Interim Progress Report for CDFA Agreement Number 12-0224-SA

Elucidating process of cell-cell communication in *Xylella fastidiosa* to achieve Pierce's disease control by pathogen confusion

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Time period covered by report: July 1, 2012 – July 10, 2013

Introduction:

Our work has shown that *Xylella fastidiosa (Xf)* uses DSF perception as a key trigger to change its behavior within plants. Under most conditions DSF levels in plants are low since cells are found in relatively small clusters, and hence they do not express adhesins that would hinder their movement through the plant (but which are required for vector acquisition) but actively express extracellular enzymes and retractile pili needed for movement through the plant. Disease control can be conferred by elevating DSF levels in grape to "trick" the pathogen into transitioning into the non-mobile form that is normally found only in highly colonized vessels. While we have demonstrated the principles of disease control by so-called "pathogen confusion", more work is needed to understand how best to alter DSF levels in plants to achieve even higher levels of disease control. Until now we have suffered from a lack of sensitive methods to detect DSF levels in plants (the *Xanthomonas*-based bioassay we have used previously is relatively insensitive to the chemically distinct forms of DSF produced by *Xf*). That is, while we showed that DSF-producing endophytes, direct application of DSF, and transgenic plants producing DSF all conferred some resistance to disease, we had no way to know why they were not more resistant, nor what would be needed to improve control measures since we could not measure the direct effect of our efforts to increase DSF levels in plants. However, in the last 2 years we have developed several new sensitive biosensors that enable us to measure *Xf* DSF both in culture and within plants. We have recently found that *Xf* apparently produces more than one molecule that can act as a DSF signal molecule and that the molecules made by *Xf* are dependent on its growth environment. We thus need to ascertain which form is most active, whether the various forms all have the same effect on regulating traits in *Xf,* and what are their fates when applied to plants in various ways. We also know that related molecules can interfere with the signaling process and thus plant susceptibility may be influenced by the suite of compounds normally present in their xylem sap. Thus the overall goals of the proposed work

is to use these new biosensors for DSF to examine how DSF levels can best be altered by the various methods we have previously identified. As disease control should be directly proportional to both the concentration of and dispersal of DSF within plants we will quantitatively explore the effectiveness of different strategies to elevate DSF levels throughout plants.

We also have made the discovery that *Xf* produces abundant extracellular membranous vesicles which are shed from the cell. Importantly, the content of outer membrane proteins including the adhesion XadA are controlled by DSF accumulation in cultures of *Xf*, and even more importantly, the shedding of these vesicles from the cell is apparently suppressed by the accumulation of DSF. We therefore are testing the model that DSF signaling in Xf involves two very different processes both of which lead to a rapid, cell density dependent change in its adhesiveness. When cells of *Xf* are found in relatively low numbers within the xylem vessel they have accumulated little DSF, and because of this they do not produce large amounts of the cell surface adhesins including XadA, HxfA, and others, yet such cells shed large numbers of vesicles. However, when cell density, and thus DSF concentrations increase membrane vesicles are not shed by the cell, and the higher concentration of afimbral adhesins would be retained on the surface of the *Xf* cells rather than being fed into the environment. Such a process would tend to maximize the adhesiveness of *Xf* when DSF levels increased. This increased adhesiveness is apparently needed for their acquisition by insect vectors but would be expected to suppress their ability to move in the plant. In contrast, the shedding of vesicles would tend to prevent access from adhering to surfaces because most cell surface adhesins would no longer be attached to the cell. In this project, we are testing the role of the membranous vesicles, since preliminary data revealed that they may interfere with the adherence of *Xf* to surfaces. That is, by shedding adhesive vesicles which themselves adhere to the surface of plants, access may prevent its own adherence to such surfaces because they are now coded would such vesicles.

Specific objectives:

1) Identify additional DSF molecules made by *Xf* that contribute to cell-cell signaling and determine their movement and stability when applied to plants in various ways to improve disease control.

2) Determine the contribution of membrane vesicles shed by *Xf* in the absence of DSF to its virulence and the utility of measurement of vesicular presence within plants as a sensitive means to assess the success of strategies of disease control by pathogen confusion.

Description of activities conducted to accomplish each objective and summary of accomplishments:

Objective 1: Finding new DSF species. We have optimized methods to use *Xf* itself to detect DSF**.** Among the several genes that we know to be most strongly regulated by DSF are genes such as *hxfA* and *HxfB* which are involved in cell-surface adhesion. Previous attempts to establish *gfp* or *inaZ*-based transcriptional fusions in *Xf* failed, presumably due to its incapability to express foreign genes properly. We now have successfully used the endogenous *phoA* gene (encoding alkaline phosphatase) as a bioreporter of gene expression in *Xf*. The PhoA-based biosensor in which *phoA* is driven by the *hxfA* promoter is quite responsive to exogenous DSF from extracts of *Xf* cultures as well as C14-cis itself. This biosensor, which we will refer to as *Xf:phoA* has been used extensively as it provides direct measures of the responsiveness of *Xf* to different molecules.

Assay of *Xf* extracts by *Xf* DSF-specific biosensors provide evidence of more than one *Xf* DSF molecule. The use of a Xanthomonas-based GCF-gfp biosensor and *Xf:pho* reveals that *Xf* produces at least 3 different compounds with DSF signaling activity. *Xf* appears to be more responsive to enoic acids of longer chain length than those produced by *Xcc*. While the

Xcc biosensor pKLN55 was maximally responsive to enoic acids of 12 Carbon in length (the size of each of the three DSF species made by *Xcc*) the GCF-gfp biosensor was maximally responsive to enoic acids of 13 and 14 Carbons in length. It thus would be expected that the various DSF molecules made by Xf would tend to be longer than those made by Xcc. We have recently published data showing that 2-Z-tetradecenoic acid (hereafter called C14-cis) is produced by *Xf*. We also have obtained circumstantial evidence that it also produces C12-cis. The relative activity of C12-cis in *Xf* is apparently somewhat less than that of C14-cis that we have more extensively studied. Importantly, an unidentified DSF species, presumably another enoic acid, is apparently much more active than either C12-cis or C14-cis. The amount of DSF, and apparently also the composition of species comprising the DSF signaling molecules are apparently strongly influenced by the environment in which *Xf* grows. A very high yield of DSF signaling molecules as detected with GCF-gfp was obtained in *Xf* cultures grown in PD3 medium compared to PWG: the signaling activity of cells *Xf:phoA* grown in PD3 as detected by the GCF-gfp biosensor was MUCH higher than that that in PWG media. Chemical analysis of these extracts revealed the presence of only very small amounts of either C14-cis or C12-cis, indicating that the very high signaling activity is due to a new compound (Figure 1). That is, induction of alkaline phosphatase activity in the *Xf:phoA* bioreporter was detected in culture extracts that contained as little as about 2 nM C14-cis, indicating that another molecular species must account for the induction of the biosensor since at least 500nM C14-cis is needed to induce this biosensor.

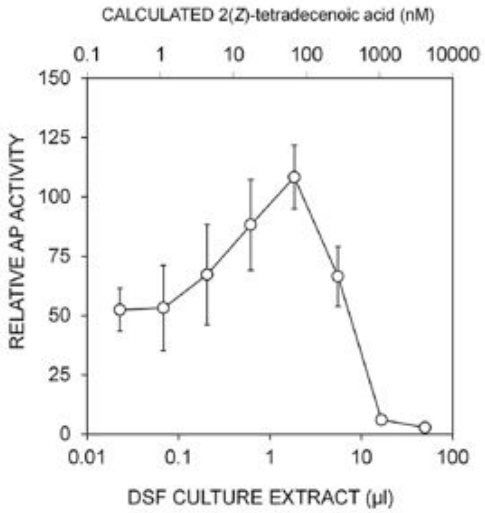


Figure 1. Alkaline phosphatase activity exhibited by Xf:phoA incubated in PD3 broth containing increasing amounts of DSF containing culture extract of PD three plate cultures of a wild type X.fastidiosa strain. The concentration of C14-cis (XfDSF1) in the extract determined by HPLC is shown by the upper X axis.

A similarly strong effect of the growth environment of *Xanthomonas* on the species of DSF molecules made has also been observed, and the DSF species originally described in *Xcc* is uncommon in some culture media. Thus our prior work on identification of DSF species in *Xf*, which has routinely relied on cultures in PWG may have inadvertently prevented us from observing a highly active DSF species present in other culture media, and presumably in plants. Chemical separation of DSF-containing chemical species from extracts of *Xf* cultures on the basis of polarity has also revealed the presence of fractions with DSF signaling activity as

detected by both GCF-gfp and *Xf:phoA* that do not correspond to those containing either C14-cis or C12-cis. Our analysis of the material collected by HPLC from these cultures using electro-spray MS revealed it to be an unsaturated C16 fatty acid which we presume to have a structure similar to that of C14-cis. We therefore chemically synthesized this presumptive derivative which we will call C16-cis or *Xf* DSF2 (Figure 2).

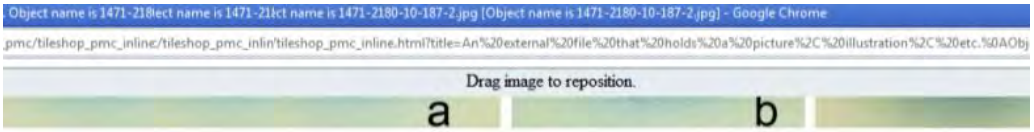
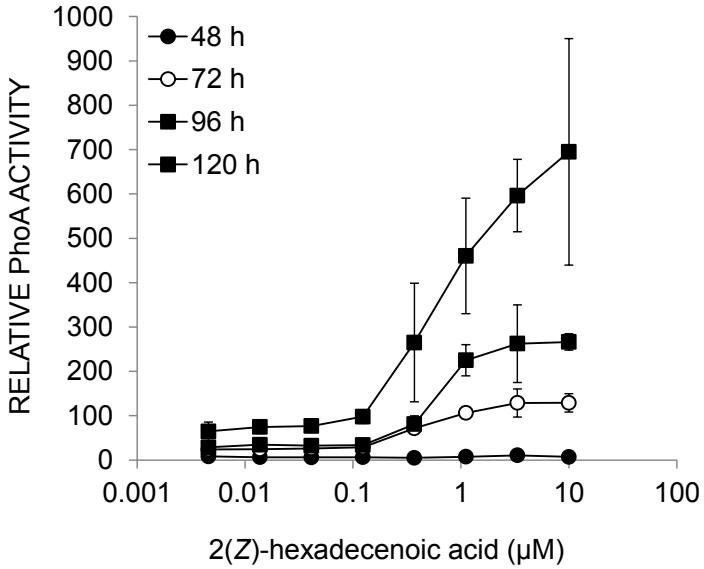


Figure 2. Structure of C16-cis (*Xf* DSF2)

The biological activity of the synthetic C16-cis was tested by the addition of this material at various concentrations to a rpfF* mutant strain of *Xf* harboring the hxfA*:phoA* reporter gene fusion and grown in PD3 medium. The rpfF* mutant is unable to synthesize DSF due to two mutations introduced into the catalytic site of the DSF synthase, yet this mutant is still able to respond to exogenous DSF. Importantly, this *Xf:phoA* biosensor exhibited very high alkaline phosphatase activity upon the addition of as little as 100 nM C16-cis in all experiments (Figure 3).



