene) and have obtained some production of DSF in this strain. This DSF-producing endophyte has been inoculated into grape to determine both its ability to move and multiply within grape as well as its ability to interfere with the disease process. The *R. etli* strain G12 was found to move within grape tissue after inoculation into either the stem or the leaves. When measured 4 weeks after inoculation by puncture inoculation into one site in the stem measurable populations of *R. etli* were seen as far as 50 cm away from the point of inoculation. While the population size away from the point of inoculation were relatively low in this short time interval since inoculation, this strain clearly has the ability to move within grape. The evidence we have obtained so far, however, suggests that this bacterium moves relatively slowly in grape, and thus such a strain would have to be inoculated into grape substantially in advance of the pathogen in order to achieve high levels of disease control. In addition, the DSF-producing R. etli strain is recombinant, and hence may face regulatory scrutiny before it might be used in production viticulture. For that reason we have initiated a study of naturally-occurring bacterial endophytes for their ability to produce DSF. This objective was possible since much effort devoted in the last two years has resulted in the development of better biosensors for the DSF produced by *X. fastidiosa* (C14-cis and related molecules) (discussed below). We now have several highly sensitive assays for *X. fastidiosa* DSF. The DSF produced by *Xcc* and that made by X. fastidiosa are slightly different from each other and the *Xcc*-based biosensor for DSF is MUCH more responsive to the *Xcc* DSF than to the C14-cis produced by *X. fastidiosa;* while the *Xcc*-biosensor can detect as little as about 100 nM of *Xcc* DSF it can detect C14-cis only in concentrations above about 1 mM. We thus previously could not easily the production of molecules in bacterial endophytes that were similar to the DSF made by *Xf*. Our new biosensor, however has allowed us to screen large numbers of bacteria for *Xf* DSF production.

Both the older *Xcc* and new *Xf*-DSF specific biosensors developed in our lab were used to screen natural bacterial endophytes recovered from BGSS insect heads and grape plants. We are executing this part of the project using two approaches; the first approach is building our own endophyte library using mainly endophytes isolated from insect head and wild grapes. The second approach involved screening an existing large grape endophyte library which was kindly made available to us by Dr. B. Kirkpatrick. BGSS adults and nymphs were surface sterilized, macerated, and diluted onto 10% TSA. The emerging colonies were harvested and transferred to KB plates where they were over-sprayed with either of the two biosensors and GFP fluorescence monitored. Our initial results reveal that 0.9% of the endophytes recovered from either insect mouthparts or from grape xylem produce either a DSF detected by the *Xcc*-specific DSF biosensor, or the *Xf* DSF-specific biosensor or both. More that twice as many strain produce a DSF detected by the *Xcc*-specific DSF biosensor, suggesting that the DSF produced by *X. fastdiosa* is not as common as that produced by other bacteria such as environmental *Xanthomonas* strains etc. Many more strains remain to be tested and a relatively large library of DSF-producing bacteria is anticipated. This collection of DSF-producing bacteria will then be assessed for their ability to grow and move within grape plants as well as their ability to reduce symptoms of Pierces disease when co-inoculated and pre-inoculated into grape before *X. fastdiosa*. The resumption of growth of grape in the field has allowed us to sample field-grown plants for bacterial endophytes; has been a focus of studies in the summer of 2011. A large number of endophytes are being collected and these will be assessed for their ability to produce DSF. Special attention will be placed on the relative portion of endophytes from grape that produce DSF detectable by the *X. fastidiosa* biosensor compared to DSF more related to that produced by Xcc.

Development of an *Xcc* biosensor efficient in detecting *Xylella* DSF. For many of the objectives of this project, an improved bioindicator for DSF would be very valuable. Such a biosensor will be needed to accurately screen transgenic plants for DSF production as well as to screen to endophytic bacteria capable of DSF production. Likewise, the direct application of DSF to plants as

well as the application of analogs of DSF for disease control will be greatly expedited by use of direct assays for DSF abundance in treated tissues. Until now, we have been using an *Xcc*-based biosensor in which the *Xcc* endoglucanase gene is linked to a GFP reporter gene. Previous studies have shown that this biosensor is able to detect the DFS made by *Xf* but that it detects *Xf* DSF with a much lower efficiency then the *Xanthomonas* DSF since the two molecules apparently differ slightly. In this reporting period we have devoted considerable attention to, and have made considerable progress in the construction and characterization of *Xanthomonas campestris* -based *X. fastidiosa*-DSF specific biosensors. In order to be able to sense *X. fastidiosa* DSF we constructed two independent *Xcc* -based DSF biosensors specific to the DSF produced by *X. fastidiosa* RpfF and which is sensed by *Xf* RpfC. In the first sensor, we replaced the Rpf-DSF detection system of *Xcc* with that of homologous components from *X. fastidiosa*. An *Xcc* mutant in which both *rpfF* and *rpfC* was deleted was transformed with a pBBR1MCS-2 based plasmid harboring *Xf rpfC* and *rpfF* genes. In a previous study, *X. fastidiosa rpfC* was introduced into an *Xcc rpfC* mutant resulting in the repression of DSF production (the *Xcc rpfC* mutant itself is a DSF hyper-producer) but not of EPS production and protease activity. Based on that observation it was concluded that the *X. fastidiosa* RpfC is capable of interacting with the *Xcc* RpfF to control its DSF production activity, but that the *X. fastidiosa* RpfC was not capable of interacting with the *Xcc* RpfG to initiate down-stream signal transduction. In that sensor the *Xcc rpfG* was left intact. *X. fastidiosa* RpfG, RpfC and RpfF are presumed to function in concert to mediate signal transduction in the following manner: *X. fastidiosa* RpfF synthesizes DSF, and we have shown that it produces DSF molecules recognizable by *Xcc* RpfC and in other bacterial hosts (*E. coli*, *E. herbicola R. etli etc.*). RpfC, upon interaction with DSF, is thought to phosphorelay to RpfG. RpfG is a cyclic di-GMP phosphodiesterase. Since cyclic di-GMP plays a regulatory role in many bacteria species, it is expected that the *X. fastidiosa rpfGCF* system, capable of reducing the levels of cyclic di-GMP, will affect the expression of genes regulated by cyclic di-GMP in any host which its gene expression relays upon cyclic di-GMP. In *Xcc*, DSF and cyclic di-GMP were shown to regulate the expression of many virulence genes including the cellulase gene *engXCA*. Therefore, the functionality of the *Xf rpfGCF* system was determined in *Xcc* based on the activity of an *engXCA'::gfp* transcriptional fusion. Introduction of *Xf rpfG* to the *Xcc rpfCF* mutant, did not affect the GFP fluorescence signal produced by the *engXCA'::gfp* fusion suggesting that *Xf rpfG* was in its inactive state. Introduction of *Xf rpfG* and *rpfC* together however, resulted in a 2-fold increase in the *engXCA* promoter activity. This strain, although it harbors *Xf rpfC*, exhibited a slight increase in *gfp* fluorescence in response to only high levels (100 µM) of 2-*Z*-tetradecenoic acid, the putative DSF molecule produced by *Xf*. In order to address this puzzling observation, *Xf rpfF* was subsequently introduced to this strain. Upon addition of *Xf rpfF* the *engXCA* promoter activity decreased to background level and was similar to that of the strain harboring *Xf rpfG* only, suggesting that it represses the RpfC-RpfG interaction. Addition of 0.1-100 µM 2-*Z*- tetradecenoic acid, de-repressed RpfC-RpfG interaction allowing *gfp* to be expressed in a dose-response manner (Figure 14).

