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ELUCIDATING PROCESS OF CELL-CELL COMMUNICATION IN *XYLELLA FASTIDIOSA* TO ACHIEVE PIERCE'S DISEASE CONTROL BY PATHOGEN CONFUSION

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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, we have now 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. Thus the overall goal of our work is to use these new biosensors 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 are 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 afimbrial 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.

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.

RESULTS AND DISCUSSION:
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. 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 (hereafter called *Xf*DSF) itself. Assay of *Xf* extracts by *Xf* DSF-specific biosensors provide evidence of more than one *Xf* DSF molecule. Our analysis of the material collected by HPLC from these cultures using electro-spray MS revealed it to be an unsaturated C16 fatty acid. We therefore chemically synthesized this presumptive derivative which we will call C16-cis or *Xf* DSF2 (Figure 2).

Figure 1. Structure of C16-cis (*Xf* DSF2)

The biological activity of the synthetic *Xf*DSF2 was tested by the addition of this material at various concentrations to an 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 (Figure 2). Importantly XfDSF2 also conferred much higher induction of hxfA, as indicated by a higher alkaline phosphatase activity at a given concentration than *Xf*DSF, and also induced other adhesins more highly. Both *Xf*DSF and *Xf*DSF2 strongly induced adhesion of wild type cells of Xf to glass tubes (Figure 2). Thus *Xf*DSF2 seems to be a particularly powerful signal molecule in *Xf*. We have applied XfDSF to plants in various ways and are in the process of evaluating changes in behavior of *Xf* and disease severity in these plants.

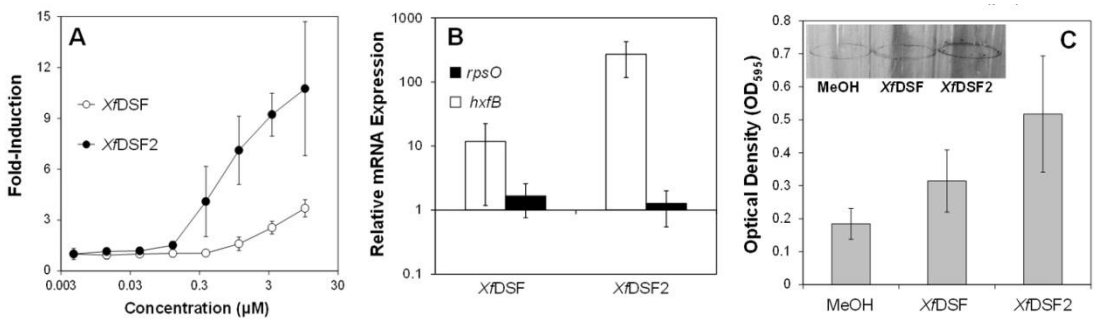


Figure 2. **(A)** *Xf*DSF and *Xf*DSF2 Dose-dependent induction of the *Xylella fastidiosa*-based DSF-biosensor (*rpfF**-*Xf*HA-biosensor). **(B)** qRT-PCR analysis of *hxfB* expression in *Xylella fastidiosa rpfF** strain after 72 h or growth in PD3 broth supplemented with 10 µM *Xf*DSF, 10 µM *Xf*DSF2 or MeOH. *rpoD* and *rpsO* were used endogenous control genes. **(C)** Biofilm formation at the liquid-air interface of shaken glass tubes by *Xylella fastidiosa rpfF** strain after 24 h of growth in PD3 broth supplemented with 10 µM *Xf*DSF, 10 µM *Xf*DSF2 or MeOH.

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. We could detect both *Xf*DSF1 and *Xf*DSF2 in culture supernatants of XF grown in PD3 media (Figure 3). 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.