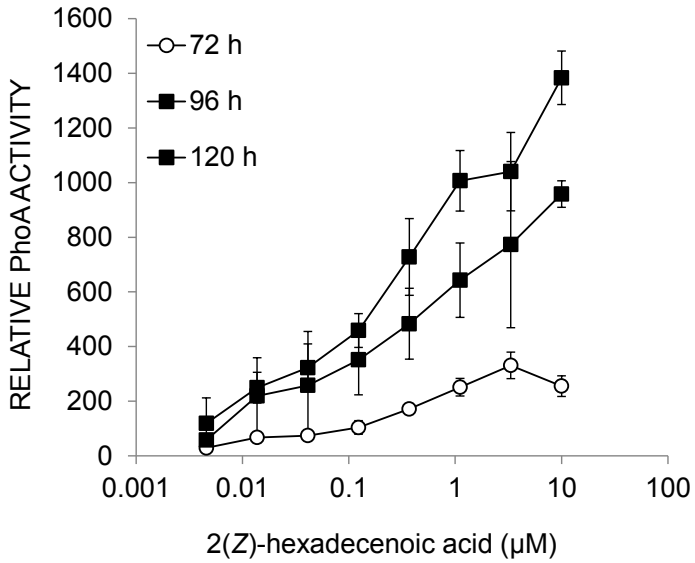


Figure 3. Alkaline phosphatase activity exhibited by the *Xf:phoA* DSF biosensor when grown in PD3 containing various amounts of C16-cis shown on the abscissa when measured at various times after inoculation in two different experiments.

Xf appears to be MUCH more responsive to C16-cis then to other DSF species such as C14-cis (*Xf*DSF1) or C12-cis (BDSF). The biological activity of the synthetic C16-cis (*Xf*DSF2) was compared with that of C14-cis (*Xf*DSF1) or C12-cis (BDSF) by the addition of this material at various concentrations to a rpfF* mutant strain of *Xf* harboring the hxfA*:phoA* reporter gene fusion and grown in PD3 medium. The alkaline phosphatase activity of this bioreporter was more than 10 times higher in cultures grown with *Xf*DSF2 than with either *Xf*DSF1 or BDSF (Figure 4). This is a very exciting results instead appears that molecules such as *Xf*DSF2 are much more active than our previously discovered *Xf*DSF1.

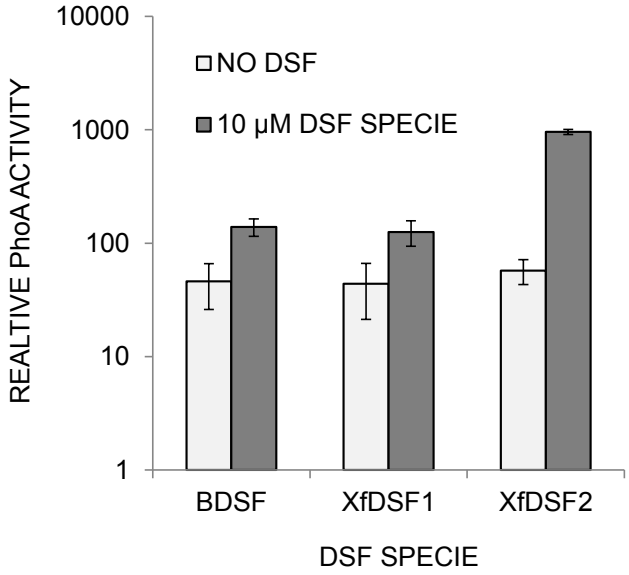


Figure 4. Alkaline phosphatase activity exhibited by the Xf:phoA DSF biosensor when grown in PD3 containing 10 µM of BDSF, *Xf*DSF1, or *Xf*DSF2 as shown on the abscissa. Light bars indicate the relatively low amounts of alkaline phosphatase activity exhibited in cultures lacking

any added DSF species while the dark bars reflect the alkaline phosphatase activity in the presence of the indicated DSF species.

Not only was *Xf* responsive to very low concentrations of added *Xf*DSF2, but more importantly, the expression of DSF-responsive genes such as *hxfA* was as much as 10 fold greater than when cells were exposed to the same concentration of either BDSF or *Xf*DSF1 (Figure 5). Thus not only does *Xf* appear to make different DSF species when grown in different environments, but our finding of extraordinarily high biological activity of *Xf*DSF2 relative to that of *Xf*DSF1 which we had previously studied extensively, suggests that *Xf*DSF2 might have great utility in altering the behavior of *Xf* when applied directly to plants. We are currently preparing plants for topical application of *Xf*DSF2 to evaluate its efficacy in the control of Pierce's disease.

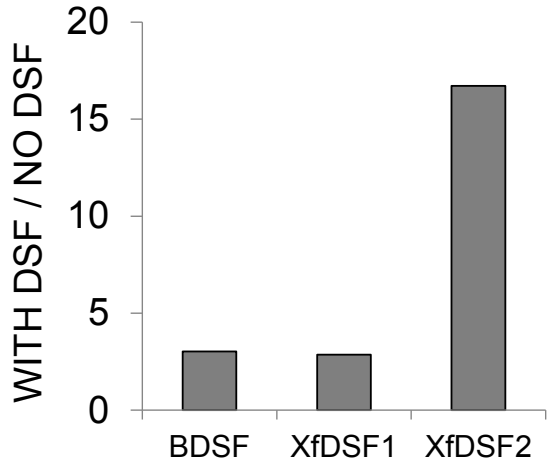


Figure 5: Ratio of alkaline phosphatase activity exhibited by an Xf-DSF biosensor when grown in PD3 containing 10 µM of BDSF, XfDSF1, or XfDSF2s compared to alkaline phosphatase activity in medium without added DSF.

To better understand the chemical species of DSF produced by *Xf* in various conditions in culture and when in infected grape plants, we examined ethyl acetate extracts of chemical species obtained from both culture and *in planta* conditions using electrospray ionization mass spectrometry (ESI-MS). It was possible to identify all of the potential DSF species which we observed in *Xf* as well as those observed by others in other bacterial species such as *Xanthomonas* species based on their highly precise and distinctive ratio of mass to charge (Figure 6). The structure of these different molecular species as shown in Figure 7.