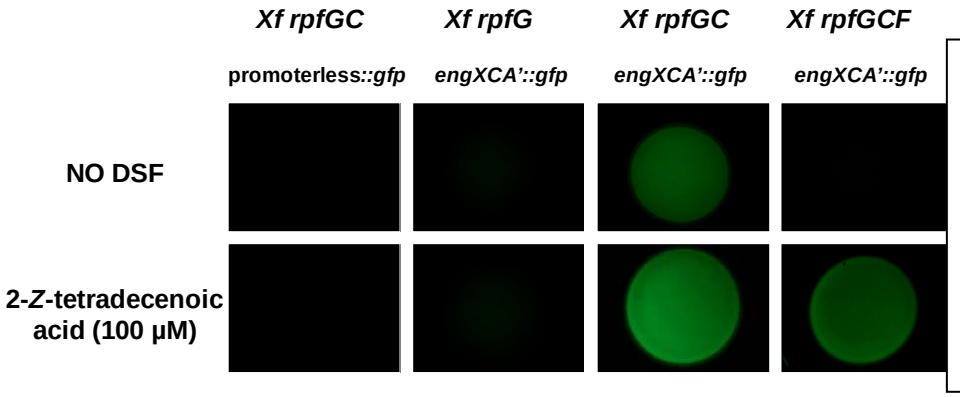
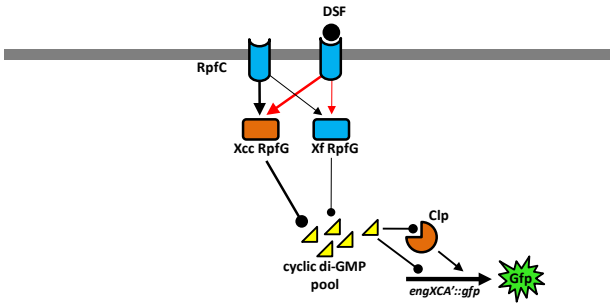


Figure 14. GFP fluorescence exhibited by a mutant strain of *Xcc* lacking its own *rpf C* and *rpfF* genes but into which the various *rpf* genes from *Xf* noted on the top of each column were added. GFP fluorescence in the presence or absence of added DSF was then visualized. Note that this biosensor yields gfp fluorescence specifically in the presence of DSF when the *XF rpfG*, *rpfC* and *rpfF* genes have been added.

The DSF production activity of *Xf* RpfF in this strain was tested, yielding no observed DSF production. Assuming that *Xf* RpfC, like *Xcc* RpfC, represses DSF production, the DSF production activity of *Xf* RpfF was further tested in the absence of *Xf* RpfC or both *Xf* RpfG and RpfC. In both cases, activity was restored suggesting that *Xf* RpfC interacts with RpfF and controls its DSF production activity.

The process by which the DSF biosensor for *X. fastidiosa* DSF was developed by substituting *X. fastidiosa* RPF constituents for their homologs in *Xcc* has provided considerable insight into the process of wrecking recognition of in response to DSF in *X. fastidiosa*. This will prove very important in our assessment of methods to alter the normal purse exception of perception of DSF by *X. fastidiosa* in our process of disease control by pathogen confusion. Models of the interaction of the different signal perception and response components in *X. fastidiosa* can now be developed. In one model (Figure 15) *X. fastidiosa* RpfC (blue), with or without DSF is active (red arrows donate a possible little enhancement in signaling activity when DSF is present in high levels), probably interacting with Xcc RpfG (in brown). *X. fastidiosa* RpfG thus acts as a dominant negative in *Xcc* cells also harboring their own RpfG, interacting with X. fastdiosa RpfC as well and by that, reducing the number of Xcc RpfG molecules that are phosphoryated and thus active. It can be assumed that Xcc RpfG is more efficient than X. fastdiosa RpfG in regulating c-di-GMP levels in Xcc cells but *X. fastidiosa* RpfG has a higher affinity to *X. fastdiosa* RpfC. Thus, the observed gfp levels seen are a result of interplay between those two forces.

Figure 15. Model of interaction of RpfC from *X. fastidiosa* with other components of the signal transduction system leading to DSF-dependent expression of genes such as *engA*.



When *X. fastidiosa* RpfF is present, the interactions are changed. RpfF clearly represses RpfC activity. They have to physically interact in order to achieve repression of DSF production and this interaction seems also changes the way RpfC signals (Figure 16). In Xcc other researchers claim that DSF abolishes RpfC-RpfF interaction, in our case, it can be suggested that RpfC and RpfF remain bound in the presence of DSF. DSF does not abolish their interaction but instead changes the conformation of RpfC to allow signaling to X. fastdiosa RpfG. This model does not, however, explain how DSF production is being initiated. To address this, the chimeric RpfC was introduced with Xf RpfG to the same Xcc mutant (below figure). It exhibits a higher background activity but also a stronger induction by DSF, probably due to the higher specificity between the cytoplasmic domain and the RpfG from Xcc. It seems that the *X. fastidiosa* RpfC trans-membrane domain determines a high level of activity that, again, was inhibited by the addition of *X. fastdiosa* RpfF. In Xcc RpfF interacts with the REC domain of RpfC. It is unclear if RpfF of *X. fastidiosa* interacts with the same domain. The REC domain present in a chimeric RpfC (see below) is the same as that in Xcc RpfC. Newman et al. reported that very little DSF was produced by *X. fastidiosa* RpfF in an *Xcc rpfF* mutant. Since we know that removal of RpfC allows about normal DSF production, it can be suggested that *X. fastidiosa* RpfF can interact with Xcc RpfC, probably with its REC domain.

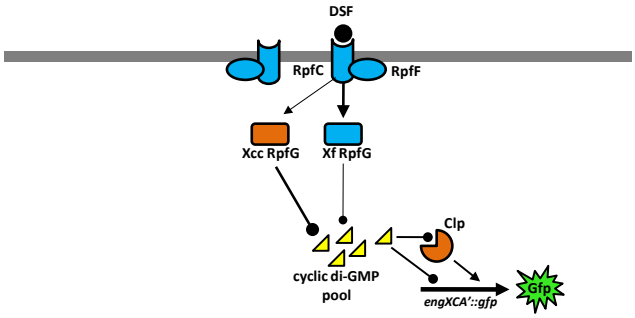


Figure 16. Model of interaction of RpfC from *X. fastidiosa* with other components of the signal transduciotn system leading to DSF-dependent expression of genes such as *engA*.

A second *Xcc*-based *Xf* DSF sensor was constructed that is composed of an *Xcc rpfF* and *rpfC* double mutant into which *Xf rpfF* and *rpfG* and a hybrid *rpfC* allele composed of the predicted trans-membrane domain of *Xf* RpfC and the cytoplasm domain of the *Xcc* RpfC has been added. The *eng'::gfp* transcriptional fusion in *Xcc* that was described above was inactive in the absence of DSF but was strongly induced by ≥0.1 µM 2-*Z*- tetradecenoic acid. We compared the activity of these two *Xf*-DSF specific biosensors to the activity of our original *Xcc*-DSF specific biosensor (*Xcc rpfF* mutant harboring an *eng'::gfp* reporting fusion) in response to diluted DSF extracts obtained from *Xcc* and from *Xf*. We named the three different sensors based on their DSF sensing element, *Xcc*-rpfC, *Xf*-RpfC and *Xf-Xcc* chimeric RpfC. While both of the *Xf*-DSF specific biosensors (*Xf*-RpfC and *Xf-Xcc*-RpfC) did not respond at all to the diluted DSF extract obtained from *Xcc* culture, the *Xcc*-DSF specific strain (*Xcc*-RpfC) exhibited a clear dose-dependent behavior to an elevated extract strength (Figure 17 left). When exposed to DSF extract obtained from *Xf* cultures, *Xf*-RpfC and *Xf-Xcc*-RpfC clearly responded to increased levels of *Xf* DSF while the *Xcc*-RpfC biosensor barely responded to the lowest dilution tested (Figure 17 right).