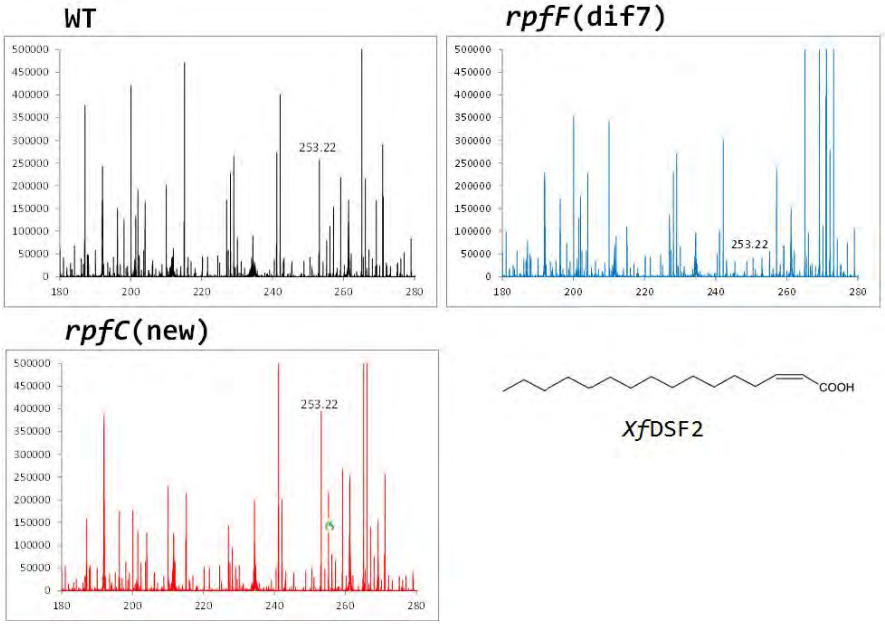


Figure 3. Abundance of ions with the M/Z ratios shown on the abscissa or culture extracts of a wild type strain of *Xylella fastidiosa* (upper left panel), of an RpfF mutant unable to produce DSF (upper right panel), of an RpfC mutant which overproduces DSF (lower left panel). Note that *Xf*DSF2 has 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). 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. Assays of this material in *Xf* revealed that it does not induce expression of genes such as *hxfA*, and instead acts as a potent inhibitor of the expression of such genes (Figure 4).

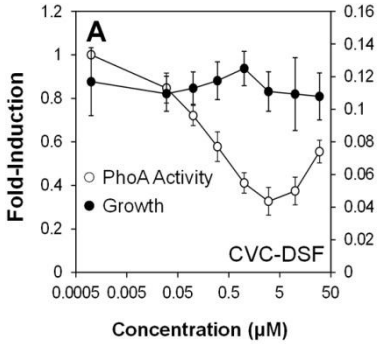


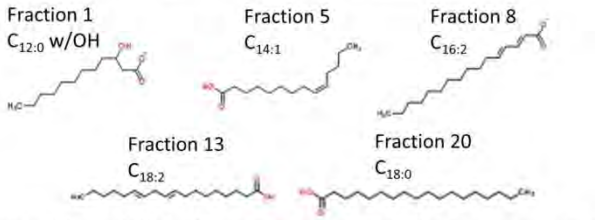
Figure 4. Silencing of the activity of the *Xylella fastidiosa*-based DSF-biosensor (*rpfF**-XfHA) by CVC-DSF. PhoA Activity units were measured at T = 96 h and is shown on the left Y-axis. Optical Density (OD_{600}) of culture at the time of PhoA Activity is shown on the right Y-axis.

To address which 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 *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. We also could detect elevated concentrations of a molecule presume to be *Xf*DSF2 in plants infected with the wild type *Xf* strain. 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.

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*. ***E. 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*. 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. A precise molecular formula for these molecules could be obtained this process and their putative structure can be obtained given the constraints that the DSF species are very likely to be 2-enoic acids (Figure 5).

Candidate molecules from m/z data

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	$C_{18}H_{36}O_2$



These candidates probably do NOT have any aromatic moieties in their structure, respectively because of NO absorbance >250nm.

Figure 5. Tentative structures for novel DSF species produced in *Erwinia herbicola* harboring RpfF from *Xylella fastidiosa*.

To better understand how promiscuous the DSF synthase RpfF from Xylella fastidiosa was, be synthesized a variety of different enoic fatty acids and assayed them with the *Xf:phoA* biosensor. A variety of related fatty acids having the site of unsaturation at the number two position but with different carbon chain lengths were assessed (Figure 6). In most cases, the double bond was constructed to be in a cis orientation, but a few corresponding trans unsaturated fatty acids were synthesized.

Chain length	Molecule name	Chain length	Molecule name
C8-cis	2-z-octanoic acid	C14-cis	5-z-tetradecanoic acid
C9-cis	2-z-nonanoic acid	C14-cis	6-z-tetradecanoic acid
C10-cis	2-z-decanoic acid	C15-cis	2-z-pantadecanoic acid
C11-cis	2-z-undecanoic acid	**C15-cis**	**CVC-DSF**
C12-cis	**BDSF (C12:1)**	**C16-cis**	**XfDSF2 (C16:1)**
C12-trans	2-E-dodecanoic acid	C17-cis	2-z-heptadecanoic acid
C13-cis	2-z-tridecanoic acid	C18-cis	2-z-octadecanoic acid
C13-cis	**DSF (C13:1)**	C19-cis	2-z-nonadecanoic acid
C14-cis	**XfDSF (C14:1)**	C20-cis	2-z-eicosanoic acid

Figure 6: List of various unsaturated fatty acids tested for their ability to induce quorum sensing in *Xylella fastidiosa*

Several different patterns of response of the *Xf:phoA* biosensor to these various fatty acids was observed. Some relatively short chain-link fatty acids such as C10-cis were toxic, and the alkaline phosphatase activity exhibited byby the *Xf:phoA* biosensor *Xf:phoA* biosensor
By the *Xf:phoA* biosensor decreased with increasing concentration of the fatty acid (Figure 7). In contrast, the alkaline phosphatase activity exhibited by the *Xf:phoA* biosensor increased with increasing concentrations of the fatty acid such as for XfDSF itself, thereby indicating a positive response, while there was no response to other fatty acids such as C19-cis (Figure 7).