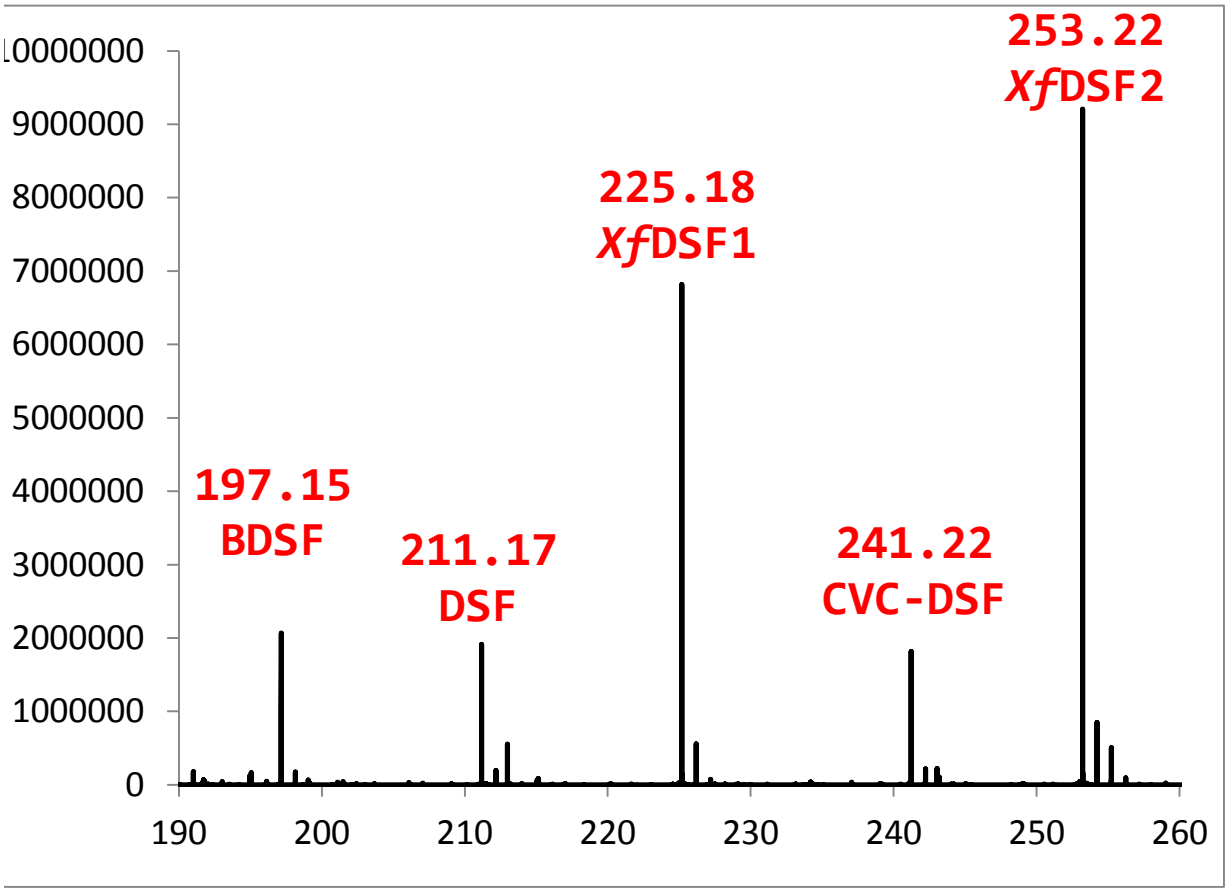


Figure 6. M/Z for ionized DSF molecular species determined by ESI-MS

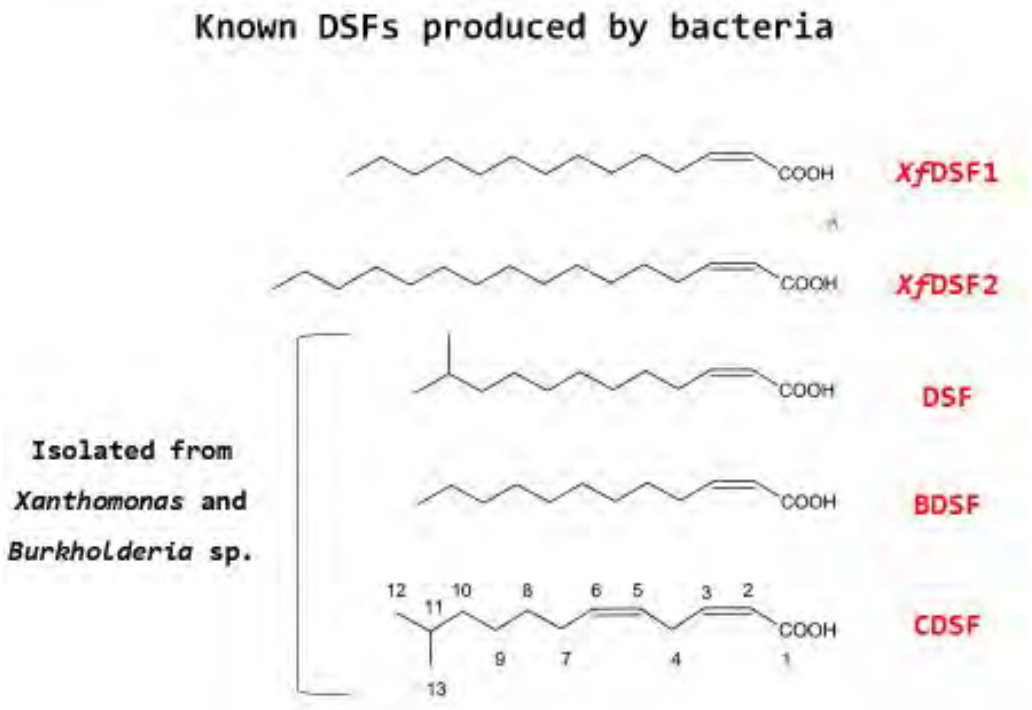


Figure 7. chemical species of DSF produced by various bacteria including *Xf*DSF1 and *Xf*DSF2 produced by *Xylella fastidiosa*.

Using ESI-MS we could detect both *Xf*DSF1 and *Xf*DSF2 in culture supernatants of XF grown in PD3 media (Figures 8 and 9). Given that *Xf*DSF2 had never been detected in PWG medium, these results confirm that the production of DSF by *Xf* is rather plastic and somewhat dependent on the growth environment of cells.

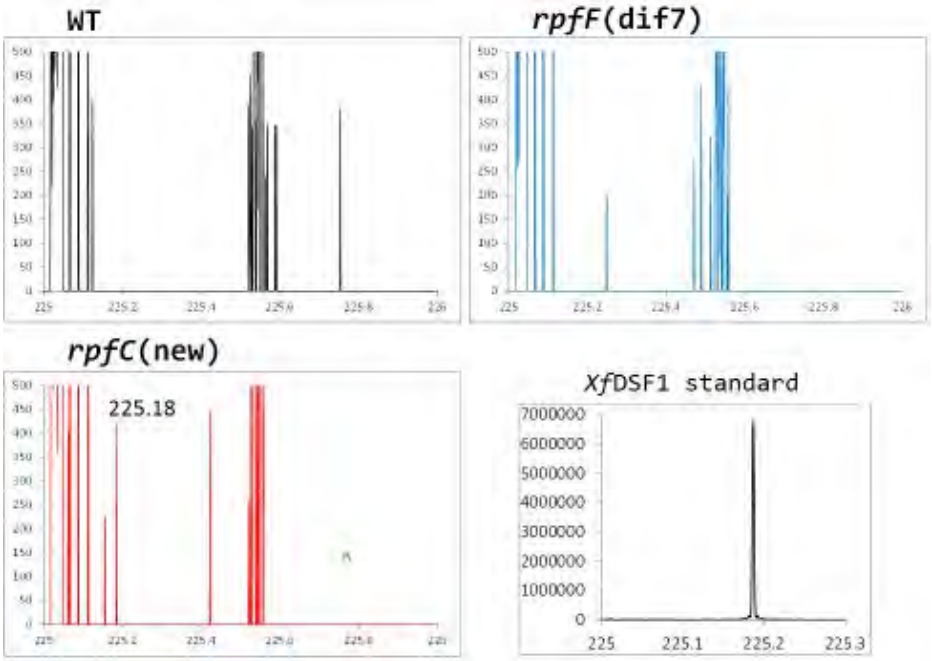


Figure 8. Abundance of ions with the M/Z ratios shown on the abscissa or culture extracts of a wild type strain of *Xylella fastidiosa* (upper left panel), of an RpfF mutant unable to produce DSF (upper right panel), of an RpfC mutant which overproduces DSF (lower left panel), as well as a standard of *Xf*DSF1 (lower right panel). Note that *Xf*DSF1 has a M/Z ratio of 225.18.

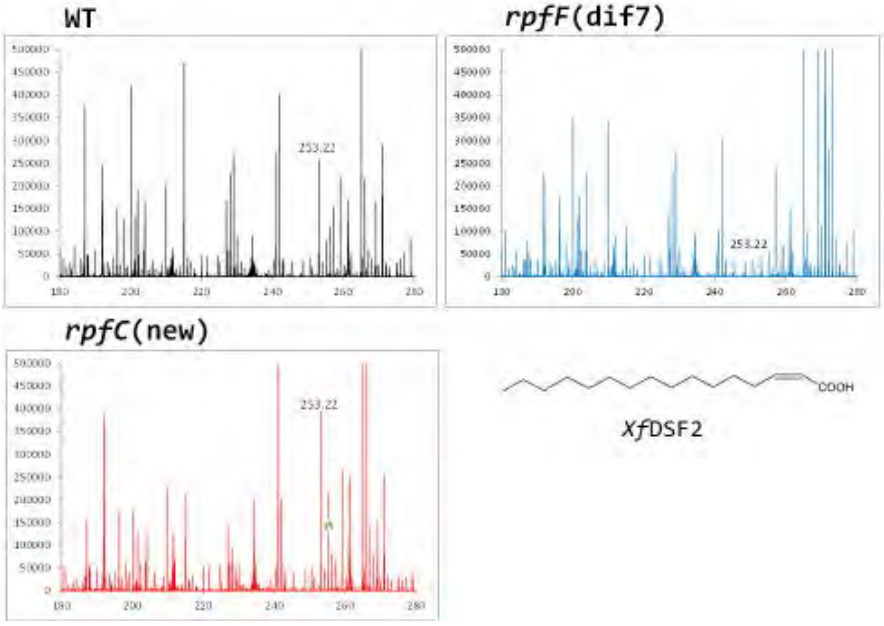


Figure 9. Abundance of ions with the M/Z ratios shown on the abscissa or culture extracts of a wild type strain of *Xylella fastidiosa* (upper left panel), of an RpfF mutant unable to produce DSF (upper right panel), of an RpfC mutant which overproduces DSF (lower left panel). Note that *Xf*DSF2 has a M/Z ratio of 253.22.

Surprisingly, we also detected small amounts of a molecule which had previously been described as the DSF from CVC strains of *Xylella fastidiosa* (which we term CVCDSF) (Figure 10). While our identification of this molecule is tentative, it is noteworthy that it was found only in wild type and a RpfC mutant of *Xf* but not in an RpfF mutant (Figure 10).

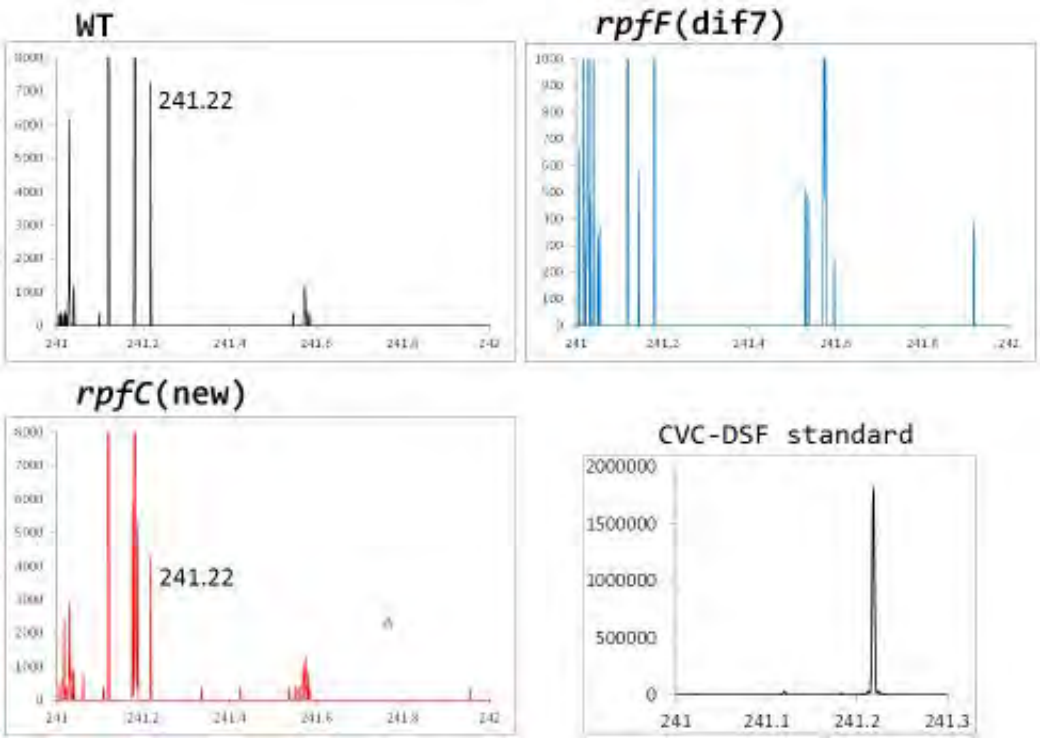


Figure 10. Abundance of ions with the M/Z ratios shown on the abscissa or culture extracts of a wild type strain of Xylella fastidiosa (upper left panel), of an RpfF mutant unable to produce DSF (upper right panel), of an RpfC mutant which overproduces DSF (lower left panel), as well as a standard of XfDSF1 (lower right panel). Note that CVCDSF1 has a M/Z ratio of 241.22.

To address the DSF species that *Xf* would produce under natural conditions such as within the xylem of grape plants, we extracted DSF-like molecules from plants infected with a wild type strain as well as from an RpfF mutant as well as from healthy plants and subjected these chemicals to identification of separation by ESI-MS. We could easily detect C14-cis (*Xf*DSF1) in plants infected with the wild type strain of XF but this DSF species was not present in either plants infected with the RpfF mutant of *Xf* or in healthy plants (Figure 11). We also could detect elevated concentrations of a molecule presume to be *Xf*DSF2 in plants infected with the wild type *Xf* strain (Figure 12). While smaller amounts of this molecule having a M/Z ratio of 253.22 were found in healthy plants and plants infected with the RpfF mutant of *Xf* (Figure 12), it seems likely that these lesser amounts in these negative control plants was associated with 16:1 unsaturated fatty acids found in plant lipids, and not with *Xf*DSF2 itself. Further characterization

of the DSF species found in plants infected with the wild type strain of XF are proceeding to identify other DSF species that are produced under these natural conditions. We find it exciting however that *Xf*DSF2 can be detected in infected plants, suggesting that this highly active DSF species is biologically relevant and may contribute to much of the cell density dependent behavior of *Xf*. For this reason, we are continuing studies to alter the abundance of *Xf*DSF2 in plants by topical applications, injections, and soil drenches.