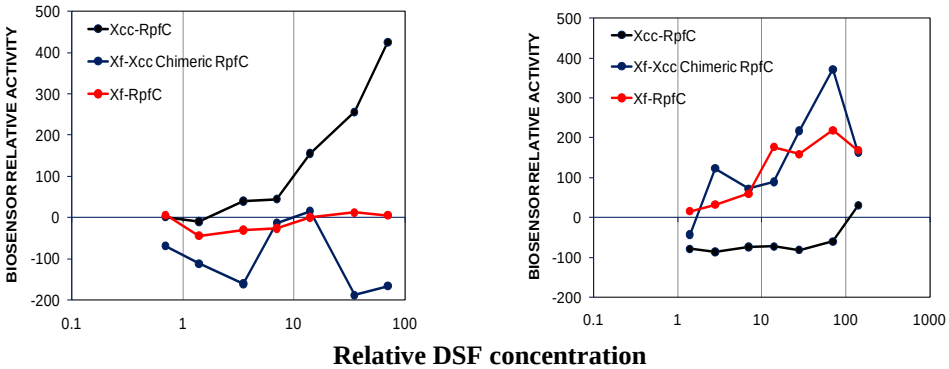
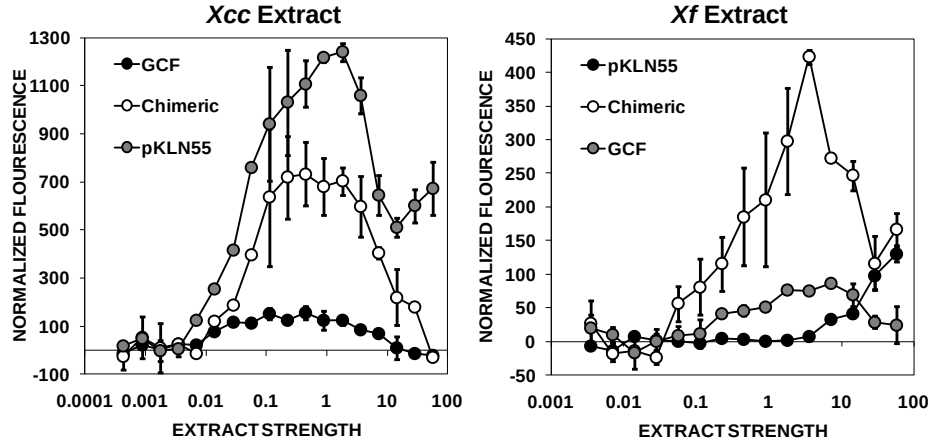


Figure 17. GFP fluorescence of the Xf DSF specific biosensors (red and dark blue) of the *Xcc*-specific DSF biosensor (black) to increasing concentrations of DSF from culture extracts of *Xcc* (left panel) or from *X. fastidiosa* (right panel).

Further tests of the relative sensitivity of the three DSF biosensors to various DSF-containing bacterial extracts were made to better understand the selectivity of the various biosensors. The three *Xcc*-based DSF biosensors (*Xcc*RpfC (also called pKLN55), *Xf*RpfC (also called GCF), and *Xcc*chimericRpfC (alsop called Chimeric)) were exposed to serial dilutions of DSF containing extracts obtained from *Xf* and *Xcc* wild type strains (Figure 18). All of the sensors exhibited similar sensitivity to the DSF containing extract from *Xcc* but in sharp contrast, *Xcc*RpfC exhibited much lower sensitivity to the DSF containing extract from *Xf* than the two *Xf*-DSF specific biosensors. These results suggest that *Xf*-RpfC can interact with a wider range of molecules then *Xcc*-RpfC. In addition, these two sensors were found to become activated even in the presence of dilutions of the *Xf* extract that contain less than the fraction contributed by a single PWG plate from which the DSF was originally extracted.



$$\text{Extract Strength} = \left[\frac{\text{Volume of growth medium}}{\text{Volume of extract}}\right] \text{X} \left[\frac{\text{Volume of extract}}{\text{Volume of growth medium}}\right]$$

EXTRACTION **APPLICATION**

Figure 18. GFP fluorescence of different DSF biosensors to increasing concentrations of DSF from culture extracts of *Xcc* (left panel) or from *X. fastidiosa* (right panel).

To test the specificity of the DSF biosensors we compared the activity of those sensors to a panel of pure DSF analogues. Inspection of the intensity of the respond (GFP level) by fluorescence microscopy revealed that *Xcc*-RpfC is activated in a stronger manner by DSF-like molecules with shorter acyl chain lengths (C11-*cis*, C12-*cis* and C13-*cis*) than by DSF homologs with either shorter or longer acyl chain lengths (Figure 19). In contrast, the *Xf* DSF-specific biosensors *Xf*-RpfC and *Xf*-Xcc-RpfC responded more strongly to C13-*cis* and C14-*cis* then to shorter chain fatty acids (Figure 19). Given that C14-cis is produced by *Xf* it appears that the RpfC-based biosensors respond more strongly to the DSF molecule to which the cell has evolved to respond. Thus the two *Xf* DSF specific DSF biosensors, particularly the *Xf*-*Xcc* RpfC biosensor is much more responsive to C14-cis, and hence will be far more useful that the original *Xcc* RpfC biosensor for assessing DSF levels in plants and bacterial cultures.

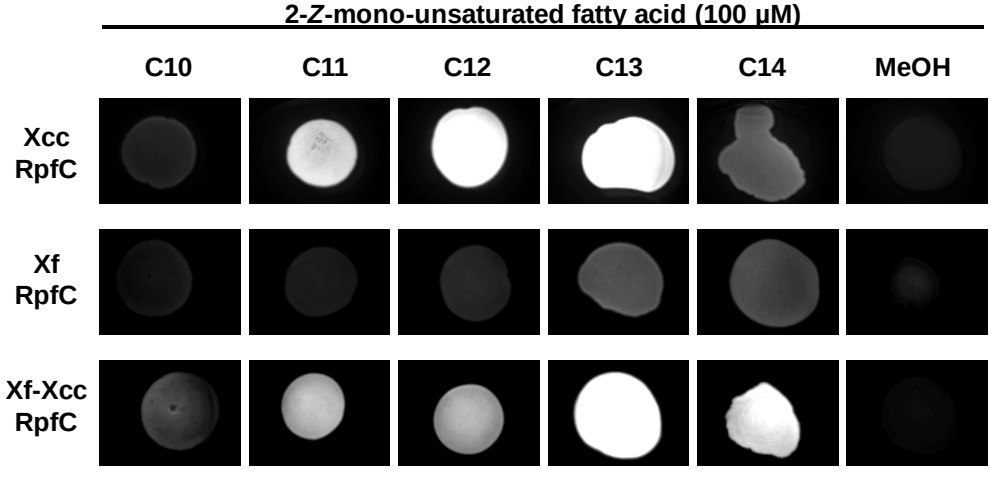


Figure 19. GFP fluorescence exhibited by the *Xcc*-based biosensors listed on each row when grown in culture media to which 100 uM of the DSF-like compounds with different acyl chain lengths shown in each column.