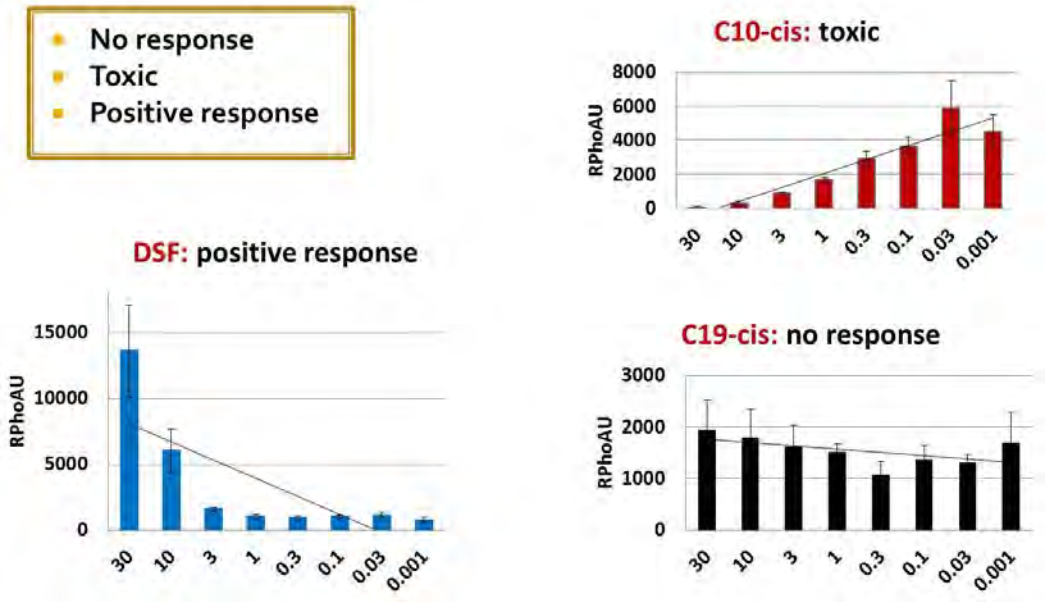


Figure 7: various responses of the *Xf:phoA* biosensor has indicated by alkaline phosphatase activity (ordinate) as a function of the concentration of various synthetic fatty acids noted on the abscissa (uM).
Unsaturated fatty acids differed greatly in their ability to induce quorum sensing in *Xylella fastidiosa*. While X.fastidiosa responded positively to unsaturated fatty acids with chain lengths from 12 to 18 carbons, as long as the site of unsaturation was at the number two carbon position, those fatty acids lesson 12 carbons in length tended to be toxic while there was no response to those greater than 18 carbons in length (Figure 8). While X.fastidiosa could respond positively to a wide range of different fatty acids, the lowest concentrations at which some response could be detected was highest for those of carbon lengths of 14-18.

Chain length	Molecule name	Xf Biosensor	Xcc-GFP biosensor
C8-cis	2-z-octanoic acid	Toxic 30	No response
C9-cis	2-z-nonanoic acid	Toxic 1	No response
C10-cis	2-z-decanoic acid	Toxic 2	10 (4.1x)
C11-cis	2-z-undecanoic acid	Toxic 3	1 (12.4x)
C12-cis	**BDSF (C12:1)**	**3 (3.2x)**	**0.1 (12.4x)**
C12-trans	2-E-dodecanoic acid	Toxic 6	3 (7.9x)
C13-cis	2-z-tridecanoic acid	Toxic 0.3	0.01 (17.9x)
C13-cis	**DSF (C13:1)**	**3 (17.5x)**	**0.01 (17.9x)**
C14-cis	**XfDSF (C14:1)**	**1 (3.3x)**	**7 (4.8x)**
C14-cis	5-z-tetradecanoic acid	No response	No response
C14-cis	6-z-tetradecanoic acid	No response	No response
C15-cis	2-z-pantadecanoic acid	10 (4.2x)	No response
C15-cis	CVC-DSF	1.5 (0.32x)	No response
C16-cis	**XfDSF2 (C16:1)**	**0.15 (8.9x)**	No response
C17-cis	2-z-heptadecanoic acid	0.3 (8.6x)	No response
C18-cis	2-z-octadecanoic acid	1 (6.5x)	No response
C19-cis	2-z-nonadecanoic acid	No response	No response
C20-cis	2-z-eicosanoic acid	No response	No response

Figure 8: responsiveness of the *Xf:phoA* biosensor (third column from left) to various fatty acids. The number given for each compound indicates the minimal concentration (in uM) at which any positive response was seen, and the number in parentheses refers to the magnitude of the induction of the *Xf:phoA* biosensor (as measured as alkaline phosphatase activity) compared to cells treated with water alone.

Given that *X.fastidiosa* appeared to be relatively promiscuous in its perception of a variety of unsaturated fatty acids, a number of different commercially available saturated and unsaturated fatty acids were evaluated for their ability to induce quorum sensing (Figure 9). While no saturated fatty acid exhibited the ability to induce DSF-mediated quorum sensing in *X.fastidiosa*, Palmitoleic acid was quite active as a signaling

molecule (Figure 10). While approximately 5 times higher concentrations of Palmitoleic acids were required to induce the *Xf:phoA* biosensor compared to XfDSF2 (C16-cis), it conferred high levels of induction of the biosensor. This is a very exciting finding as it will allow us to proceed with tests to apply such as exogenous sources of DSF as a signal molecule to plants.

