Final Report for CDFA Contract Number 05-0588

Project Title: Management of Pierce's disease of grapevine by interfering with cell-cell communication in *Xylella fastidiosa*

Time period: July1, 2002 to July 1, 2008

PRINCIPAL INVESTIGATOR
Steven E. Lindow
Department of Plant and Microbial Biology
University of California, Berkeley 94720
Cooperator:
Subhadeep Chatterjee
Department of Plant and Microbial Biology
University of California, Berkeley 94720

OBJECTIVES:

1) Pathogenicity tests on grapevines colonized by DSF-interfering bacteria to determine potential for Pierce's disease control
2) Isolation of mutant strains of DSF-degrading and DSF-producing bacteria that can no longer interfere in cell-cell signaling to verify that disease control is linked to cell-cell signal interference
3) Molecular identification of genes conferring DSF-degrading activity
4) Engineer the grapevine endophytes *Alcaligenes xylosoxidans denitrificans* and *Agrobacterium vitis* to express genes conferring DSF-degradation and DSF-synthesis activities and test whether the resulting transgenic endophytes are capable of disease control
5) Creation of grapevines expressing genes conferring DSF-degradation and DSF-synthesis activity to test for PD resistance
6) Evaluate topical application of DSF-degrading and DSF-producing bacteria with penetrating surfactants for PD control

RESEARCH ACTIVITIES AND ACCOMPLISHMENTS:

Objective 5. Production of DSF in transgenic plants for disease control.

We have expressed the *rpfF* gene in several different plant species to investigate whether DSF excess can lead to reduced disease caused by *Xf*. In addition to grape, we have transformed genes conferring DSF production into tobacco since this species is colonized by *Xf* and disease symptoms can be produced (Fig. 1). Because transformation of tobacco is much quicker than grape, we have used studies of *Xf* infection of tobacco as a surrogate for studies in grape to speed our assessment of different ways to produce DSF in grape. The various mutants of *Xf* that are hyper and hypo virulence on grape yield similar reactions on tobacco (Fig. 2).

Figure 1. Symptoms caused by Xf on SR1 tobacco

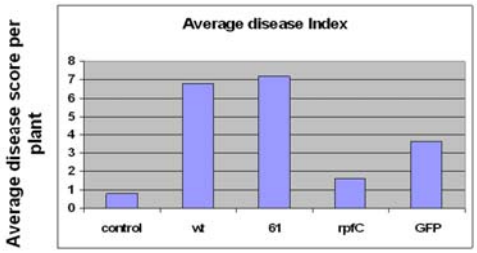

Figure 2 Disease fromWT Xf, rpfF- (61) an rpfC- mutant, and a gfp-marked strain on SR1

Grape has been transformed at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis. Initially, we submitted a tested but un-optimized *rpfF* construct to the facility. These plants produced only very low levels of DSF. Importantly, the *rpfF*-expressing grape are MUCH less susceptible to Pierce's disease. (Fig. 3). 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. We thus expect to find that *Xf* is limited in its movement in plants having even higher levels of DSF due to the expression of *rpfF*, in a manner similar to what we have observed in DFS-overproducing strains of *Xf*.

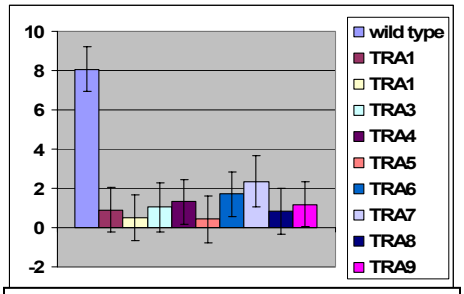

Fig. 3. Disease severity (# symptomatic leaves/plant) on Freedom grape transformed with the r*pfF* gene encoding DSF production and inoculated with *Xf*.

Enhancing the DSF levels in plants. We have recently transformed tobacco and *Arabidopsis* with an *rpfF* gene that has been modified to direct the protein product to the chloroplast where fatty acid synthesis (and DSF synthesis) should be much enhanced compared to its production in the cytosol, the presumed location of RpfF in the current transformed plants. Assay of DSF in transgenic SRI tobacco plants-where the RpfF is targeted to the chloroplast, indicates that the DSF levels as well as expression of *rpfF* are much higher as compared to the plants in which the RpfF is expressed in the cytosol. Transcription analysis of the chloroplast targated *rpfF* transformed plants indicates high level expression of the *rpfF* gene (Fig. 4). We have generated seeds from the transgenic SRI tobacco plants and we are conducting pathogenicity assays with *X. fastidiosa* comparing these enhanced producing plants with normal and untargeted RpfF plants.

