

EXPLOITING PATHOGEN SIGNAL MOLECULES FOR CONTROL OF PIERCE'S DISEASE

Principal Investigator:

Steven Lindow
Dept. of Plant & Microbial Biology
University of California
Berkeley, CA 94720
icelab@berkeley.edu

Cooperators:

Dirk Trauner
Department of Chemistry
University of California
Berkeley, CA 94720
trauner@cchem.berkeley.edu

Ellen Beaulieu
Department of Chemistry
University of California
Berkeley, CA 94720
trauner@cchem.berkeley.edu

Reporting Period: The results reported here are from work conducted July 1, 2008 to October 1, 2008.

ABSTRACT

The movement of *Xylella fastidiosa* (*Xf*) in plants and insect transmission is controlled by a small diffusible signal factor (DSF) that accumulates when cells are at high cell densities. Pathogen behavior can be dramatically changed and disease reduced by altering the abundance of DSF in plants in a form of "pathogen confusion." To enable new strategies of pathogen confusion we have chemically characterized the DSF produced by grape strains of *Xf* as 2-Z-tetradecenoic acid (hereafter called C14-cis). The DSF is structurally related to, but distinct from, the DSF made by *Xanthomonas campestris* pv. *campestris* (*Xcc*). While an *Xcc* *eng:gfp* based biosensor for DSF can detect as little as about 1 μ M of DSF produced by *Xcc*, more than about 100 μ M of C14-cis is required for detection. Biological assays for the presence of C14-cis are being developed in *Xf*. As the expression of genes conferring type IV pili and thus twitching are suppressed while those involved in extracellular polysaccharide (EPS) production and production of various cell adhesins are induced in the presence of DSF in *Xf*, we are developing bioassays for C14-cis using an *rpfF* mutant of *Xf* that cannot produce DSF but which can respond to exogenous C14-cis. Twitching motility of the *rpfF* mutant was suppressed in the presence of as little as 1 μ M exogenous C14-cis while cell-cell adhesiveness and cell-surface adhesiveness was enhanced. Preliminary results indicate that *Xf* responds to C14-cis concentrations that are at least 10-fold less than that of the DSF produced by *Xcc* suggesting that the responsiveness of different DSF-producing bacteria is likely species specific; eg. they respond best to the DSF that they produce. Further bioassays based on immunological detection of cell surface adhesins or EPS as well as by quantifying mRNA associated with these genes in *Xf* are being developed. Sufficiently large amounts of C14-cis, as well as the sodium salt of this fatty acid, which is highly water soluble, have been produced and have been used as topical and injected treatments of grape that have subsequently been challenge inoculated with *Xf* for tests of disease control. We have designed and will soon initiate synthesis of DSF-analogs and test them for their ability to alter pathogen gene expression and behavior in culture as well as control disease.

INTRODUCTION

Research in the Lindow lab has provided considerable evidence for a diffusible signal factor (DSF) encoded by *rpfF*, which was considered likely to be a fatty acid derivative, that operates in quorum sensing and biofilm initiation in *Xylella fastidiosa* (*Xf*). *Xf rpfF*- mutants, blocked in production of DSF, exhibit increased virulence to plants, however, they are unable to spread from plant to plant by their insect vectors. We found that *Xf* colonizes grapevine xylem extensively, with many vessels harboring relatively few *Xf* cells and only a minority blocked by *Xf*. We thus believe that *Xf* has evolved as an endophyte that colonizes the xylem; blockage of xylem would reduce its ability to multiply and thus the DSF-mediated virulence system in *Xf* constrains virulence when cell density increases to high levels in the plant. Preliminary data indicate that DSF perception is central to the expression of a large number of genes in *Xf*, including those that are involved in virulence to plants as well as acquisition by insect vectors. DSF accumulation results in the expression of several fimbrial and afimbrial adhesins, resulting in the cells becoming "sticky" in the plant. DSF accumulation also results in the suppression of expression of extracellular enzymes such as polygalacturonases and endoglucanases that are required for erosion of pit membranes and hence movement through the plant. As the pathogen apparently acquires substantial nutrition from the degradation products of the pit membranes, DSF thus suppresses the multiplication in vessels as cell numbers, and hence DSF, accumulate. *Xf* thus appears to coordinate its behavior in a plant to have both an "exploratory" phase (non-sticky cells highly expressing pit membrane-macerating enzymes) that enable it to spread widely through the plant but not be easily acquired and transmitted by insect vectors, that occurs until cells start to become locally abundant. This phase is followed by an "acquisition phase" (sticky cells that no longer express extracellular enzymes) in a subset of the cells that are maximally transmitted, by insects. Thus, because the plant lifestyle (as an endophyte) conflicts with its ability to adhere to insects and be transmitted the pathogen apparently takes on a "bi-polar" lifestyle of two different physiologies that are adapted for plant invasion and insect transmission, respectively. DSF serves as the switch to coordinate the plant lifestyle and convert cells into the insect acquisition phase.