Chain length	Common name	Chemical name
C14:0	Myristic acid	tetradecanoic acid
C14-cis	Myristoleic acid	9-z-tetradecenoic acid
C14-cis	Physeteric acid	5-z-tetradecenoic acid
C16:0	Palmitic acid	hexadecenoic acid
C16-cis	Palmitoleic acid	9-z-hexadecenoic acid
C16-cis	Sapenic acid	6-z-hexadecenoic acid
C16-trans	Palmitelaidic acid	9-E-hexadecenoic acid

Figure 9: commercially available fatty acids tested for their ability to induce alkaline phosphatase activity by the *Xf:phoA* biosensor.

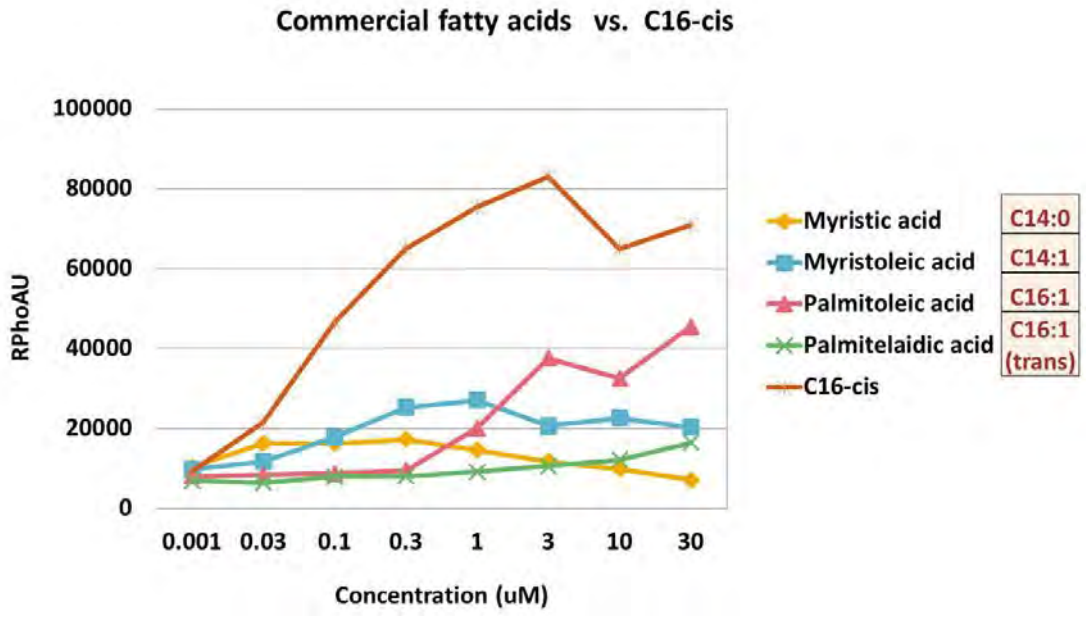


Figure 10: alkaline phosphatase activity exhibited by different commercially available saturated and unsaturated fatty acids tested at various concentrations in the *Xf:phoA* biosensor.

Given that a commercially available unsaturated fatty acid is a potent inducer of quorum sensing in *X. fastidiosa*, we further investigated the extent to which DSF signaling could be interfered with by the presence of other dissimilar saturated and unsaturated fatty acids. As noted above, the DSF from CVC strains of X that fastidiosa are powerful inhibitors of signaling in grape strains of X.fastidiosa in the presence of its own DSF, C16-cis (Figure 11). In the presence of one micromolar C16-cis the induction of the *Xf:phoA* biosensor

decreased steadily with increasing concentrations of CVC DSF in the range from 0.1 to 30 micromolar (Figure 11).