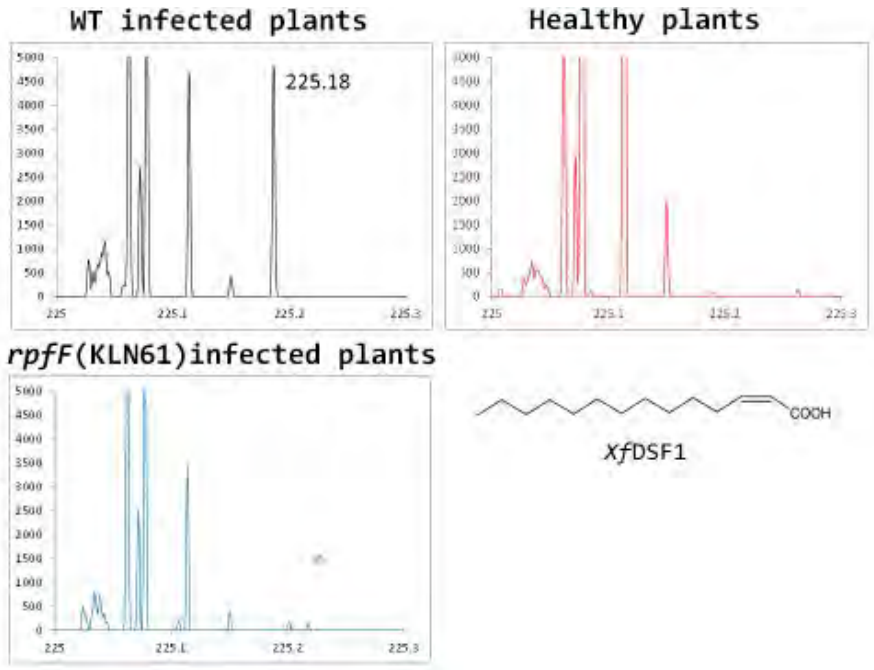


Figure 11. Abundance of ions with the M/Z ratios shown on the abscissa in extracts of plants infected with a wild type strain of *Xylella fastidiosa* (upper left panel), infected with a RpfF mutant unable to produce DSF (lower left panel), or from a healthy plant (upper right panel). Note that *Xf*DSF1 has a M/Z ratio of 225.18.

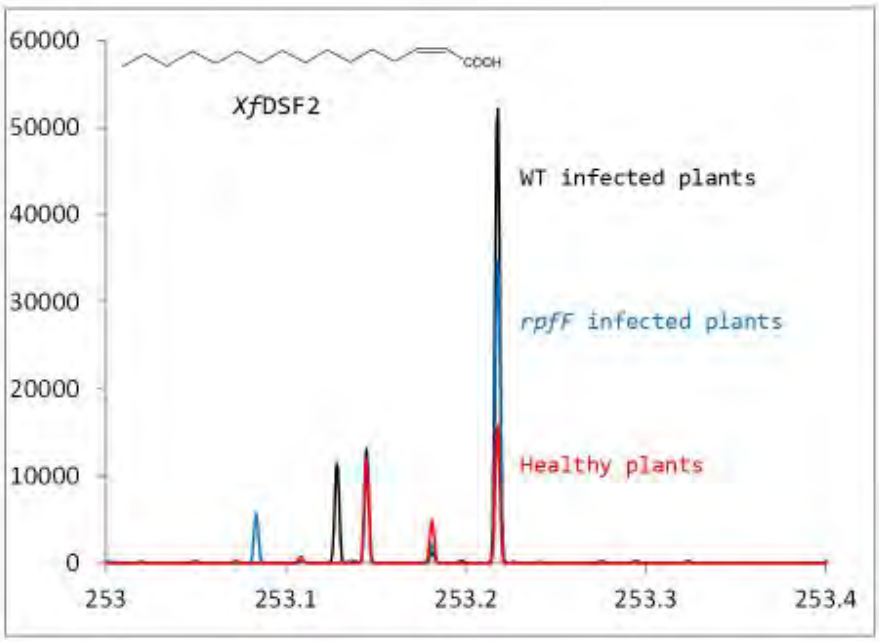
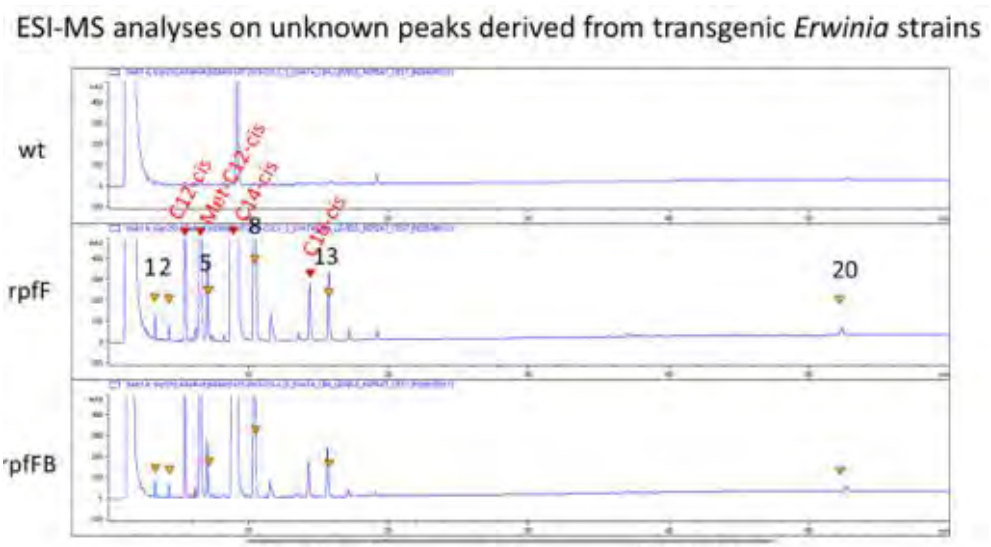


Figure 12. Abundance of ions with the M/Z ratios shown on the abscissa made from extracts of plants infected with a wild type strain of Xylella fastidiosa (black), from plants infected with an RpfF mutant of XF (blue), or from healthy plants (red). Note the much higher concentration of the ion with a M/Z ratio of 253.22 corresponding to XfDSF2.

To better understand the variety of different DSF species that might be produced by Xf, we chemically fractionated cultures of *Erwinia herbicola* harboring RpfF, the DSF synthase from *Xf*. *Erwinia herbicola* was chosen as a surrogate host for the DSF synthase because much larger numbers of cells of the species can be produced than that of *Xf* itself. Ethyl acetate extracts of culture supernatants were separated using HPLC. Several chemical entities were identified in extracts of *Erwinia herbicola* containing RpfF or containing both RpfF and RpfB from *Xf* but not in control cultures of *Erwinia herbicola* lacking these genes from *Xf* (figure 13). To obtain putative chemical structures for these chemical species that were present only in the presence of the DSF synthase we physically collected fractions of the separated material and subjected to ESI-MS to obtain high-resolution M/Z ratios for these materials (Figure 13). While a precise molecular formula for these molecules could be obtained this process, it is not possible to obtain an unambiguous structure, given that there are often several solutions that would match the particular formula weight. However, given the constraints that the DSF species are very likely to be fatty acids, we have proposed tentative structures for these novel DSF-like species (Figure 14). The location of the double bond in these molecules however cannot be placed unambiguously based on their mass alone, and further work is underway to obtain sufficient amounts of these molecules to enable them to be subjected to NMR and other techniques that would localize the unsaturation.



Fraction No.	*m/z*	Formula
1	215.164747	$C_{12}H_{23}O_3$
5	225.185257	$C_{14}H_{26}O_2$
8	251.200399	$C_{16}H_{27}O_2$
13	279.23184	$C_{18}H_{32}O_2$
20	283.261797	$C_{18}H_{36}O_2$

Figure 13. Optical absorption patterns (lamda = 220 nm) for chemical species separated on a methanol: water gradient by HPLC. Note peaks that are found in extracts of Erwinia herbicola harboring RpfF from XF (center panel), or harboring both RpfF and RpfB (bottom panel) but which are not found in extracts of Erwinia herbicola lacking these heterologous genes from XF (top panel). The M/Z ratios of the numbered peaks and the molecular formula for molecules in each peak are noted below.

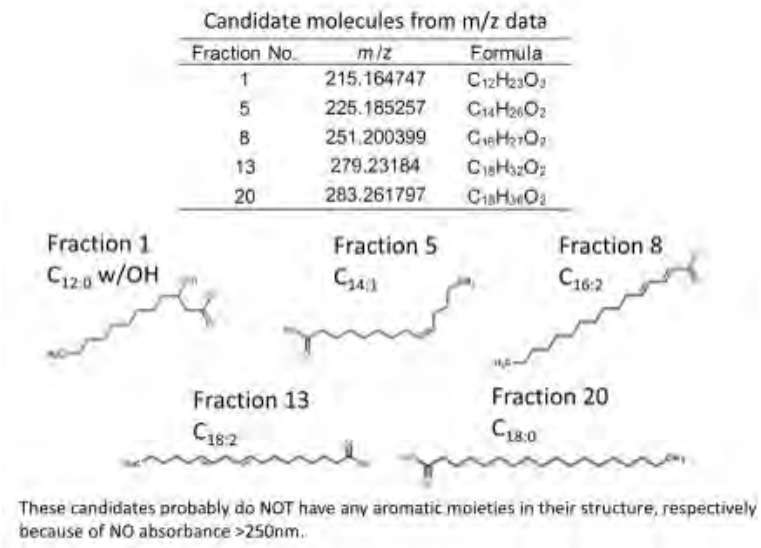
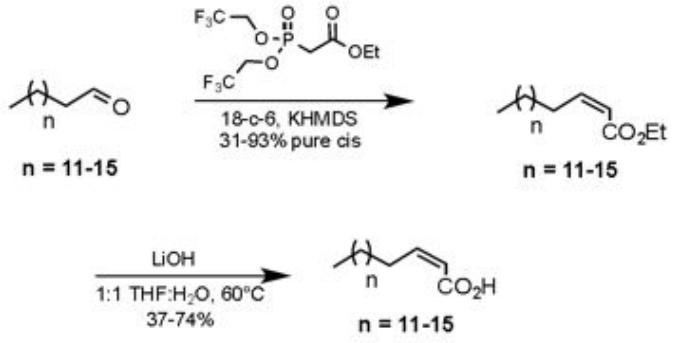


Figure 14. Tentative structures for novel DSF species produced in *Erwinia herbicola* harboring RpfF from *Xylella fastidiosa*.