Our earlier work demonstrated that the severity of Pierce's disease is reduced when the levels of DSF are increased in the plant in various ways. For example, the severity of Pierce's disease is greatly reduced when DSF-producing bacteria are co-inoculated with *Xf* into grape or when DSF expression is enhanced in *Xf* itself. In a direct approach to altering DSF levels in plants we have transformed grape with the *rpfF* gene from *Xf*. Large numbers of clonal *rpfF*-expressing grapes have been produced and inoculated with *Xf* to test for susceptibility to Pierce's disease. In very exciting results, the DSF-expressing grapes are MUCH less susceptible to Pierce's disease. The severity of disease was reduced over 10-fold compared to non-

transformed plants. While *Xf* spread throughout non-transformed plants causing disease on petioles located great distances from the point of inoculation, disease was observed only very close to the point of inoculation in *rpfF*-expressing plants. A major goal of this proposal is to determine the structure of *Xf* DSF so that it and analogs can be evaluated in a strategy of control of diseases caused by *Xf* that rely on “pathogen confusion”. Synthetic DSF and analogs will be made and tested for efficacy in controlling Pierce’s disease by introducing these materials on or into the plant in various ways.

OBJECTIVES

1. Identification and characterization of low molecular weight signaling molecule (DSF) central to behavior of *Xf*.
2. Design and synthesize low molecular weight compounds capable of interfering with signal molecule function in *Xf*.
3. Evaluate efficacy of signal analogs for control of disease and insect transmission of *Xf*.

RESULTS AND DISCUSSION.

Objective 1. Characterization of DSF. We determined the conditions that led to optimum production of DSF by *Xf* and surrogate hosts. An *rpfC* mutant of *Xf* that is de-repressed for DSF production was cultured in defined media for the harvest of signal molecules. We found that an *RpfC*- mutant of *Xf* produces about 11-fold more DSF than a wild type strain. We also expressed *rpfF* from *Xf* in *E. coli* and *Erwinia herbicola* strain 299R under strong promoters. The yield of DSF from these surrogate hosts was much larger than even from the *rpfC* mutant of *Xf* because of the much larger number of cells that could be produced in culture. We obtained more than 100-fold more DSF than normally produced by a comparable number of *Xf* cells in such surrogate hosts, and found that that *E. herbicola* is a superior surrogate host.

The scheme depicted in **Figure 1** was used to isolate and characterize the DSF from *Xf*. Initial characterization of DSF was made from the large amounts of DSF produced in these surrogate hosts. DSF was extracted from culture media using ethyl acetate partitioning. Among several fractions from separations of materials made from these crude extracts made by flash column chromatography, the fraction containing organic acids showed higher activity in an *Xcc* DSF bioassay than other fractions above the background. The *Xf* DSF isolated from reverse phase HPLC of the active fraction showed NMR spectral data consistent with a fatty acid containing one site of unsaturation. The DEPT 135 indicates that this is a straight chain acid with no branching. Spectral data suggest the *Xf* DSF has a molecular formula of $C_{14}H_{26}O_2$. The methyl ester was synthesized for GCMS analysis. The methyl ester has a molecular formula of $C_{15}H_{28}O_2$ which means the *Xf* DSF has a formula of $C_{14}H_{26}O_2$. DSF was then extracted from *Xf* and used to verify that the compounds made by *Xf* and the surrogate hosts are the same. *Xf* was grown on periwinkle wilt (PW) gel in solid culture. From 200 plates (~4 L volume), we were able to obtain 0.8 mg of the *Xf* DSF. The gel medium was cut into 0.4 x 0.4 cm squares and sonicated with twice the volume of ethyl acetate. Extracts were purified by flash column chromatography and HPLC as described above. The isolable active compound (DSF) from *Xf* was identified as 2-*Z*-tetradecenoic acid (hereafter called C14-*cis*). Isolates from an *rpfF* mutant of *Xf* strain did not produce C14-*cis*. The putative *Xf* DSF was synthesized using a Still-Gennari olefination followed by saponification (**Figure 2**). The spectral data for the acid isolated from *E. herbicola* match those obtained for the synthetic 2-*Z*-tetradecenoic acid.