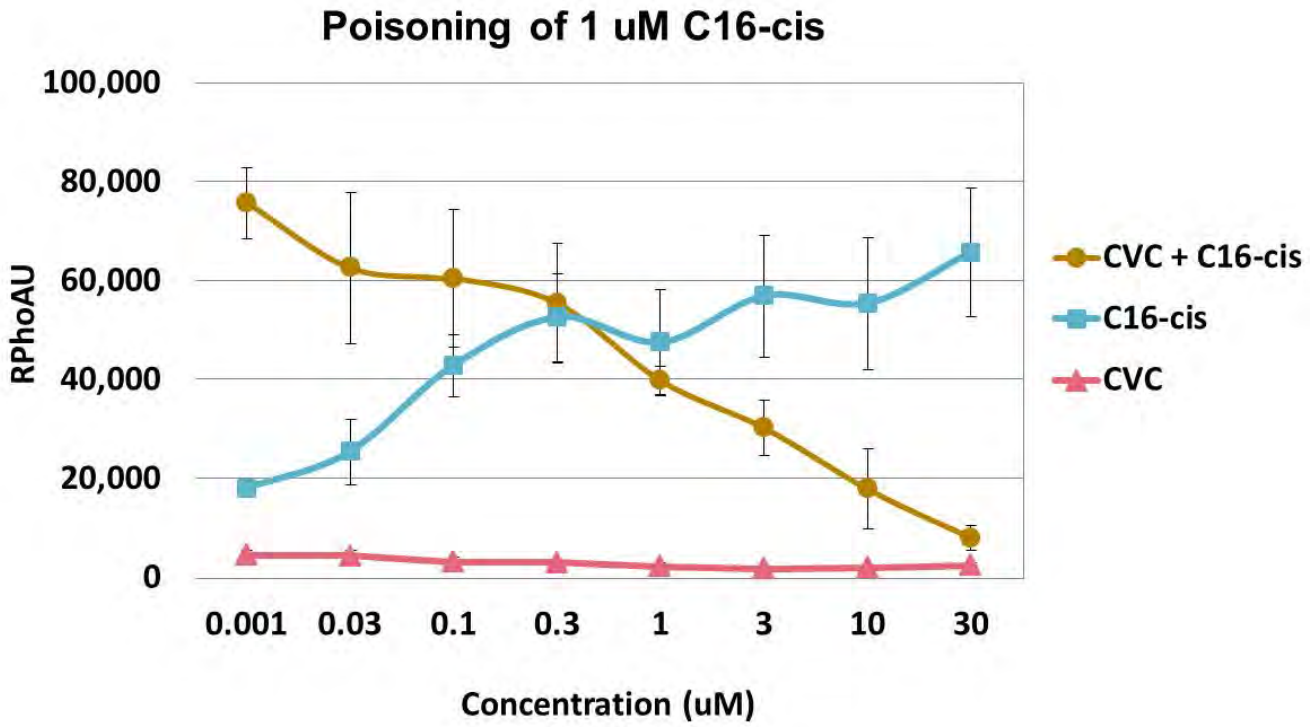


Figure 11: alkaline phosphatase activity exhibited by cells of the *Xf:phoA* biosensor exposed to different concentrations of CVC DSF (red line), C16-cis (blue line), or to a combination of 1 uM C16-cis and different concentrations of CVC DSF as shown on the abscissa (Brown line).

Given that Palmitoleic acid is a promising commercially available fatty acid that can serve as a signaling molecule in X.fastidiosa, we tested to what extent its ability to act as a signaling molecule could be blocked in the presence of other fatty acids (Figure 12). Not only did the saturated fatty acids Palmitic acid (C16) and Myristic acid (C14) interfere with signaling induced by C16-cis or C14-cis, but it also interfered with signaling induced by Palmitoleic acid as measured by the *Xf:phoA* biosensor (Figure 12).

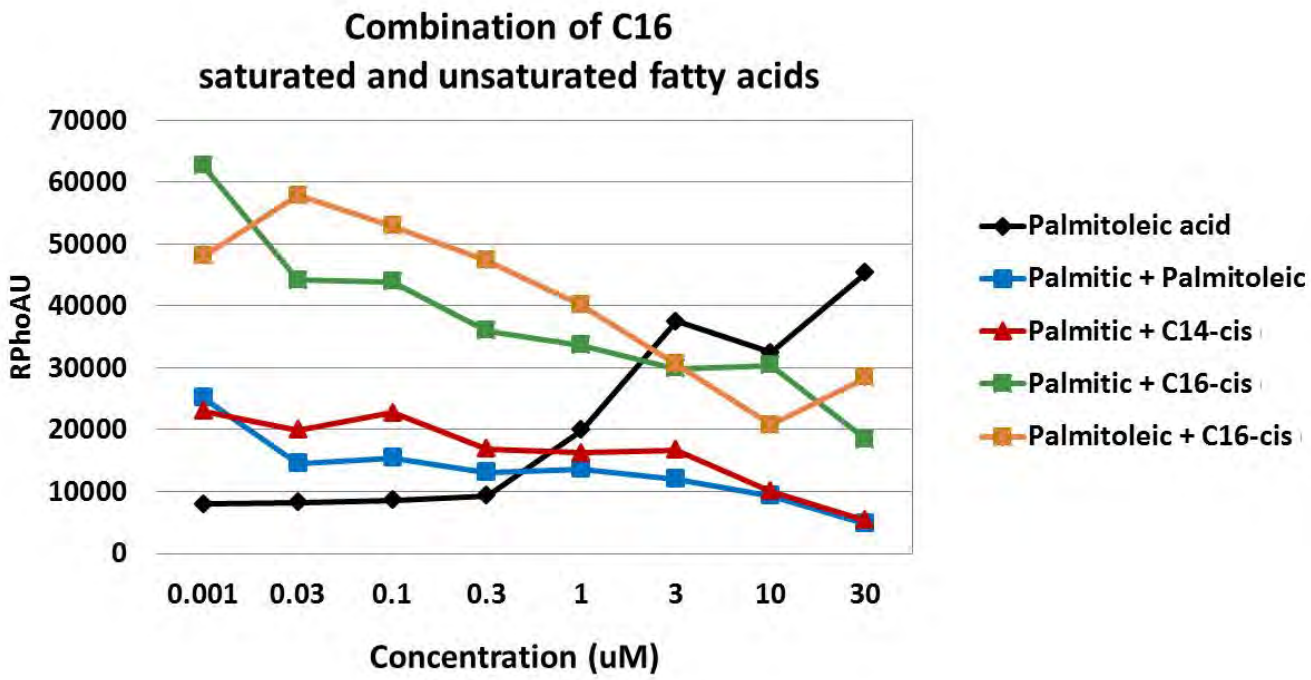


Figure 12: alkaline phosphatase activity exhibited by the *Xf:phoA* biosensor exposed to different concentrations of Palmitoleic acid alone (black line), or to one micromolar Palmitoleic acid and various concentrations of Palmitic acid (blue line).