Since X*f*DSF2 was highly biologically active in Xf, we have synthesized sufficient amounts of this material for use in plant inoculations. (*Z*)-hexadec-2-enoic acid that was produced by *Xylella* was potentially more active than (*Z*)-tetradec-2-enoic acid that was previously characterized. Fatty acids were synthesized by the route shown in Scheme 1 (Figure 15). A Still-Gennari olefination provided the *Z*-enoates and saponification afforded DSF products. Yields of these reactions decreased with increasing chain length presumably due to differences in solubility that made purifications more challenging.



Scheme 1. General synthesis of DSF analogs.

Figure 15. Synthesis route for X*f*DSF2

A total of about 2 grams of synthetic X*f*DSF2 has been provided by our collaborator at SRI International. We are in the process of evaluating XfDSF2 for its ability to alter the behavior of Xf in grape vines. This DSF species is being applied in two different ways to plants before inoculation with Xf, as well as in weekly applications following inoculation of Xf. In one method, *Xf*DSF2 is being solubilized in 75% methanol and sprayed onto leaves. Because of the very low solubility of XfDSF2 in water, we had to use organic solvent such as methanol to solubilize it so that it could be applied to the foliage of grape plants. In an alternative approach, *Xf*DSF2 solubilized in 75% methanol is being applied directly to xylem vessels by a similar droplet puncture procedure used to inoculate *Xf* itself. In this method, a small “cup” capable of containing 20 µl of DSF solution is made using silicon adhesive on the side of the stem, and a needle is used to puncture a 20 µl droplet of *Xf*DSF2, allowing it to be taken up into the xylem. Studies were initiated in July, and we would expect symptom development by September. Plants will be maintained at a height of no more than 1.5 m so that relatively small amounts of *Xf*DSF2 can be applied to the plant (2.5 mg/plant).

Since both XfDSF1 and XfDSF2 are active as signaling molecules in Xf, we have explored whether other related 2-enoic fatty acids will also be active as a signaling molecule in this pathogen. We therefore have synthesized 2-enoic fatty acids with chain lengths of 17, 18, 19, and 20. Preliminary results of their biological activity using the *Xf*:phoA DSF biosensor reveal that the 17 and 18 carbon enoic acids have nearly as much biological activity as *Xf*DSF2, while the 20 carbon enoic acid, having substantial activity, is less active than *Xf*DSF2. We will continue studies to determine whether Xf can produce any of these molecules.

To better assess the relative amounts of different 2-enoic fatty acids produced by *Xf* in different culture conditions and *in planta* we have explored new technologies in cooperation with the Lawrence Berkeley national laboratories. Dr. Trent Northern has developed a novel NI-MS

technologies that should allow the detection of picomole concentrations of 2-enoic acids. While this technology does not enable the identification of novel molecules, it should be able to verify and quantify the presence of known 2-enoic fatty acids. Since we now have a large collection of synthetic 2-enoic acids we have approached Dr. northern and will be attempting to quantify *Xf*DSF1 and *Xf*DSF2 in various samples as well as other 2-enoic acids, with a particular interest in understanding the amount and type of 2-enoic acids made by Xf while growing in grape. We presume that such a chemical species would be the most biologically active and relevant for our purposes of pathogen confusion.

Objective 2: Role of extracellular vesicles

Our continuing work reveals that *Xf* is a very prolific producer of extracellular vesicles. For a large numbers of vesicles (>400/cell) can be associated both with the surface of the bacterial cell, as well as a high portion that are shed by the cells to the extracellular environment (Figure 16). The vesicles are generally quite small, ranging in size from about 0.01 to 0.1 μm in diameter.

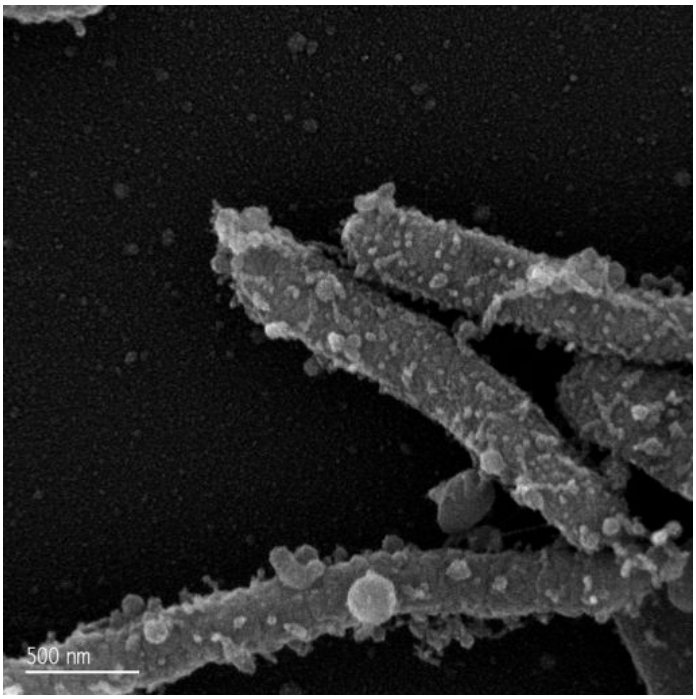


Figure 16. Visualization of membranous vesicles forming on the surface of cells of a wild type strain of *Xylella fastidiosa*. Not shown to the method of preparation are those vesicles that have been shed from cells.

Our ability to quantify membrane vesicles and to determine those factors which control their production and release from cells has been facilitated by our finding that a major outer membrane adhesins XadA is a significant component in these membranous vesicles. Since we have obtained antibodies specific to XadA, it is possible to visualize membrane vesicles using light microscopy (Figure 17). Using anti-XadA antibodies with a red fluorescent tag it is clear that the surface of *Xf* cells harbors a large constellation of membranous vesicles which surround the cell has somewhat of a “cloud”. In addition to those vesicles which are relatively closely associated with the cell (although apparently not physically linked), are vesicles that can be

found at further distances away from cells (Figure 18). These distantly located vesicles are clearly not simply “mini-cells” of *Xf* since they do not contain DNA as determined with a DNA-binding dyes such as DAPI (Figure 19).

To utilize the outer membrane protein XadA as a molecular marker for the presence of extracellular vesicles it was necessary to demonstrate that it is not secreted as a free protein into the extracellular environment, and is always found associated with membranous vesicles. To demonstrate this we isolated total proteins from vigorously washed cells of an RpfF mutant of *Xf,* from a pallet of extracellular material could be recovered after high-speed centrifugation (150,000 x g), as well as free protein that was not pelleted after high-speed centrifugation. While small amounts of XadA were associated with the attack cells of *Xf,* large amounts of XadA were found in membranous vesicles that could be obtained after high-speed centrifugation, and importantly, no XadA was free and solution of cultures of *Xf* (Figure 19). These results clearly show that XadA is abundantly released in the form of membranous vesicles from cells of *Xf*, and since it is always associated with membranes, it makes an excellent marker for membranous vesicles.

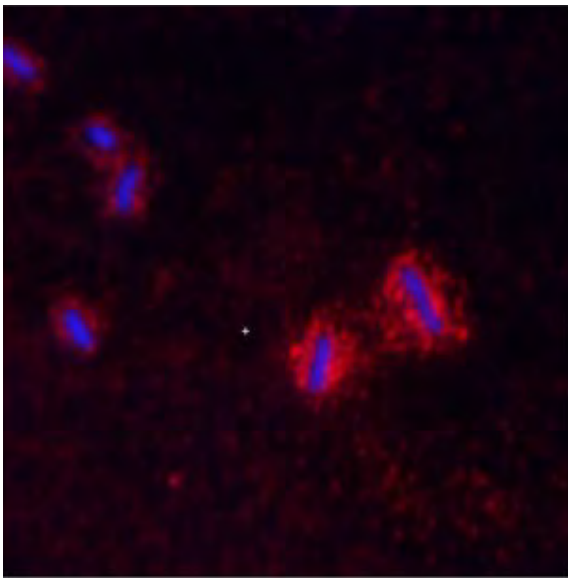


Figure 17. Visualization of membranous vesicles produced by cells of *Xylella fastidiosa* whose DNA is stained with DAPI and appearing blue in this image. Vesicles are read in this image due to their binding to fluorescently labeled anti-XadA antibodies.

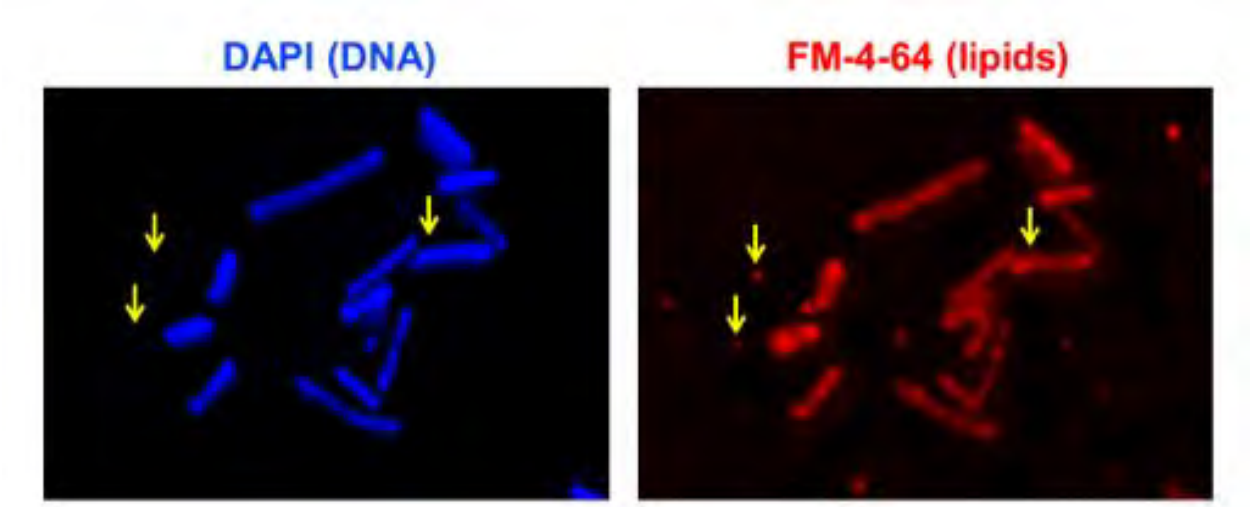


Figure 18. Visualization of cells of *Xylella fastidiosa* stained with the DNA-binding stain DAPI (left panel) as well as with the lipid binding stain FM-4-64 (right panel). Note the location of small circular red objects indicating presence of membranous material distal to the location of adjacent Xylella fastidiosa cells.