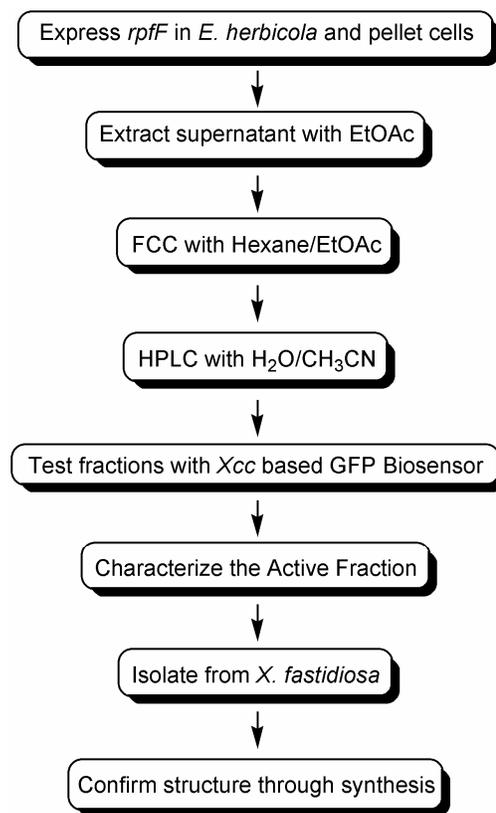


Figure 1. Process by which *Xf* DSF was isolated and characterized.

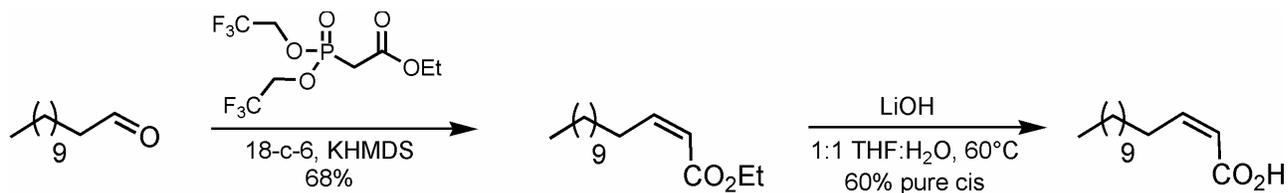


Figure 2. Synthesis of C14-cis.

Based on the finding that the DSF from the *E. coli* and *E. herbicola* surrogate hosts harboring *Xf rpfF*, and that isolated from *rpfC* mutants of *Xf* were the same and that all matched that the synthetic material, we conclude that DSF from *Xf* is C14-cis (**Figure 3**). The putative DSF from *Xf* differs somewhat from the DSF made by *Xcc* in that it has a longer, but unbranched acyl chain (**Figure 4**).

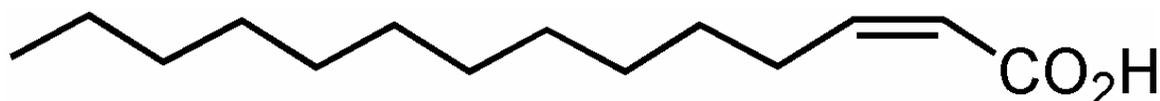


Figure 3. Putative structure of C14-cis, the DSF made by *Xf*.