Quantification of the response of the three biosensors to various fatty acid molecules revealed a substantial difference in their selectivity to compounds of different chain length. The *Xf*RpfC and *Xcc*ChimericRpfC biosensors, based on the use of an *X. fastidiosa* RpfC receptor were clearly more responsive than the XccRpfC biosensor for fatty acid molecules greater than 12 Carbon atoms long (Figure 20). Given that the DSF produced by *Xcc* is 12 Carbons in length, while at least one of the molecules made by *Xf* is apparently 14 Carbons in length, it appears that the RpfC receptor has evolved to bind fatty acid signal molecules of a particular length. This also suggests that while *Xcc* may be relatively unaffected by exposure to DSF made by *X. fastidiosa*. The converse is probably not the case.

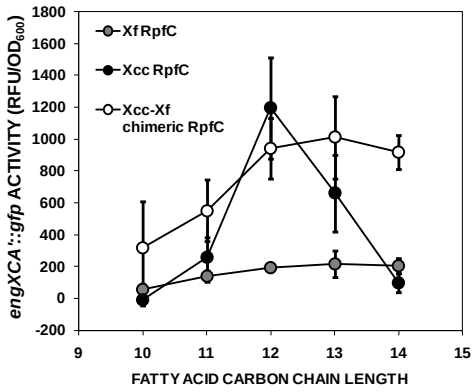


Figure 20. Relative responsiveness of three different DSF biosensors to unsaturated fatty acids of different chain lengths.

Since the various DSF sensors appeared to have high specificity and sensitivity for DSF, they appear suitable for large-scale testing of DSF molecules and transgenic plants as well as for DSF produced by antagonistic bacteria. Methods were therefore tested to ascertain the most expedient way in which these biosensors could be deployed for the detection of DSF. Nearly all bioassays using these biosensors to date have used solid media on which DSF had been placed or in which DSF had been dispersed. The application of the biosensor to the surface of the plate would then be followed by its removal at different times and subsequent assessment of GFP fluorescence. This method however is not optimal for high throughput screening of the many samples that will be needed to assess the large collection of transgenic plants for example. We therefore assessed the use of a broth culture assay using the same biosensors to detect DSF. DSF was dispersed into either phosphate buffer or into minimal culture medium such as PIM6 and the GFP fluorescence of the biosensors monitored as a function of time in a 96 well fluorescence plate reader. We were pleased to find that GFP fluorescence and hence DSF responsiveness of the biosensor could be easily monitored in such a high throughput manner (Figure 21).

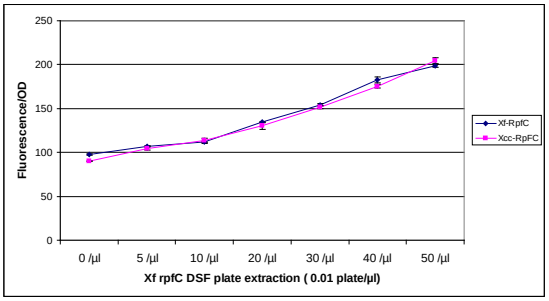
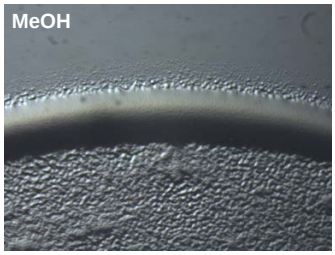
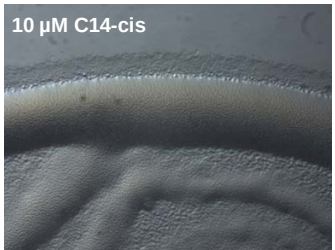


Figure 21. GFP fluorescence of two different *Xcc*-based DSF biosensors to increasing concentrations of DSF from culture extract of Xf rpfC added to cells suspended in phosphate buffer when measured after 24 hours incubation using a Biotek multi-well plate reader.





Since RpfF seems to play a dual role, synthesizing DSF and sensing DSF with RpfC it can be suggested that in an rpfF mutant RpfC cannot sense DSF. Therefore, we constructed an rpfF mutant allele that cannot produce DSF. We presume that the protein has preserved its native structure and can still interact with RpfC. The mutant with the altered RpfF was subjected to growth on PIM6 media supplemented with 10 uM C14-cis. The effect was very pronounced: DSF induced apparent EPS production that covered the culture (Figure 22). Thus, this mutant not only helps confirm our models of interaction of X. fastidiosa with DSF, but also should be valuable tool, serving as a biosensor for DSF, since it cannot produce, but can respond to DSF.

Figure 22. Appearance of colonies of an *X. fastidiosa* strain harboring a variant RpfF that cannot produce DSF when grown on PIM6 media without (top) or with added DSF (C14-cis).

Development of reporter gene systems for use in *X. fastidiosa*. The study of the response of *X. fastidiosa* to DSF as well as other studies of its response to other plant compounds etc. would be greatly enhanced by the ability to easily monitor gene expression via the use of reporter genes. Previous attempts to establish *gfp* or *inaZ*-based transcriptional fusions in *Xf* failed, presumably due to its incapability to express foreign genes properly (a phenomena though to be related to *Xf* peculiar genetic codon usage. We have now successfully developed a reporter gene system by utilizing *Xf* endogenous *phoA* gene (encoding alkaline phosphatase) as a reporter gene. *Xf phoA* was cloned under the control of *E. coli lacZ* promoter and its activity was confirmed in *E. coli*. Six different promoter::*phoA* transcriptional fusions were established, each harboring an *Xf* promoter of a gene associated with virulence (*hxfA*, *hxfB*, *pglA*, *pilB* and *rpfF*) and as a control, the 16S-rRNA gene promoter (Figure 23). Three of these constructs, cloned in the broad host range vector pBBR1MCS-5 were transformed to an *Xf phoA* mutant along with the promoterless *phoA* vector that serves here as a negative control. *Xf* PhoA relative level in these four strains (*Xf* Δ*phoA* harboring *hxfA'::phoA*, *hxfB'::phoA*, 16S-rRNA'*::phoA* or promoterless*::phoA*) which reflects promoter activity, was determined following 4 days of growth on PWG plates supplemented with 15 ug/ml gentamicin (to force maintenance of the plasmids). Activity of the promoterless*::phoA* construct was subtracted from the activity of the promoter*::phoA* fusions. As expected, induction of the 16S-rRNA promoter was always the strongest; *hxfA* promoter induction was ca. 20-fold higher than that of *hxfB* but ca. 10-folds lower than that of the 16S-rRNA promoter (Figure 24). These results suggest that the *phoA* reporter gene system will be quite valuable for assessing gene expression in vitro. Preliminary data that reveals that the PhoA-based biosensor in which *phoA* is driven by the *hxfA* promoter is quite responsive to exogenous DSF. We are currently conducting extensive tests of this *X. fastidiosa*-based biosensor to screen various compounds produced by *X. fastidiosa* to determine those that are active as DFS signaling molecules (see below)

Figure 23 Expression vector harboring *phoA* from *X. fastidiosa* that is fused to promoters from several virulence genes in *X. fastidiosa*.