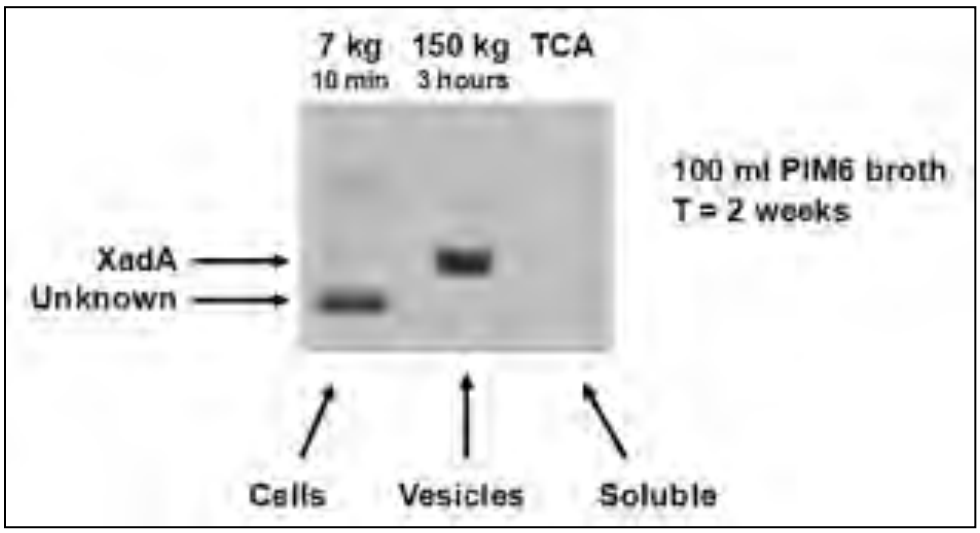


Figure 19. Quantification of the outer membrane protein XadA by Western blot analysis using anti-XadA antibodies from washed cells of *Xylella fastidiosa* (left lane) as well as from material recovered from high-speed centrifugation of culture supernatants (center lane), or in proteins precipitated from culture supernatants after high-speed centrifugation by TCA (right lane).

Quantification of vesicles by both deconvolution fluorescence microscopy as well as flow cytometry reveals that the higher number of vesicles (normalized for the number of *Xf* cells) are present in RpfF mutants, suggesting strongly that DSF accumulation suppresses the release of such vesicles. Deconvolution microscopy coupled with staining with lipid specific dyes enabled us to quantify the number of vesicles associated with cultures of either a wild type or an *rpfF* mutant of *Xf*. The *rpfF* mutant produced up to three times more vesicles as measured by this methodology than the wild type strain (Fig. 20). Deconvolution microscopy however is limited to visualizing vesicles larger than approximately 200 nm.

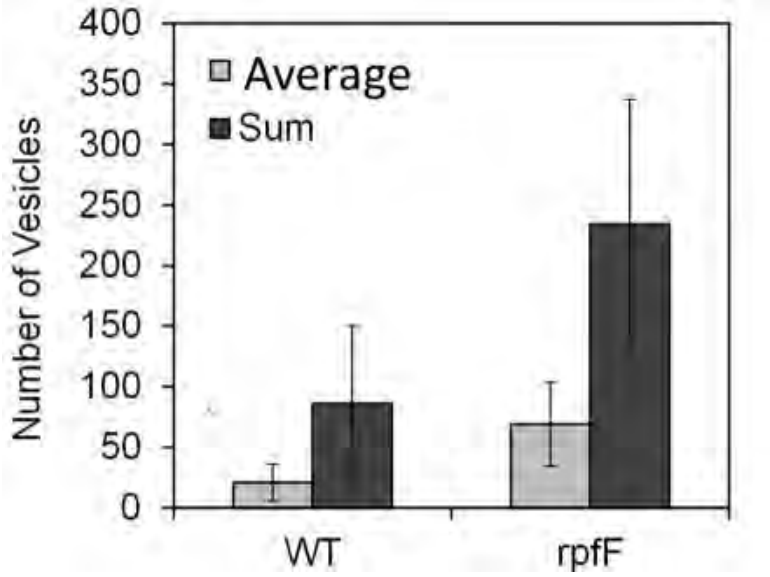


Figure 20. Enumeration of vesicles produced by wild type and an *rpfF* mutant of *Xf* grown for two days in PIM6 minimal media. Cells were removed by low-speed centrifugation and the cell free culture supernatant was visualized using a deconvolution microscope following staining with a lipid dye.

To better understand the total number of vesicles and their relative sizes we utilized a device known as a Nanosight particle counter. This device can enumerate particles as small as about 5 nm and also is highly accurate in enumerating particles of all sizes. Use of this device on both purified vesicles collected by ultracentrifugation of cell free culture supernatants of cultures of WT and an *rpfF* mutant of *Xf* confirmed that the *rpfF* mutant produces as much as three times more vesicles (Figure 21). To ensure that merger of vesicles did not occur during ultracentrifugation, and thus to obtain a better estimate of the original size of vesicles produced by *Xf* we directly determine the numbers and sizes of vesicles in cell free culture supernatants of wild type and *rpfF* mutants grown in PIM6 medium (Figure 22). Again, the results reveal that the *rpfF* mutant produced nearly 3 times more vesicles than the wild type strain. While some vesicles were as large as approximately 1000 nm, the average diameter of vesicles was only approximately 150 nm (Figures 21 and 22). We can estimate that each *Xf* cell has shed approximately 100 to 1000 vesicles of different sizes. It is thus clear that vesicles constitute a major extracellular factor produced by *Xf.*

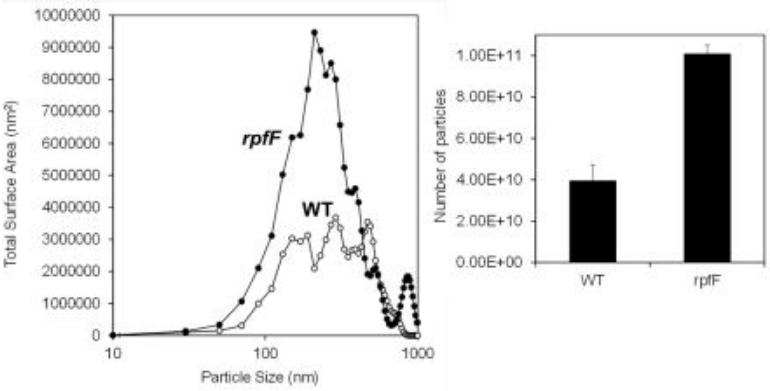


Figure 21. Distribution of sizes and abundance of vesicles of different sizes produced by a wild type and an *rpfF* mutant of *Xf* when grown for two days in PIM6 minimal media when assessed with a Nanovision device. Vesicles were concentrated by ultracentrifugation following removal of whole cells by low-speed centrifugation. The absolute number of vesicles of all sizes are shown in the right-hand panel. The vertical bars represent the standard error of the estimate of the number of vesicles produced by a given strain.

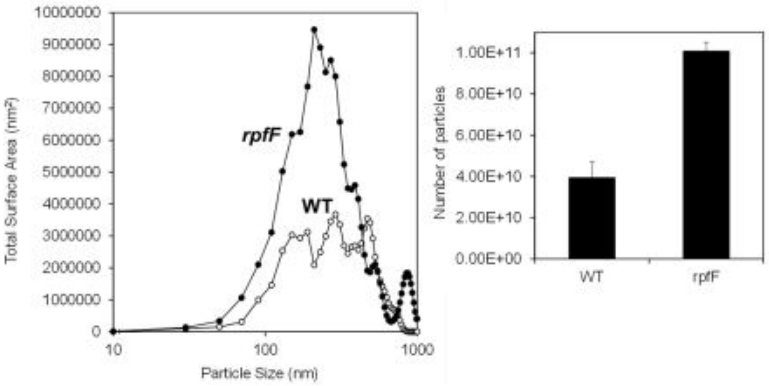


Figure 22. Distribution of sizes and abundance of vesicles of different sizes produced by a wild type and an *rpfF* mutant of *Xf* when grown for two days in PIM6 minimal media when assessed with a Nanovision device. Vesicles were determined directly in culture supernatants after removal of whole cells by low-speed centrifugation. The absolute number of vesicles of all sizes are shown in the right-hand panel. The vertical bars represent the standard error of the estimate of the number of vesicles produced by a given strain.

Evidence was obtained that XadA, as an outer membrane protein of *Xf* also can act as an adhesin. T to test this hypothesis the gene encoding XadA was cloned into an *E. coli* strain lacking strong surface adhesins. The comparative ability of this wild type *E. coli* and *E. coli* overexpressing *Xf* XadA to adhere to glass surfaces was then assessed by quantifying the number of bounds cells by their ability to bind crystal Violet. A much higher number of E. coli harboring XadA adhered to glass services, forming a biofilm (Figure 23), than of *E. coli* itself.

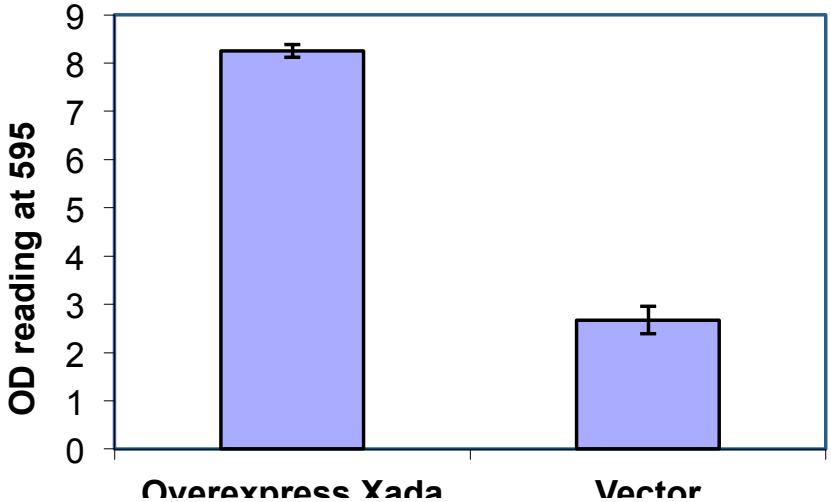


Figure 23. Attachment of E. coli harboring the cloning vector pVSP61 alone (right bar) and harboring the cloned *xadA* from *Xylella fastidiosa* (left bar) to glass as measured by intensity of crystal Violet staining. The vertical bars represent the standard error of the mean optical density measured at 595 nm.

To better understand the sites to which XadA prefers to attach, we examined the attachment of cultures of E. coli harboring a cloned *xadA* to glass surfaces over time. We hypothesize that if *XadA* preferentially mediated adhesion of *Xf* cells to itself that while initial adhesion of *Xf* to glass surfaces would require it to bind to glass, that subsequent binding events with preferentially occur at sites where *Xf* had previously attached. We found that the majority of subsequent binding events of *E. coli* harboring *Xf* XadA were to glass, and not to other cells, since the average number of cells bound per site on the glass surface remains very low (<2 cells/site) (Figure 24). That is, most commonly *E. coli* harboring XadA found as individual cells to the glass surface and less commonly found in other cells to form larger sized aggregates. The use of E. coli as a surrogate to harbor XadA prove useful in assessing the role of XadA in attachment, since *Xf* harbors several different adhesins, and the specific role of XadA would have been hard to discern from that of other, perhaps more tenacious adhesion such as HxfA and HxfB.