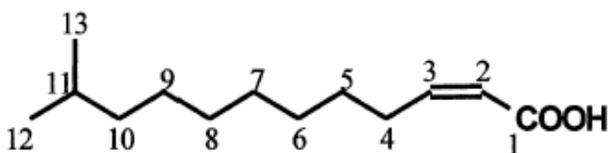


Figure 4. Structure of DSF made by *Xanthomonas campestris*.

The biological activity of C14 cis was assessed using the *Xcc* based biosensor *Xcc* 8523 (pKLN55). In this biosensor *gfp* fluorescence conferred by cells harboring an *eng:gfp* reporter gene fusion that is responsive to *Xcc* DSF is measured. While the *Xcc* based biosensor for DSF can detect as little as about 1 μ M of DSF produced by *Xcc*, more than about 100 μ M of C14-cis is required for detection. (**Figure 5**). It is important to note that the biological activity of C14-cis was much less than that of *Xcc* DSF; this was expected as earlier work had revealed that while the *Xcc* biosensor could detect DSF from *Xf* the signal was much lower than from a corresponding amount of cells of *Xcc*. It is also clear that the trans form of the C14 enoic acid has no biological activity in this assay (**Figure 5**).

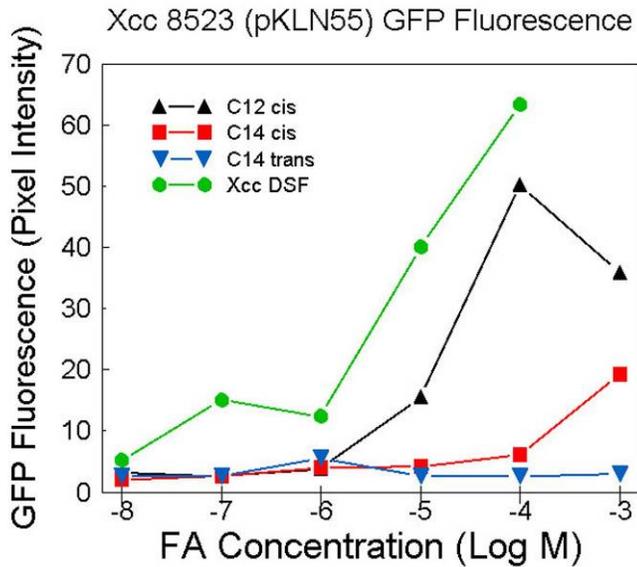


Figure 5. Dose response relationship for DSF from *Xcc* and that from *Xf* as well as other related enoic acids.

Biological assays for the activity of C14-cis are also being developed in *Xf*. As the expression of genes conferring type IV pili and thus twitching are suppressed while those involved in EPS production and production of various cell adhesins are induced in the presence of DSF in *Xf*, we are developing bioassays for C14-cis using an *rpfF* mutant of *Xf* that cannot produce DSF but which can respond to exogenous C14-cis. Twitching motility of the *rpfF* mutant was suppressed in the presence of as little as 1 μ M exogenous C14-cis while cell-cell adhesiveness and cell-surface adhesiveness was enhanced (Figure 6). Preliminary results indicate that *Xf* responds to C14-cis concentrations that are at least 10-fold less than that of the DSF produced by *Xcc* (Figure 7) suggesting that indicating that the responsiveness of different DSF-producing bacteria is likely species specific; eg. they respond best to the DSF that they produce.