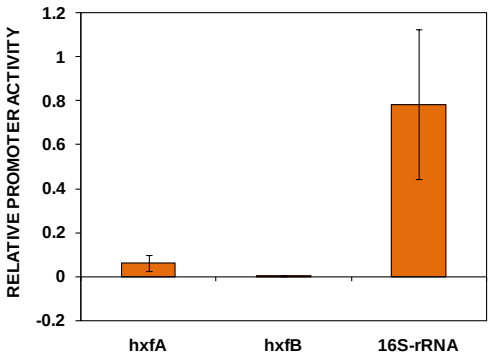


Figure 24. PhoA activity measured in *X. fastidiosa* harboring fusions of a promoterless *phoA* reporter gene with the promoters of the genes noted on the abscissa.

We have made much effort in this reporting period to developing methods to use X. fastidiosa itself to detect DSF**.** Among the several genes that we know to be most strongly regulated by DSF include *pil* genes involved in twitching motility, several genes such as *fimA* and *hxfA* and *HxfB* which are involved in cell-surface adhesion, and gum genes involved in production of EPS. We thus have examined the phenotypes of an *rpfF*- mutant and WT strain of *X. fastidiosa* exposed to different amounts of DSF to determine if it can be used to bioassay for the presence of DSF. Initial results are encouraging. An important discovery during this reporting period was that the responsiveness of any strain of X. fastidiosa, including the wild type strain, or an RpfC mutant all are much more responsive when cells are grown in a minimal medium such as PIM6. In contrast, the addition of DSF to cultures in other media, including all medium containing BSA, provides relatively little responsiveness as measured in cell adhesion to the surfaces of tubes or wells. For example, cells of the WT strain as well as *rpfF*- and rpfC- mutants which are not adherent in culture, and thus which do not form cell-cell aggregations became much more adherent to each other when DSF was added to shaken broth cultures. The increased adherence is readily visualized as an enhanced ring of cell-cell aggregates that forms at the liquid-air interface of shaken cultures (Figure 25). The attachment of *X. fastidiosa* cells to tubes or wells can be readily measured by estimating the number of attached cells by their ability to bind crystal violet. Tubes are treated with crystal violet after cells are attached and then the amount of crystal violet retained on cells is measured spectrophotometrically. A substantial increase in the number of cells, and hence of crystal violet binding, is seen in both the RpfC mutant and the wild type strain to which 3 uM of C14-cis was added to PIM6 medium (Figure 26). The amount of cells bound to the surface of tubes increased with increasing concentration of C14-cis above about 1 uM. The concentrations of C14-cis at which cell binding was increased as estimated by this assay was similar to that as measured in an *Xcc*-based bioassay (Figure 25). Cells of *X. fastidiosa* exposed to DSF in PIM6 minimal medium also appear to adhere to each other more than those in the medium with added DSF (Figure 27). Clearly, exogenous DSF stimulates an adhesive state in *X. fastidiosa.* The X. *fastidiosa*-based cell binding assay therefore appears to be a very valuable and rapid method by which DSF response in *X. fastidiosa* can be assessed. Thus monitoring the adhesiveness of cells of the wild type strain of X fastidiosa in a minimal medium appears to be a very rapid and convenient assay for its responsiveness to DSF.

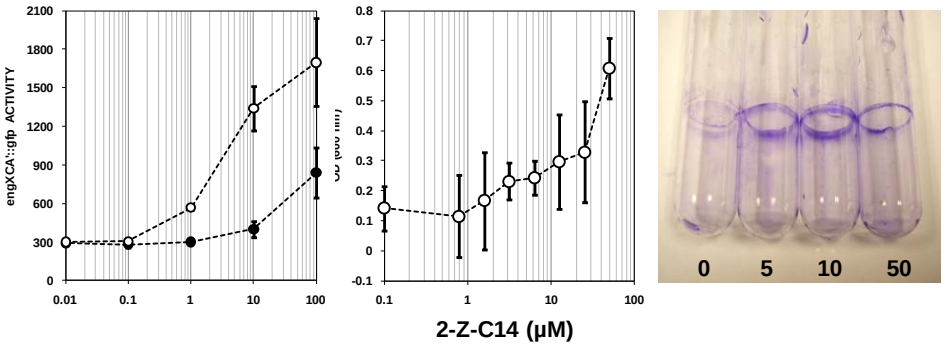


Figure 25. Binding of cells of *X. fastidiosa* to the walls of glass tubes at the air-medium interface in cells grown in PIM6 medium containing up to 50 micromolar C14-cis as visualized by crystal violet staining (right panel). The quantification of cell binding as measured by crystal violet absorption is shown in the center panel for the cells. The responsiveness (gfp fluorescence) of an *Xcc*-based biosensor grown in PIM6 medium containing up to 50 micromolar C14-cis is shown in the left panel.

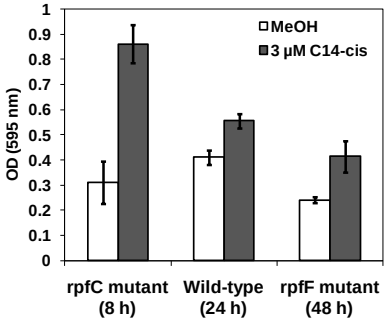


Figure 26. Attachment of cells of the RpfC mutant, wild type strain, and RpfF mutant of *X. fastidiosa* to the walls of glass tubes to which 3 uM C14-cis had been added when assayed with a crystal violet binding procedure.

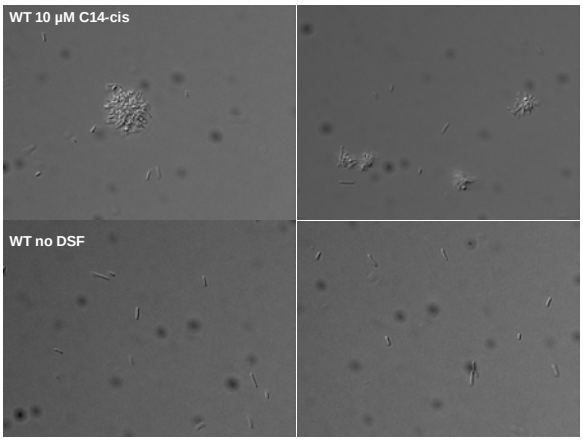


Figure 27. Microscopic depiction of cells of wild type *X. fastidiosa* grown in PIM6 medium for three hours to which 10 uM C14-cis had been added (top panels) and cells grown in PIM6 medium without added DSF (bottom). Note the cellular aggregates found in the top panels in the presence of DSF.

In addition to estimating the transcriptional expression of genes known to be regulated in response to accumulation of DSF, we are also exploring ways of measuring the amounts of gene products (proteins) or EPS that are made in response to the presence of DSF. For example, we are exploring whether we can detect EPS production by *Xf* both in culture and in plants by use of antibodies that recognize the EPS of *Xf*. Such antibodies have recently been described by the group of Bruce Kirkpatrick. Our initial results suggest that DSF-deficient RpfF- mutants of *Xf* exhibit little or no EPS production as monitored by use of fluorescently-labeled antibodies directed against EPS. A gfp-marked RpfF- strain of *Xf* could be used as a DSF detector both in culture and *in planta* by examining co-localization of constitutive GFP fluorescence and red fluorescence when a red-fluorophore-labeled anti-EPS antibody is applied to a sample; GFP fluorescent cells that were not also labeled with the antibody stain would indicate lack of DSF availability while cells that were both GFP and red fluorescent would indicate the presence of DSF.