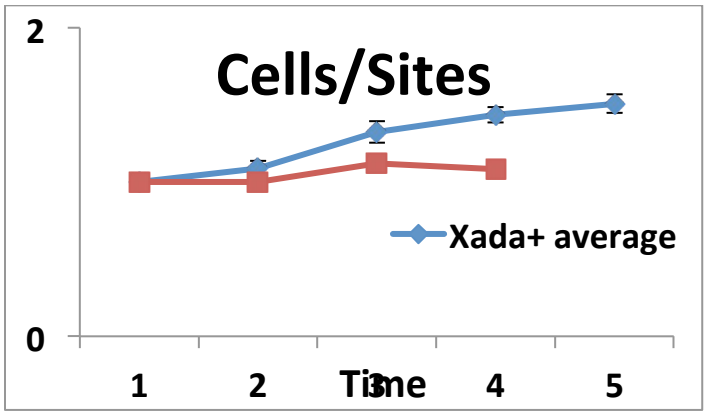


Figure 24. The number of cells of E. coli harboring Xf XadA (blue) or E. coli itself (red) that were attached to glass surfaces at various times after exposing slides to suspensions of each culture. Cell numbers were determined after DAPI staining and fluorescence microscopy. The vertical bars represent the standard error of the mean number of cells found in a site that had at least one bacterial cell present.

Strong evidence has been obtained that *Xf* releases factors into the xylem fluids of plants that it is colonizing that prevents their adherence to surfaces. XadA that was not associated with intact cells of *Xf* (and hence associated with membranous vesicles), was readily detected in the xylem sap of plants infected with both the wild type strain as well as an RpfF mutant of *Xf* (Figure 25). Substantially more vesicles were apparently present in the sample plants infected with the RpfF mutant, consistent with our finding that such a mutant produces more membranous vesicles in culture.

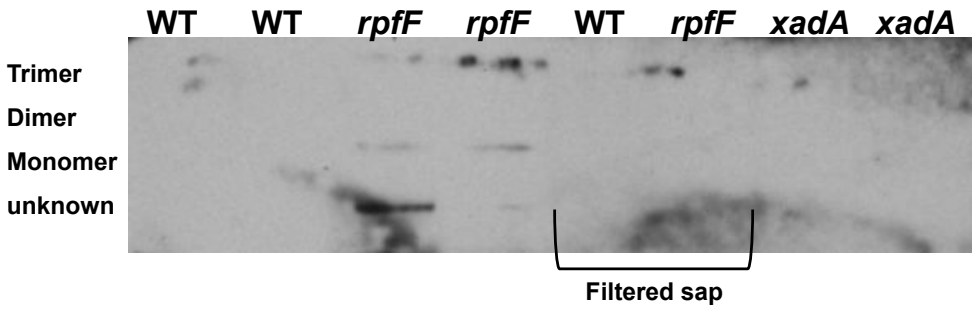


Figure 25. Abundance of XadA, indicative of the presence of membranous vesicles, as determined by Western blot analysis in xylem sap of Thompson seedless grapes infected with either a wild type or an RpfF mutant of Xf. Total XadA in sap which had not been filtered to remove intact cells are shown in the leftmost four lanes, while that in sap that had been filtered to remove intact cells (retaining only membranous vesicles) are shown in lanes 5 and 6. Note the high abundance of XadA in filtered xylem sap, especially from plants infected with the RpfF mutant.

Xylem sap containing membranous vesicles was shown to reduce the adherence of various bacteria to surfaces. Xylem fluid was collected by pressure bomb from healthy Thompson seedless grape, as well as from plants infected with a wild type strain of *Xf* or with an RpfF mutant of *Xf*. To test the differential adherence of wild type cells of *Xf* to surfaces such as insects in the presence of these different sample collections, we immersed small sections of hind wings of glassy winged sharpshooter in each of these xylem sap samples to which we also added a wild type strain of *Xf* (final concentration 10^8 cells/ml). Cell suspensions in these various sap samples were allowed to incubate with the wings for various times, the wing segments were then removed from the cell suspension in the sap, washed briefly to remove non-adhering cells, and the wing then macerated to release the adhering cells which were then enumerated by dilution plating. While relatively small numbers of cells had attached to wings suspended either of the sap suspensions after a short incubation period (30 min.), a much larger number of cells had attached after a two-hour incubation period. Importantly, many more cells that had been suspended in samples from healthy plants attached them from cells suspended in sap from plants infected with the wild type Xf strain, particularly from sap infected with an RpfF mutant of *Xf* (Figure 26). Over 20-fold more bacterial cells were attached when suspended in sap from the healthy plant compared to that of sap from the plant infected with the RpfF mutant of *Xf* (Figure 26). It is thus clear that the xylem sap environment of plants infected with an RpfF mutant of XF is much less conducive to the adherence of *Xf* to surfaces than that of healthy plants. In fact, the cells sap environment of plants infected with the wild type strain of *Xf* is also somewhat less conducive to adherence.

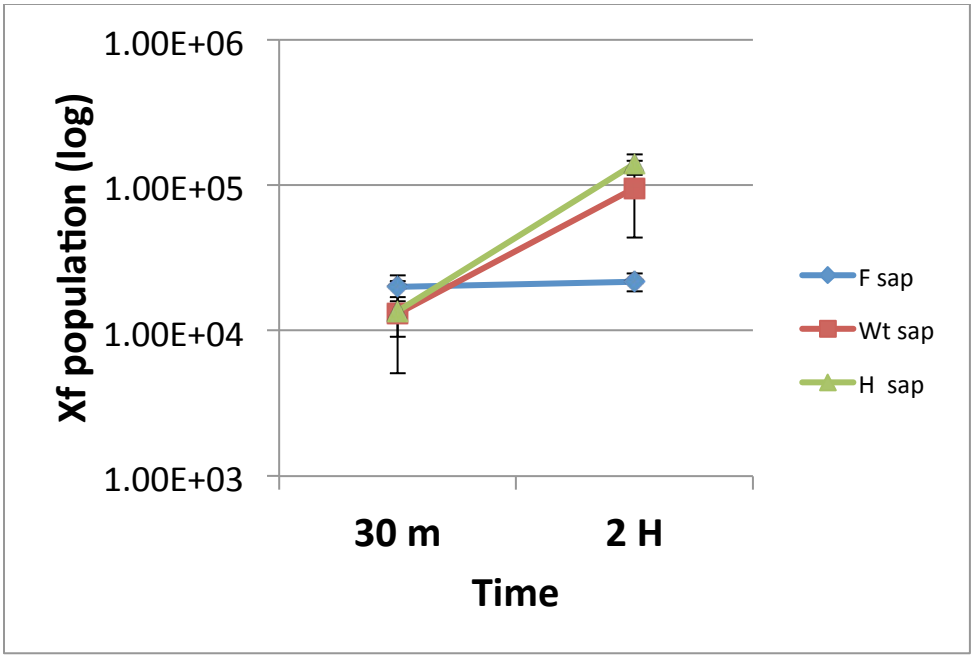


Figure 26. The number of cells of a wild type strain of *Xylella fastidiosa* that had adhered to wings of the glassy winged sharpshooter suspended in xylem sap from plants infected with an RpfF mutant (blue), or a wild type strain of *Xylella fastidiosa* (red), or from healthy plants (green) after incubation for either 30 min. or two hours.

The adhesion of a wild type strain of *X. fastidiosa* to vector wings when suspended in culture supernatants of a wild type strain and an *rpfF* mutant was also compared. The number of *X. fastidiosa* cells that had attached to wings that was suspended in un-inoculated culture media was much greater (ca. 14-fold) than that of cells suspended in culture supernatants of an *rpfF* mutant after 24 hours incubation (Fig. 27). Cells suspended in a culture supernatant of a wild type strain of *X. fastidiosa* also exhibited (ca. 5-fold) less adherence to wings than those suspended in culture media itself. These results indicate that an extracellular product of *X. fastidiosa* is sufficient to account for the interference of adherence of cells in xylem sap of plants infected with this pathogen.

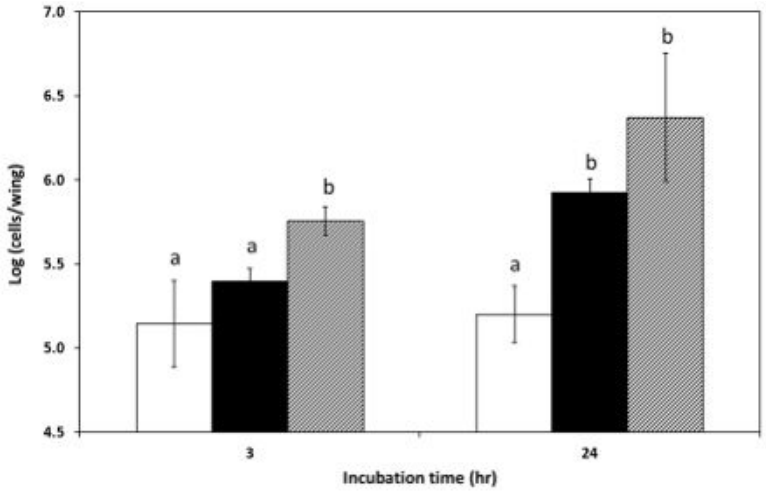


Fig. 27. Attachment of cells of wild type *X. fastidiosa* strain Temecula to hind wings of glassy-winged sharpshooters when suspended in cell-free supernatants of cultures of a wild type strain (black bars) or an *rpfF* mutant (white bars) that had been grown in PIM6 medium, or in un-inoculated PIM6 medium (striped bars) when incubated for the various times shown on the abscissa. Vertical lines represent the standard error of the determination of the mean. Means indicated by the same letter do not differ ($P < 0.05$).