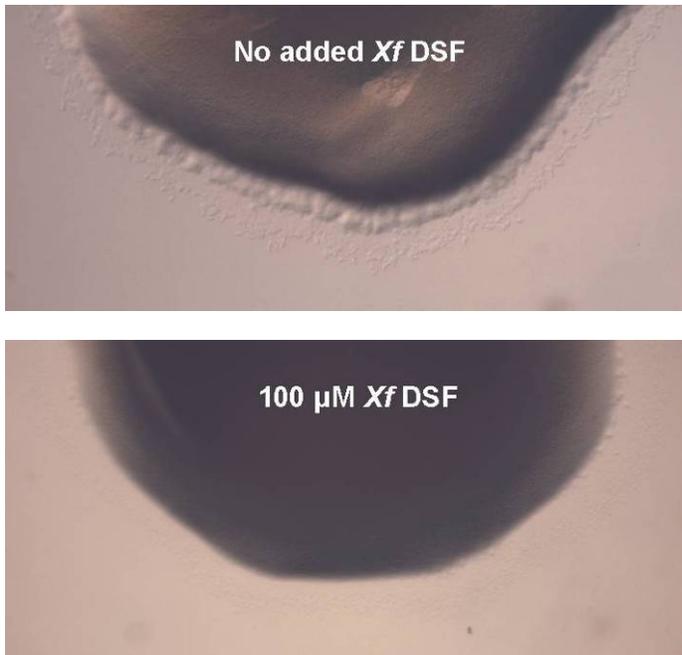


Figure 6. Twitching motility of *Xf* evident as a fringe around the colony of an *rpfF* mutant (top) on PWG medium but not around the colony when grown on medium containing C14-cis.

Activity in Xylella Fastidiosa

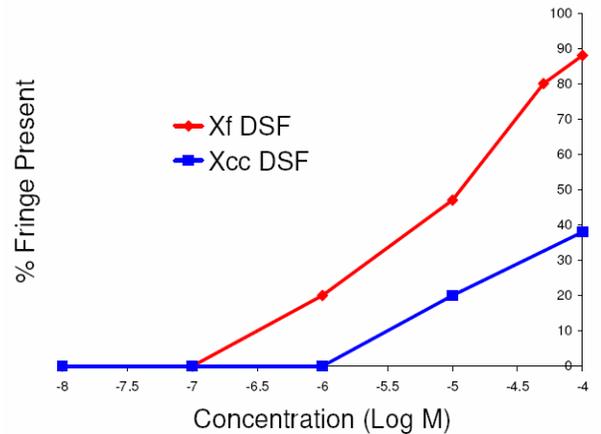


Figure 7. Inhibition of twitching activity of an *rpfF* mutant of *Xf* in the presence of different concentrations of DSF from *Xf* and *Xcc*.

Further bioassays based on immunological detection of cell surface adhesins or EPS as well as by quantifying mRNA associated with these genes in *Xf* are being developed to better assess the activity of DSF and synthetic analogs in future

experiments. The current bioreporter for DSF that we developed earlier is based on an *eng:gfp* fusion that is expressed in *Xanthomonas campestris* pv. *campestris* (*Xcc*) (it was known that the endoglucanase gene of *Xcc* was induced in the presence of DSF). The *Xcc* DSF biosensor (8523/PKLN55) will detect DSF of *Xf* but we have now shown it to be much less responsive to C14-cis. This may be due to considerable differences in the components involved in DSF sensing like *RpfC* and *RpfG* which are hybrid two component sensor and response regulators in *Xcc* and *Xf*. We thus will produce improved biosensors by two different means: A) The *rpfC* and *rpfG* genes from *Xf* that are believed to be required for signal transduction in the presence of DSF will be used to replace these homologs in *Xcc*. To increase the sensitivity of *Xcc* biosensor for *Xylella* DSF, we will express the whole DSF signal transduction component (*RpfC*, *RpfG* and *RpfE*) of *Xf* in an *rpfF*-*Xcc* mutant background. In this strategy, we will clone the entire operon of *rpfC*, *rpfG* and *rpfE* of *Xf* and insert the operon in a construct containing the flanking sequence of the *Xcc* *rpf* genomic region. The entire region will be recombined in the *rpfF*- and wild type *Xcc* background. We have already made constructs which can express high levels of *Xf* *rpfC*. Thus this *Xcc* bioreporter should respond more efficiently to DSF from *Xf*; and B) as an alternative, we will take advantage of the fact that we now know what genes in *Xf* are induced in the presence of DSF. For example, we now know that *gumJ*, involved in extracellular polysaccharide (EPS) biosynthesis is strongly induced in the presence of DSF from *Xf* and that DSF-deficient strains produce noticeably less EPS in culture. We will fuse this gene to a *gfp* reporter gene and introduce it into the genome of *Xf* by homologous recombination to yield cells of *Xf* that will become green fluorescent in the presence of DSF. Such cells should be much more responsive to *Xf* DSF and be useful in assaying biochemical fractions for DSF in the purification processes below and in assaying DSF analogs. Alternatively, we can detect EPS production by *Xf* both in culture and in plants by use of antibodies that recognize the EPS of *Xf*. Such antibodies have recently been described by the group of Bruce Kirkpatrick. We expect that DSF-deficient *RpfF*- mutants of *Xf* will exhibit little or no EPS production as monitored by use of fluorescently-labeled antibodies directed against EPS. A GFP-marked *RpfF*- strain of *Xf* could be used as a DSF detector both in culture and *in planta* by examining co-localization of constitutive GFP fluorescence and red fluorescence when a red-fluorophore-labeled anti-EPS antibody is applied to a sample; GFP fluorescent cells that were not also labeled with the antibody stain would indicate lack of DSF availability while cells that were both GFP and red fluorescent would indicate the presence of DSF.