We have also explored the use of immunofluorescence to detect other DSF-regulated proteins in *X. fastidiosa*. Antibodies to XadA were provided by Dr. Alessandra Souza who had developed this tool to detect a homologous protein in CVC strains of *X. fastidiosa*. The antibodies cross-reacted strongly to the XadA from grape strains of *X. fastidiosa*. In preliminary experiments we find that cells of

an *rpfF* mutant of *X. fastidiosa* harbor very little XadA when grown on XFM minimal medium without added DSF, but that significant amounts of XadA is detected with the antibody when DFS-containing extracts from an rpfC mutant of *X. fastidiosa* were added (Figure 28). These are very exciting results in that it suggests that such a biosensor would be very useful within plants to monitor the temporal and spatial patterns of DSF production within plants, as well as allow us to monitor the dispersal and stability of DSF that has been applied to plants, or of DSF which has been produced by transgenic plants themselves. We also observed a very curious effect of DSF on XadA; while some of this protein is cell-associated, a much lower proportion of this protein is released to the outside of the cell when cells are exposed to DSF (Figure 29). Quite importantly, the proportion of XadA that is retained by the cell is strongly influenced by the amount of DSF that the cell is exposed to. Addition of DSF from an extract of a DSF-producing strain of *X. fastidisoa* reduced its secreted portion in both the wild type and the *rpfF* mutant (Figure 29). The DSF-dependent retention of XadA is being exploited as a measure of DSF content of samples in further analysis of DSF analogs as well as further reexamination of related DSF molecules that are being made by *X. fastidiosa* and other bacteria.

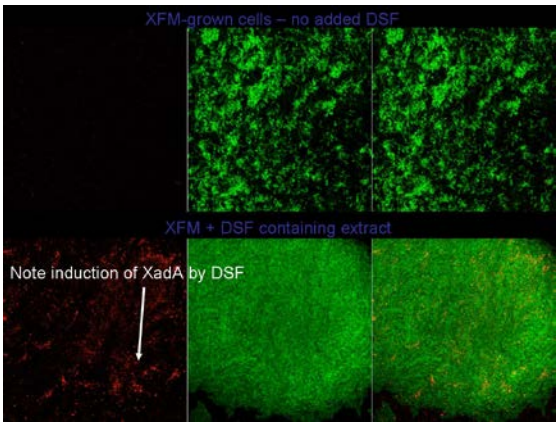


Figure 28. Cells of rpfF- mutant of *X. fastidiosa* grown on a minimal medium and then stained with Syto-9 (green) and probed with rhodamine-labeled anti-XadA antibody (red). Cells were grown in XFM minimal medium without added DSF extract (top) or with added DSF-containing cell culture extract (Bottom).

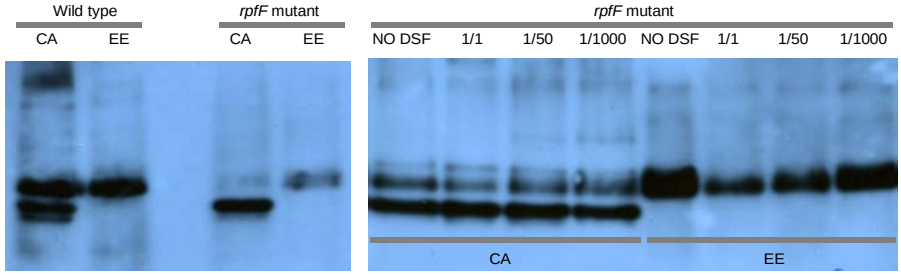


Figure 29: Left panel: Expression of XadA in wild type and rpfF mutant (CA = Cell-Associated; EE = Extracellular Environment). Right panel: Effect of DSF-crude extract (1/1 = 1 % strength [200 µl / 20 ml], 1/50 = diluted by 50 and 1/1000 = diluted by 1000) on XadA localization to the extracellular environment in *Xf rpfF* mutant. A non-specific band appears under the XadA band and can be seen only in the CA samples.

The phenomenon by which XadA is secreted from cells of X fastidiosa has been investigated in more detail. One question that has been addressed is whether XadA is secreted as a naked protein or whether it is released from cells as part of a vesicle. Preliminary data suggests that XadA is found in very small particles or as single molecule since it cannot be pelleted by such vacation that on hundred and 50,000 G for several hours. We are exploring the possibility that XadA serves as an anti-adhesive factor. We had observed that blue green sharpshooter vectors feeding on plants infected with an RpfF mutant were unable to acquire or transmit a wild type strain of *X. fastidiosa*. Likewise, sharpshooters fed on xylem sap from plants infected with the RpfF mutant were unable to acquire *X. fastidiosa in vitro*. The lack of acquisition and transmission of *X. fastidiosa* by blue-green sharpshooters was associated with a reduced ability of cells of *X. fastidiosa* to adhere to the surface of the insect as measured by attachment to wings or by retention in the mouth parts of the vectors fed cells of wild type *X. fastidiosa* suspended in sap from plants infected with the RpfF mutant. We can detect substantial amounts of XadA in the sap from plants infected with the RpfF mutant. The hypothesis that we are investigating is that XadA, being an adhesive protein that is released from cells, attaches to surfaces in the vicinity of *X. fastidiosa*. Thus once colonizing the plant and when relatively low cell concentrations when DSF concentrations are apparently low in the plant, XadA would be released from the cell and thus free to attached to the walls and pit membranes of plants. In so doing, XadA may coat the surface of plants preventing the attachment of *X. fastidiosa* itself. Presumably, as cell density increases and DSF concentrations also increase, the accumulated DSF would tend to suppress the secretion of XadA, thereby reducing the protective coating of plant surfaces as well as increasing the presence of this adhesive protein on the surface of cells where they could then mediate attachment to other surfaces such as those of insect vectors. XadA therefore may play two important roles in the lifestyle of *X. fastidiosa*: firstly, it may facilitate movement to the plant by reducing the propensity of *X. fastidiosa* to sticks to cells of the plant, and secondly by promoting adhesiveness of cells at the time transmission to new plants by sharpshooter vectors. Demonstration of this role of XadA may provide a new strategy of disease control based on blockage of transmission of *X. fastidiosa* from infected plants to healthy plants. Conceivably, it should be possible to express XadA in transgenic plants constitutively. Such plants need only to be plants other than grape on which sharpshooter vectors feed and thereby acquire XadA. Such “trap plants" might be dispersed within grape vineyards. Sharpshooter vectors would be expected to feed on such plants and, in so doing, coat their mouthparts with XadA, preventing them

from acquiring *X. fastidiosa* from any infected plants on which they might feed. Further work is being performed on XadA to determine whether this strategy of disease control is feasible.