The presence of factors that interfere with insect transmission of *X. fastidiosa* is consistent with a developing model of the disease process in which DSF signaling is used to partition the population of cells of this pathogen to optimize fitness in the plant while enabling it to be transmitted to new host plants. *X. fastidiosa* is present in a large number of xylem vessels in infected grape stems and petioles, yet the population size varies from only a few cells to many thousands of cells per vessel. It is presumed that the concentration of DSF within a given vessel is proportional to the number of cells of *X. fastidiosa* present in that vessel, although some dispersal of DSF might occur, leading to low concentrations of DSF even in un-colonized vessels. It is unlikely that high concentrations of DSF would be found in the vicinity of cells unless they were present in high local concentration in a colonized vessel. DSF accumulation is associated with traits such as induction of fimbrial and afimbrial adhesins as well as suppression of type IV pili and the production of extracellular enzymes, all of which would tend to restrict the ability of the pathogen to move within the plant. Given that the majority of vessels in infected plants harbor relatively few cells of *X. fastidiosa*, it is presumed that most do not experience sufficient concentrations of DSF to suppress their movement through the plant, and thus can be considered to be actively exploring the plant. Only in those vessels in which high cell concentrations are achieved (and in which DSF accumulates) will cells become sufficiently adhesive to be efficiently acquired by insect vectors. High adhesiveness associated with DSF signaling appears to be essential for acquisition of *X. fastidiosa.* Thus cells of *X. fastidiosa* in infected plants appeared to be partitioned into subpopulations that are either 1) actively moving and multiplying in plants or 2) are incapable of movement but which can be acquired by insect vectors. We postulate that unknown factors are preferentially released from those cells of *X. fastidiosa* that are present in low numbers in vessels, and which are therefore most highly motile. Such factors would logically be suppressed by DSF signaling, and would be maximally produced by an *rpfF* mutant. The highest concentration of such an anti-adhesive factor would be present in the xylem sap of plants infected with an *rpfF* mutant of *X. fastidiosa* not only because such mutants would maximally produce such a factor, but also because they are in higher numbers due to their unrestricted growth compared to the wild type strain. Thus, xylem sap recovered from plants infected with a wild type strain would be a mixture from xylem vessels colonized by low numbers of cells of *X. fastidiosa* (in which the anti-adhesive factor would be maximally produced on a per cell basis) as well as from vessels harboring high populations of the pathogen, although the blockage of such vessels by cells and tyloses may reduce the recovery of sap from such vessels. In this model those most adhesive cells of *X. fastidiosa* (present in vessels having the highest numbers of cells of this pathogen) could be most efficiently retained and thereafter vectored by sap-sucking insects without the interference that an anti-adhesive factor produced by more solitary cells in the plant would have on the transmission process. The presumptive anti-adhesive factor could play a major role in facilitating the movement of *X. fastidiosa* throughout the plant, and further work to elucidate its nature and contributions to this process are warranted.

Strong circumstantial evidence suggests that the release of extracellular membranous vesicles by the RpfF mutant is responsible for the suppression of adherence of *Xf* to surfaces. Since the RpfF mutant of *Xf* does not accumulate DSF, which in turn suppresses the release of extracellular vesicles, a higher concentration of extracellular vesicles would be expected in

plants infected with the RpfF mutant. At least some extracellular vesicles would also be expected in the sap of plants infected with the wild type strain as well. A higher concentration of extracellular vesicles, as estimated by the abundance of XadA (which we can use as a marker protein for these membranous vesicles), is found in plants infected with the RpfF mutant of *Xf* compared to that of the wild type strain (Figure 28). These results further support our model of a "Teflon mechanism" of virulence of *Xf* whereby it releases adhesive vesicles, especially one found at relatively low cell densities where DSF would not have accumulated. These vesicles would be expected to attached to surfaces such as that of the walls of the xylem vessels. By so attaching, these vesicles would prevent the attachment of *Xf* cells themselves to such surfaces. Only upon reaching relatively high cell concentrations in a particular vessel would DSF concentrations increase to a level that would suppressed the release of the membranous vesicles, thereby retaining adhesive molecules on the surface of *Xf* cells themselves, thus allowing the bacterial cells themselves to attached to surfaces, such as that of insect vectors (Figure 28).

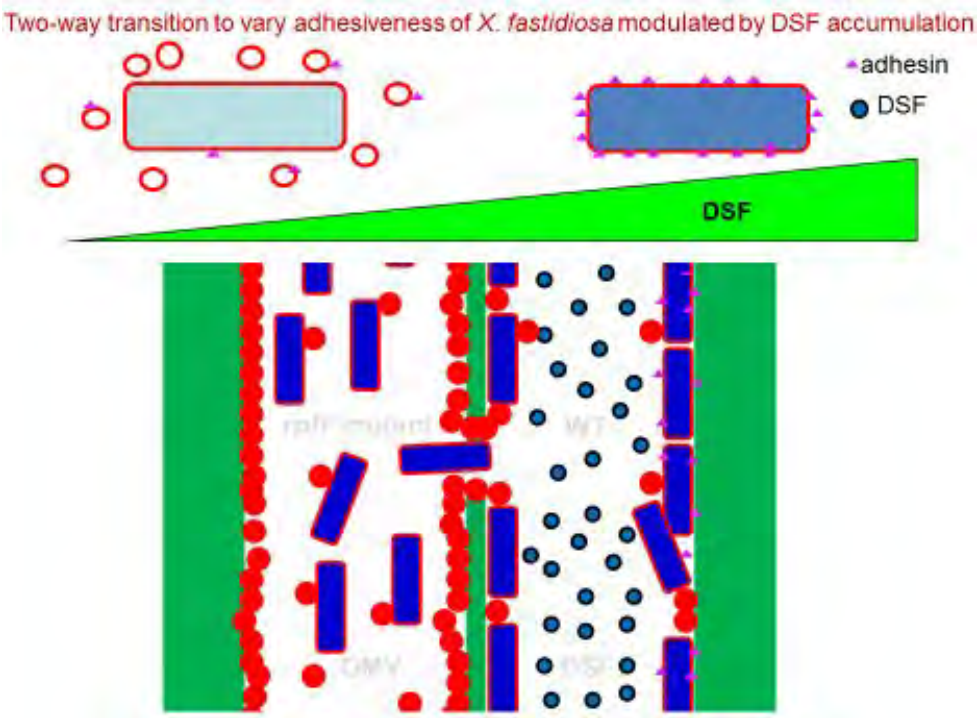


Figure 28. "Teflon Model" of DSF regulated virulence involving DSF-mediated control of shedding of extracellular vesicles in *Xylella fastidiosa*.

Reports and presentations:

1. Beaulieu, E., M. Ionescu, S. Chatterjee, K. Yokota, D. Trauner, and S.E. Lindow. 2012. Characterization of a diffusible signaling factor from *Xylella fastidiosa*. mBio 4(1): doi:10.1128/mBio.00539-12
2. Wang, N., Li, J.-L., and Lindow, S.E. 2012. RpfF-dependent regulon of *Xylella fastidiosa*. Phytopathology 102:1045-1053.

3. Almeida, R.P.P., Killiny, N., Newman, K.L., Chatterjee, S., Ionescu, M., and Lindow, S.E. 2012. Contribution of *rpfB* to cell-to-cell signal synthesis, virulence, and vector transmission of *Xylella fastidiosa*. MPMI 25:453-462.

4. Baccari, C., Killiny, N., Ionescu, M., Almeida, R.P.P., and Lindow, S.E. 2013. DSF repressed extracellular traits enable attachment of *Xylella fastidiosa*to insect vectors and transmission. Phytopathology 103: (in press).

Presentation at the University of Florida, Department of plant pathology entitled "the complex lifestyles of Xylella fastidiosa coordinated by cell- cell signaling: achieving disease control by pathogen confusion" presented on February 14, 2013.

Presentation at the headquarters of the American society for microbiology for "microbes after hours" webcast entitled "the complex lifestyles of *Xylella fastidiosa* coordinated by cell-cell signaling: achieving disease control by pathogen confusion" presented on January 28, 2013.

Research relevance statement:

We are very excited about results to date that show that several means of elevating DSF levels in plants have provided disease control via a strategy of "pathogen confusion". Given the limitations in standard methods of disease control, we are optimistic that DSF interference represents a promising strategy for PD control. Control of Pierce's disease by direct application of DSF is a very attractive disease control strategy since it could be quickly implemented and would utilize commonly used agricultural equipment and methods and would not require the use of transgenic technologies. Our earlier work had shown that C14-cis, a component of *Xf* DSF, conferred some reduction of disease after topical application, but less than might have been expected compared to application of crude DSF-containing extracts of *Xf*. Our recent studies using biosensors more responsive to the DSF molecules made by *Xf* reveal that at least 2 additional molecules related to C14-cis are biologically active in *Xf,* at least one of which may be much more active than C14-cis. We have now found several additional DSF species that are synthesizing them in testing their biological activity. We will determine which of these molecules are most biologically active, whether they all have similar effects on gene expression in *Xf*, and which are most abundant within plants infected with *Xf*. Our new sensitive biosensors will be used to document the absorption and translocation of these molecules by grape after application in various ways. This should enable us to greatly increase disease control by direct application of the most appropriate molecule. (Obj. 1). Our recent results indicate that *Xf* changes the nature of the xylem sap during its colonization of plants to restrict its adhesion to plants via its shedding of outer membrane vesicles and that DSF accumulation restricts such shedding. Such vesicles represent important virulence factors for the pathogen, presumably by preventing *Xf* adhesion to pit membranes because the vesicles themselves will adhere to and coat plant targets. The presence of such vesicles would also be easily assessed indicators of the cell-cell signaling status of *Xf* in the plant. Our work to date has shown that these vesicles are both very abundant and have very strong effects on the behavior of *Xf* within plants. We thus will continue to further explore the role of vesicles as virulence factors and as tools to determine the effects of strategies of pathogen confusion by elevating DSF in plants.

Layperson summary:
X. fastidiosa produces an unsaturated fatty acid signal molecule called DSF. Accumulation of DSF in *Xf* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in the pathogen, but the overall effect is to suppress its virulence in plants by increasing its adhesiveness to plant surfaces and also suppressing the production of enzymes and genes needed for active movement through the plant. We have investigated DSF-mediated cell-cell signaling in *X.fastidiosa* with the aim of developing cell-cell signaling disruption (pathogen confusion) as a means of controlling Pierce's disease. Elevating DSF levels in plants r artificially educes is movement in the plant. In this study we have investigated the variety of different fatty acid molecules that can serve as cell cell signaling agents in Xf. Several new DSF species have been found including a 16 carbon unsaturated fatty acid appears to be far more active than the 14 carbon unsaturated fatty acid that we have previously investigated. The release of extracellular membranous vesicles by Xf is responsible for the suppression of its adherence to surfaces. These vesicles attach to surfaces such as that of the walls of the xylem vessels. By so attaching, these vesicles prevent the attachment of *Xf* cells themselves to such surfaces. Only upon reaching relatively high cell concentrations in a particular vessel would DSF concentrations increase to a level that would suppressed the release of the membranous vesicles, thereby retaining adhesive molecules on the surface of *Xf* cells themselves, thus allowing the bacterial cells themselves to attached to surfaces, such as that of insect vectors

Status of funds:
While this project was approved as of July 1, 2012, because of delays in the sponsored projects office at the University of California, Berkeley, this contract was not formally completed until February, 2013. Since some funding remained in closely related project 10-0276 for which a no cost extension was granted through June 30, 2013, much of the work presented here was conducted with carry-forward funding that project. For that reason, as of July, 2013, a sizable amount of the originally budgeted funding remains, and is anticipated that a no cost extension will be requested at the end of the currently scheduled budget period.
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Summary and status of intellectual property associated with the project:
A patent application (12/422,825) entitled "biological control of pathogenicity of microbes that use alpha, beta unsaturated fatty acid signal molecules" had been submitted March 13, 2009. While many of the claims had been rejected earlier, the University of California patent office has filed on March 13, 2012 a motion requesting reconsideration of the application with clarification of, and justification for, claims related to the production of transgenic plants transformed with the *rpfF* gene from *Xylella fastidiosa*. This petition was approved in June, 2012 and patent US 8,247,648 B2 was issued on August 21, 2012.