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 13). The vesicles are generally quite small, ranging in size from about 0.01 to 0.1 μm in diameter.

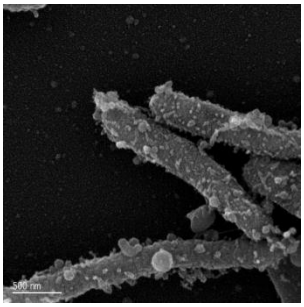


Figure 13. Membranous vesicles forming on the surface of cells of a wild type strain of *Xylella fastidiosa*. Not shown due to the method of preparation are those additional vesicles that have been shed from cells.

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 14). 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 7). 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 14).

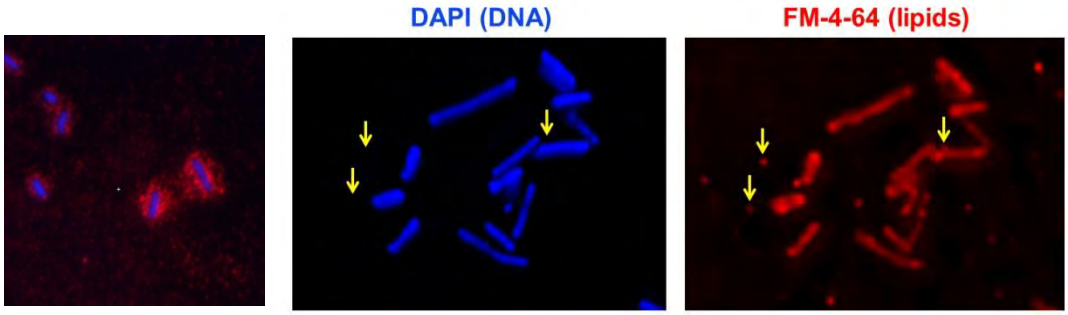


Figure 14. (left panel) Visualization of membranous vesicles produced by cells of *Xf* whose DNA is stained with DAPI and appearing blue in this image. Vesicles are red in this image due to their binding to fluorescently labeled anti-XadA antibodies. (Center panel) Visualization of cells of *Xf* stained with DAPI 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 *Xf* cells.

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. 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 attached to the *Xf* cells, 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 15). 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.

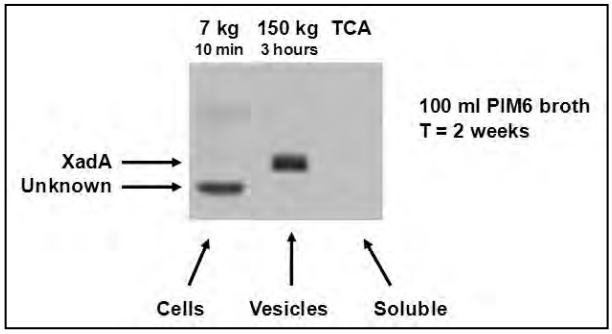


Figure 15. Quantification of the outer membrane protein XadA by Western blot analysis using anti-XadA antibodies from washed cells of *Xf* (left lane) as well as from material recovered from high-speed centrifugation of culture supernatants (center lane), or in proteins precipitated from culture supernatants after high-speed centrifugation by TCA (right lane).

Quantification of vesicles by both deconvolution fluorescence microscopy, flow cytometry, and a Nanovision particle counter 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. The *rpfF* mutant produces as much as three times more vesicles (Figure 16). While some vesicles were as large as approximately 1000 nm, the average diameter of vesicles was only approximately 150 nm (Figure 16. 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*.

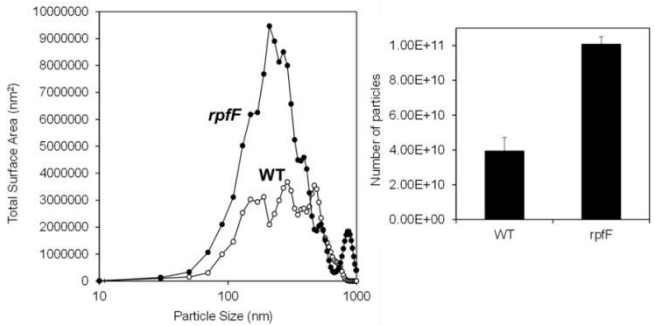


Figure 16. Distribution of sizes and abundance of vesicles of different sizes produced by a wild type and an *rpfF* mutant of *Xf* when grown for two days in PIM6 minimal media when assessed with a Nanovision device. Vesicles were determined directly in culture supernatants after removal of whole cells 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. 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 than of *E. coli* itself.

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 17). Substantially more vesicles were apparently present in the sap of plants infected with the RpfF mutant, consistent with our finding that such a mutant produces more membranous vesicles in culture.