Identification of additional DSF molecules. Since we were successful in developing Xcc-based DSF biosensors that are much more responsive to the DSF produced by *Xf* (compared to the original DSF biosensor that was much more responsive to *Xcc* DSF than to *Xf* DSF) as well as determined in vitro assays of monitoring the effects of DSF on *X. fastidiosa* itself (eg. cell adhesion assay) we have continued studies to determine the structures of the various molecules made by *X.f astidiosa* that are involved in rpfF-mediated signaling. It is clear that *X. fastidiosa* produces C14-cis and that this molecule is active in regulating the behavior of *X. fastdiosa*. As shown in Figure 7, preliminary evidence has been obtained, that like *Xcc*, *X. fastidiosa* may produce more than one related signal molecule. That is, DSF may not be a single molecule, but instead may be a family of closely related molecules. We expect that the molecules will be closely related to each other structurally, but they might have different effects on the cell. The *Xf*-RpfC sensor was used to quantify biologically active fractions of crude extracts of *Xf* cultures of a wild type strain (500-series of fractions) and from an *Xf rpfC* mutant (900-series of fractions) that were separated on the basis of polarity by HPLC. Fractions No. 504 and 901 (not assayed here) had previously been shown to contain C14-cis and thus perhaps other fatty acids and therefore, were sub-fractionated here. In both extracts, a more polar fraction (fractions 507 and 905) was found to contain an active compound as assayed by the *Xf-Xcc* RpfC biosensor. Fraction 507 was further fractionated and its sub-fraction 2.2 was found to be active (Figure 30). Thus more than one molecule besides C14-cis with biological activity appears to be active in both extracts. Work is underway to chemically characterize these additional biologically active compounds. Larger amounts of crude DSF containing culture filtrate are being further fractionated and exposed to several different bioassays for DSF presence. DSF activity is being measured in three different *Xcc*-based bioassays as well has the *hxfA:phoA* based *X. fastidiosa* biosensor as well as the *X. fastidiosa* wild type cell adhesion assay. We expect that the presence of DSF in more polar fractions will be soon elucidated.

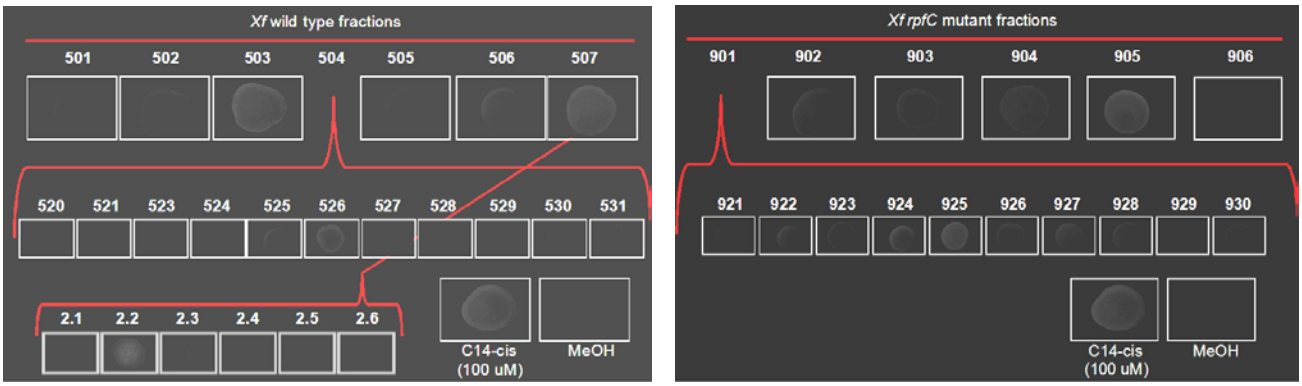


Figure 30. GFP fluorescence detected in various fractions of an ethyl acetate extract of a WT *Xf* culture (left panel) or of a culture of an RpfC mutant (right panel). Sub-fractionation of active fractions is noted with the brackets.

The new biological sensors also allow us to identify potential antagonists of signaling in *X. fastidiosa*. During the characterization of the new *Xf*-specific DSF-biosensors two DSF-like molecules were found to interfere with the sensors ability to induce the DSF-dependent *engXCA'::gfp* transcriptional fusion. The first is a version of the agonistic DSF compounds (C14-*cis*), in which the *cis* double bond is located between C4 and C5 rather than between C2 and C3. Exposure of the Chimeric sensor (but not of the Xf *rpfC* sensor) to a mixture of the two C14-*cis* compounds, reduced the *gfp* activity normally observed in the presence of the agonist alone (Figure 31 A and B). We studied this phenomenon in two different agonist concentrations (100 and 10 µM) and several agonist:antagonist molar ratios. Interestingly, strong inhibition of the *engXCA'::gfp* activity was observed only when the agonist was provided in a concentration of 10 µM, despite the similar agonist:antagonist ratio. The second compound is a saturated C14 with a branched methyl on C13. The molecule exerted the same biological activity, but now on both sensors (Figure 31 C). Both antagonistic compounds did not affect the activity of the sensors when applied alone. These particular antagonists thus appear to be relatively weak antagonists, but other, stronger antagonists might well be found. The fact that antagonists of the DSF signaling system in *X. fastidiosa* can be found spurs us to examine plant compounds and other natural products to determine to what extent the behavior of *X. fastidiosa* is influenced by the chemical environment in which it is found.

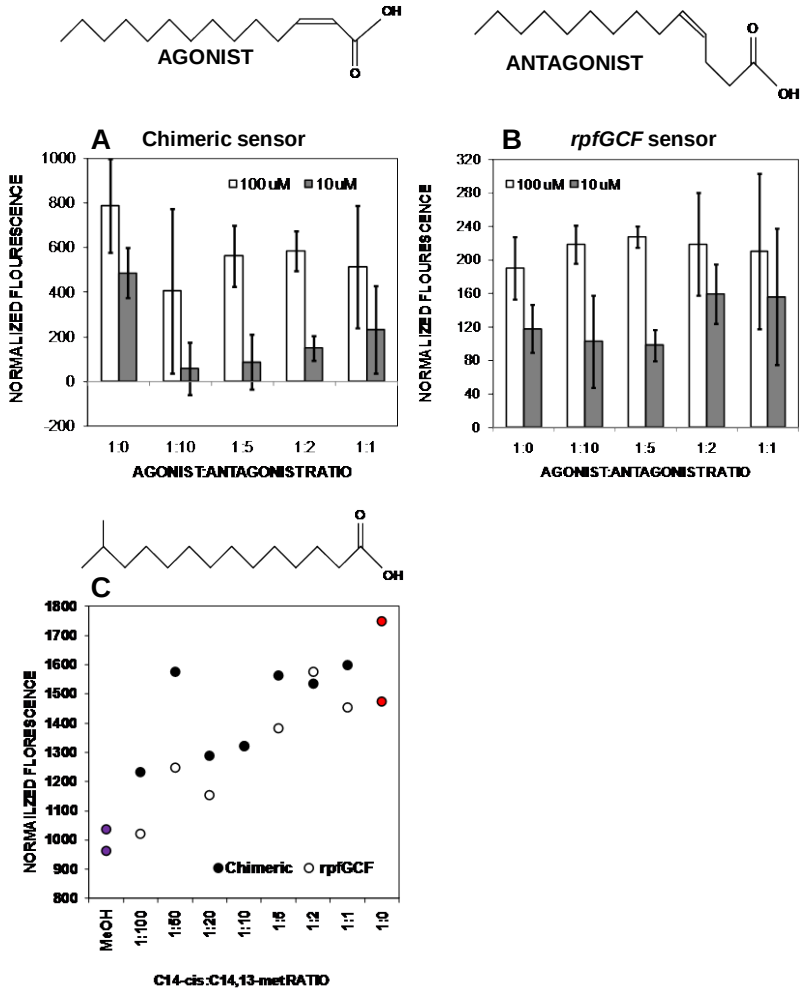


Figure 31. Activity of the *engXCA'::gfp* fusion in response to various mixtures of two C14-*cis* molecules differ only in the position of their *cis* double bond (A and B). Activity of the same sensors in response to a mixture of 10 µM C14-*cis* with various concentrations of C14,13-met (C). Red dots are activities in response to the agonistic 14-*cis* only; Purple dots are the background activity without any of the compounds. C

Reassessing the signal transduction pathway leading to cell-cell communication in *X. fastidiosa*

Based on studies in *Xcc*, signaling through the Rpf system is suggested to be a linear process in which RpfF produces DSF, which is sensed by RpfC which in turn phosphorylates RpfG. Activated RpfG, hydrolyzes cyclic di-GMP to 2 GMP molecules. In *Xcc*, cyclic di-GMP was reported to bind to Clp, a transcription regulator; when Clp is associated with cyclic di-GMP, it was shown to be dissociated from its operator (Clp box) and vice versa.