Objective 2. Design and synthesize DSF analogs. We have made several synthetic analogs of C14-cis for testing for biological activity in *Xf* (**Figure 8**). As these materials have only recently been synthesized, the biological activity of most have not yet been assayed. As noted above and as expected, the trans variant of the C14 enoic acid exhibited no activity in any of the biological assays performed today. In addition to the DSF analogs noted in **Figure 8**, various halogenated variants will also be synthesized.

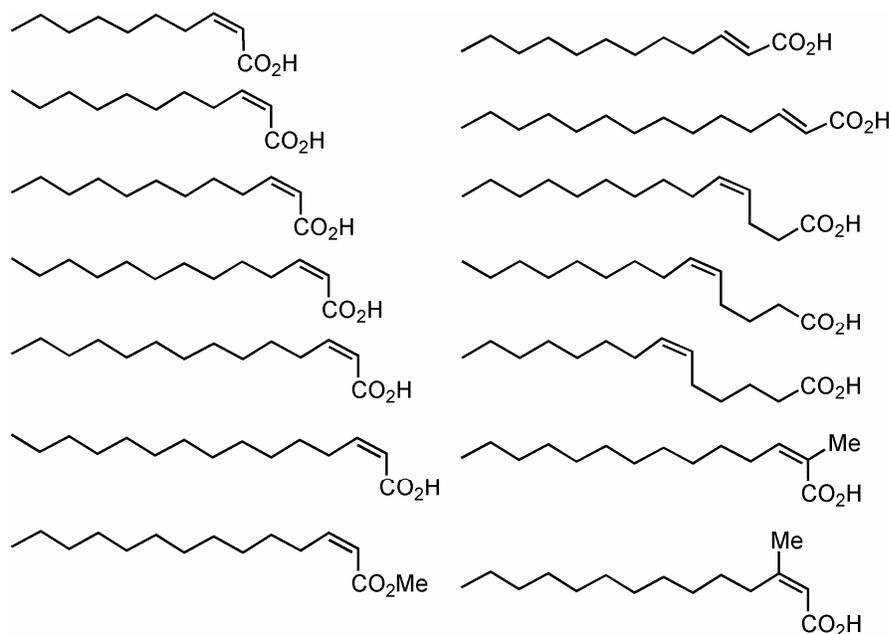


Figure 8. Analogs of the DSF produced by *Xf* that have been synthesized to date.

Objective 3. Synthesis of sufficient DSF analogs for *in planta* evaluations. We have synthesized gram quantities of C14 cis as well as the Sodium salt of this fatty acid which is highly water soluble. These quantities are sufficiently large for initial greenhouse studies. These materials have been sprayed onto leaves as well as injected into stems and used as a soil drench in

initial studies to determine their efficacy for disease control. After treatment plants have been challenge inoculated with *Xf* and disease incidence will be measured; the first disease symptoms are expected by mid-November.

CONCLUSIONS

The DSF produced by grape strains of *Xf* has tentatively been characterized by C14-cis. Both its relatively higher biological activity as assessed in *Xf* than that of the DSF from *Xcc* and lesser activity in an *Xcc* bioassay is as expected, indicating that there is considerable specificity in the structure-function relationships between different bacterial DSF signal molecules. The production of sufficient *Xf* for testing for pathogen confusion has been shown to be possible and we are anxiously awaiting initial tests to determine if topical applications of the material can lead to disease control via pathogen confusion.

FUNDING AGENCIES

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

***Section 5:
Crop Biology
and
Disease Epidemiology***