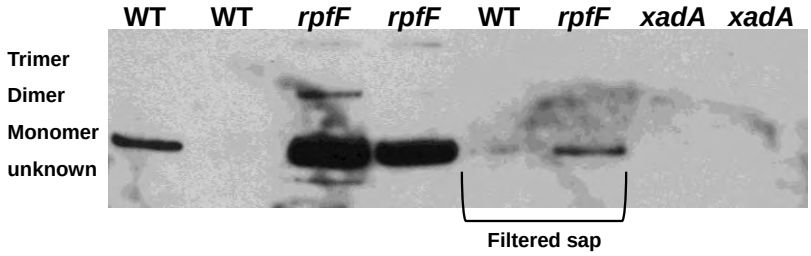


Figure 17. Abundance of XadA, indicative of the presence of membranous vesicles, as determined by Western blot analysis in xylem sap of Thompson seedless grapes infected with either a wild type or an RpfF mutant of Xf. Total XadA in sap which had not been filtered to remove intact cells are shown in 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*. 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 18). 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 18). 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 sap environment of plants infected with the wild type strain of *Xf* is also somewhat less conducive to adherence.

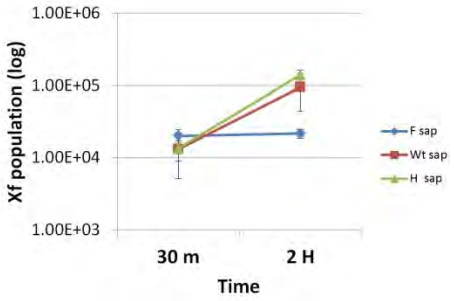


Figure 18. The number of cells of a wild type strain of *Xylella fastidiosa* that had adhered to wings of the glassy winged sharpshooter suspended in xylem sap from plants infected with an RpfF mutant (blue), or a wild type strain of *Xylella fastidiosa* (red), or from healthy plants (green) after incubation for either 30 minutes or 2 hours.

The ability of vesicles to interfere with binding of X.fastidiosa to surfaces such as insect wings also suggested that it would interfere with binding to plant surfaces, such as xylem vessels, thereby better enabling the movement of the pathogen through the plant. This was investigated by introducing cells of X.fastidiosa to grape stem segments in the presence or absence of purified membrane vesicles. Vesicles were collected by ultracentrifugation of cell free supernatants. Vesicles were than either resuspended in buffer or in culture media and cells of X.fastidiosa were then introduced into surface sterilized, 2 cm stem segments in buffer or culture media alone, or in such solutions containing membrane vesicles. After introduction into the stem segments and incubation for one hour, stem segments were flushed with sterile buffer to remove any unattached cells of X.fastidiosa. Population size of the attached X.fastidiosa were then determined by dilution plating. When cells were co-inoculated into stem segments with membrane vesicles suspended in buffer, there was a dramatic reduction (>20-fold) and the fraction of those cells which attached to the xylem vessels compared to that of cells introduced in buffer alone (Figure 19).

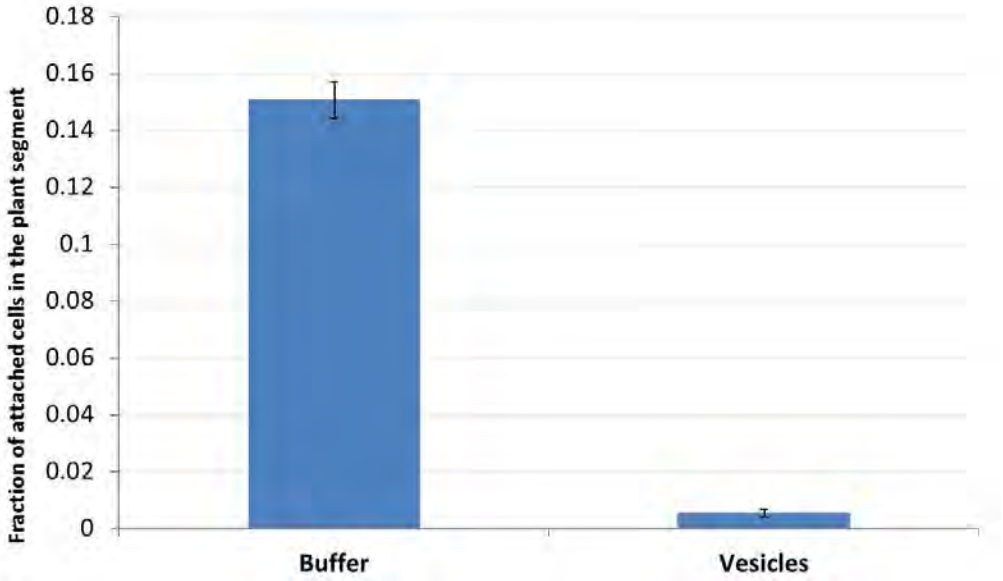


Figure 19: The fraction of total cells introduced into xylem vessels that were retained after one hour incubation when introduced in buffer alone or in phosphate buffer containing membranous vesicles of *X. fastidiosa*. The vertical bars represent the standard error of the mean of the fraction of attached cells.

This inhibition of binding of cells of X.fastidiosa to plant tissue also occurred in the presence of culture media. Again, a much reduced fraction of the total number of cells of X fastidiosa introduced into a stem segments in PIM6 medium containing membrane vesicles compared to that of cells introduced into PIM6 medium alone was observed. (Figure 20).