In *X. fastidiosa*, an *rpfF* mutant is hyper virulent to grape while a *rpfC* mutant is hypo virulent (Figure 32 A and B). This observation, supported also by these mutants opposing biofilm forming capabilities and gene expression profiles, suggests that the signaling cascade in *X. fastidiosa* might be non-linear. An *rpfG* mutant was found to be hyper-virulent, much like the *rpfF* mutant (Figure 32) suggesting that either DSF regulates RpfG activity through a different pathway (e.g. a hypothetical cytoplasmic DSF sensor) or that RpfC interacts with more than a single response regulator. From that it seems that high level of cyclic-di GMP is associated in *X. fastdiosa* with increased virulence. An *X. fastidiosa clp* mutant was found to have characteristics similar to those of an *rpfC* mutant. It is hypo-virulent and forms a massive biofilm. Given that virulence is dependent upon both cyclic di-GMP and its receptor Clp, it can be suggested that in contrast to *Xcc*, *X. fastdiosa* Clp is associated with promoters when cyclic di-GMP is bound to it (as occurs in *E. coli*, where its homologue Crp interacts with cAMP to allow DNA binding). In addition to the plant colonization process, Clp was found to be essential for plant to plant transmission mediated by the insect vector (Figure 32 C). Its importance to the life cycle of *Xylella* has driven us to characterize its regulon, a knowledge we hope will shed light on new genes which are involved in virulence and vector transmission.

Virulence (given as the averaged number of symptomatic leaves per plant) was found to coincide (compare Figure 32 A with B) with bacterial population found in the xylem vessel (50 cm away from the point of inoculation). Like the *rpfF* mutant, the *rpfG* mutant was found to be less transmissible by the insect vector (Figure 32 C) and it was also attenuated in its capacity to colonize the insect vector mouth parts (Figure 32 D).

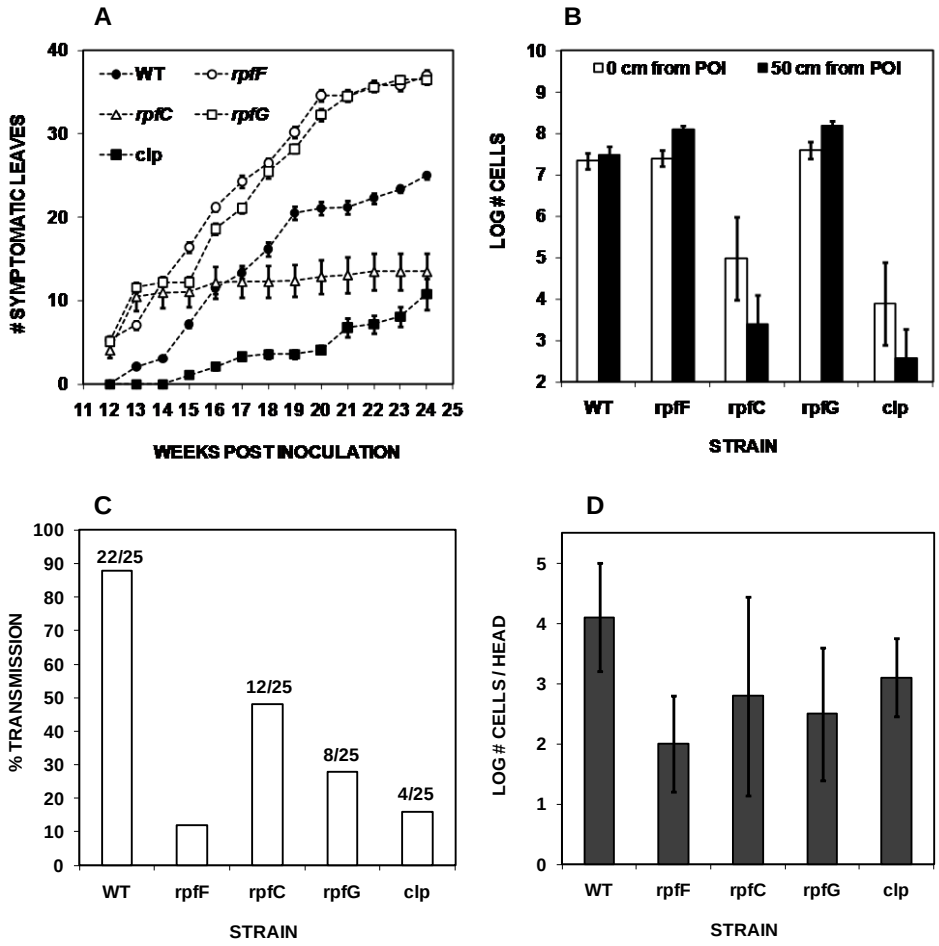


Figure 32. (A) Disease progress as reflected by symptomatic leaves accumulation with time on grape inoculated with various mutants of *X. fastidiosa*, (B) *Xf* population within xylem vessels 25 weeks post inoculation in the point of inoculation and 50 cm away, (C) Insect vector transmission rate of the various mutants and, (D) bacterial population within the colonized insect heads.

Conclusions:

Since we have shown that DSF accumulation within plants is a major signal used by *X. fastdiosa* to change its gene expression patterns and since DFS-mediated changes all lead to a reduction in virulence in this pathogen we have shown proof of principle that disease control can be achieved by a process of "pathogen confusion". Several methods of altering DSF levels in plants, including direct introduction of DSF producing bacteria into plants, and direct application of DSF itself to plants appear promising as means to reduce Pierce's disease. Transgenic DSF-producing plants appear particularly promising and studies indicate that such plants provide at least partial protection when serving as a rootstock instead of a scion. Based on work done on other plant species in which a chloroplast-targeted DFS synthase has provided much higher levels of DSF production, we are hopeful that such a construct in grape will provide even higher levels of Pierce's disease control in our current studies. While the principle of disease control by altering DSF levels has been demonstrated, this work addresses the feasibility of how achieve this goal, and what are the most practical means to achieve disease control by pathogen confusion. Our continuing work will address which method is both most practical and efficacious. The tools we have developed to better detect the specific DSF molecules made by *X. fastidiosa* will be very useful in our on-going research to test the most efficacious and practical means to alter DSF levels in plants to achieve disease control. We are still optimistic that chemically synthesized DSF molecules might also ultimately be the most useful strategy for controlling disease. The presence of more than one DSF base signal molecule suggests that perhaps more than one molecule might be needed to achieve changes in pathogen behavior. Our major advances in the development of biosensors to detect the responsiveness of *X. fastidiosa* to signal molecules is a major breakthrough that hopefully will allow us to make rapid progress in ascertaining those transgenic plants most capable of altering pathogen behavior as well as in formulating synthetic molecules suitable for use in disease control. The biological sensors also have proven useful in screening naturally occurring bacteria associated with grape that might also be exploited to produce signal molecules

Funding Agencies:

Funding was provided by the Pierce's Disease and Glassy-winged Sharpshooter Board.