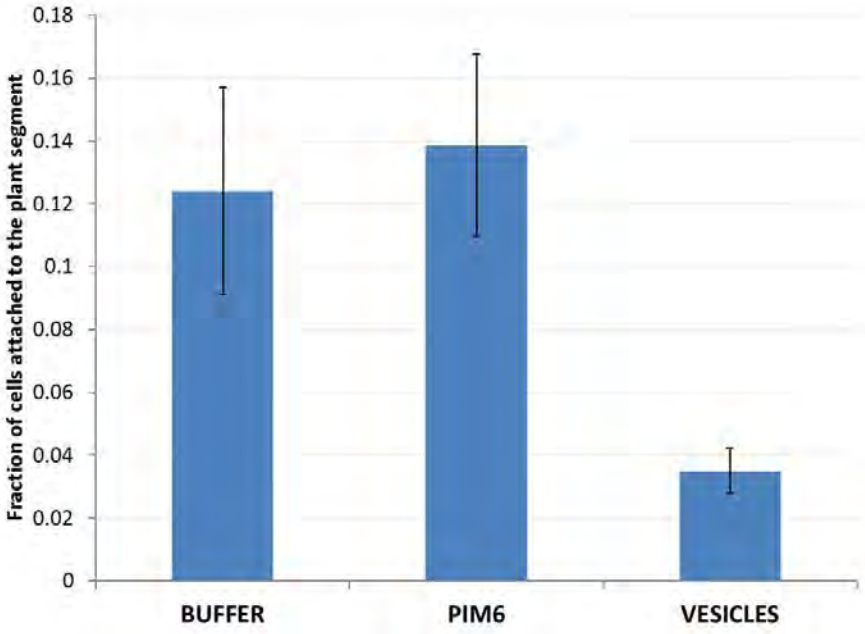


Figure 20: The fraction of total cells introduced into xylem vessels that were retained after one hour incubation when introduced in PIM6 medium or buffer alone or in PIM6 medium containing membranous vesicles of *X. fastidiosa*. The vertical bars represent the standard error of the mean of the fraction of attached cells.

To better assess the process by which the vesicles were interfering with the attachment of *X. fastidiosa* to plant tissues, we conducted similar experiments as above, but introduced the membrane vesicles in buffer alone, incubated the vesicles with the tissue for one hour before then flushing the vesicles out with buffer, before then introducing bacterial cells in buffer to the same stem segments. In this way, we enabled membrane vesicles to interact with plant tissue before, or instead of, bacterial cells themselves. This design enabled us to determine whether the process of finding of vesicles to plant tissues led to the blockage of finding of *X. fastidiosa* to the plants, or whether binding of the vesicles to the bacterial cells then prevented their binding to the plant tissue. It was clear however that prior treatment of the plant tissue with the vesicles conferred the same dramatic reduction in the ability of X.fastidiosa to bind to plants as well as the case when the cells and *vesicles were coinoculated into the plant (Figure 21).* It thus seems clear that vesicles prevent binding of X. fastidiosa to plant by preferential binding to the surfaces that the bacteria themselves might otherwise have bound to.

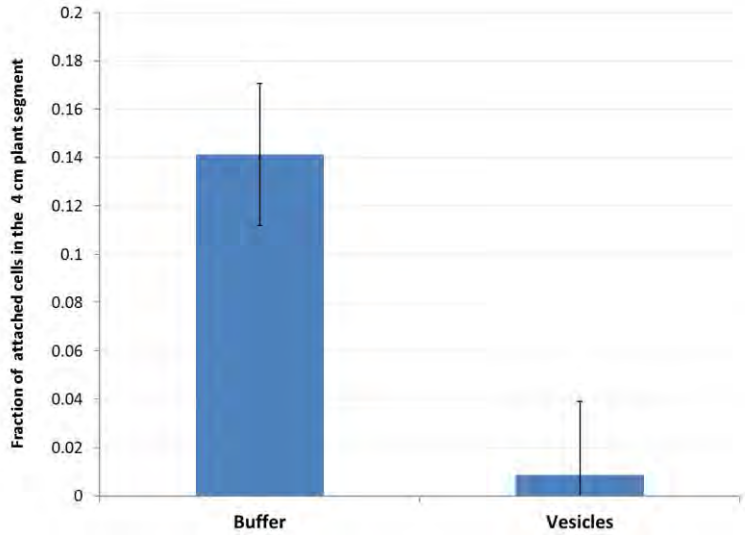


Figure 21: The fraction of total cells introduced into xylem vessels that were retained after one hour incubation when introduced into plants treated one hour earlier with either buffer alone or phosphate buffer containing membranous vesicles of *X. fastidiosa*. The vertical bars represent the standard error of the mean of the fraction of attached cells.

Publications:

Lindow, S.E., Newman, K., Chatterjee, S., Baccari, C., Lavarone, A.T., and Ionescu, M. 2014. Production of *Xylella fastidiosa* diffusible signal factor in transgenic grape causes pathogen confusion and reduction in severity of Pierce's disease. Molec. Plant-Microbe Interact. 27:244-254.

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 are very excited to find that a cheap, commercially available molecule palmitoleic acid also is quite active as a DSF signal molecule. 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 March, 2014, 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.

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.