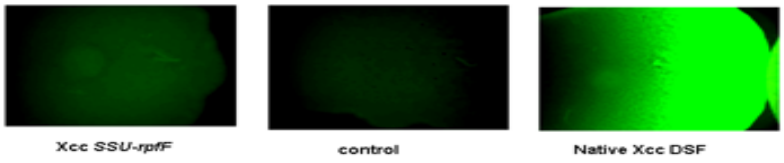

Fig. 4. DSF extracted from transgenic tobacco SR1 plants expressing the chloroplast-targeted RpfF, compare to nontransgenic SR1 and native DSF extracted from Xcc. The DSF is spotted at the right hand side on a filter disc and the *Xcc* DSF bioindicator is streaked on the left side of the spot. The green GFP fluorescence is indicative of DSF production

We have also initiated transformation of grapes with the improved chloroplast targeted *rpfF* constructs at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis. We expect to receive the transformed plants by mid-October, 2008, and then will grow them to larger sizes, make green cuttings to produce enough plants for pathogenicity

testing by early 2009. Although RpfB is not required for DSF synthesis in *Xf*, it presumably aids in DSF synthesis by encoding long chain fatty acyl CoA ligase which might increase availably of the appropriate substrates for DSF synthesis by RpfF. We expect that co-expression of RpfB and RpfF in the chloroplast will further enhance the DSF levels in plants. We have produced transgenic *Arabidopsis* plants with such a construct and find evidence of high levels of DSF production. Pathogenicity assays with the *rpfF* mutant of *Xanthomonas campestris* pv. *campestris* (Xcc) indicated that the transgenic plants can complement the virulence of the non pathogenic *rpfF* mutant of *Xcc* (Table 2). Importantly, transgenic plants expressing both *rpfB* and *rpfF* were more susceptible to the *rpfF* mutant of *Xcc*, indicating enhanced DSF levels. Given this evidence of enhanced DSF production in transgenic *Arabidopsis*, and recent results with similarly-transformed tomato, we are initiating transformation of grape with similar constructs.

Table 1. Disease severity from topical application of bacteria varying in DSF production to Arabidopsis. Bacteria were inoculated on different *Arabidopsis* genotypes transformed with *rpfF* or with both-*rpfB* and *rpfF*

Arabidopsis Genotype	**Xcc strains**	
	Wild type	*rpfF*
Col (wild type)	++++	-
rpfF transformed	++++	+
rpfF and *rpfB* transformed	++++	++

Direct application of DSF to plants. We have tested whether application of DSF directly to non-transgenic grape can confer control of Pierce's Disease. While we are in the process of identifying the chemical structure and synthesis of DSF, for these studies we used crude ethyl acetate extracts of a DSF-producing *E. herbicola* strain as a source of DSF. The DSF was either topically applied as well as needle inoculated into the stems of grape either once a day before inoculation with *Xf* or weekly. While a single needle application of DSF reduces disease index, a weekly application of DSF into the stem of the plant was much more effective (Fig. 5). These results are exciting in that they suggest that disease control from external applications of DSF might be a practical means of disease control. We have recently been successful in determining the structure of *Xf* DSF and have synthesized gram quantities of DSF. Plants have recently been treated with topical and injected synthetic DFS and then inoculated with *Xf*; disease assessment will commence in mid-October.

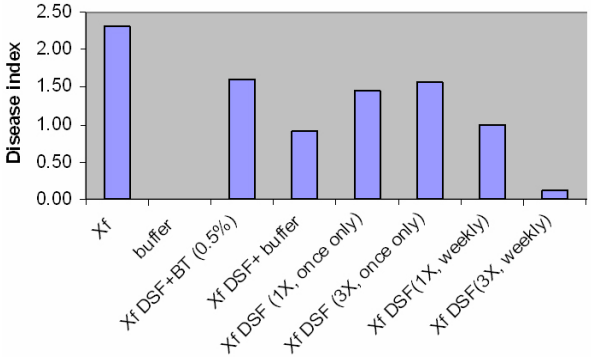

Figure 5. Disease incidence-severity relation (disease index) for grape inoculated with *Xf* and to which DSF was topically applied or introduced into the stem

Graft transmissibility of DSF. To test whether DSF is mobile within the plant we are performing grafting experiments in which DSF-producing tobacco transformed with the rpfF of *Xf* are used as rootstocks to which normal SR1 tobacco is grafted as a scion (Fig. 6). Over 100 of such grafted plants have now been made, and they have been inoculated with *Xf* to test whether normal SR1 scions on DSF-producing rootstocks have a lower susceptibility to *Xf* colonization;

disease will be rated within about 6 weeks. Non-chloroplast targeted RpfF-expressing transgenic Freedom grape plants are currently being propagated to obtain enough plants to serve as rootstocks to test with *Xf* inoculations in larger scale studies. Over 100 such plants have now been propagated and green-grafting of Cabernet Sauvignon is being conducted to produce grafted plants with a normal Freedom and a DSF-producing Freedom rootstock. Initial attempts at green grafting of grape produced a low frequency of successful grafts, but a new procedure is providing a satisfactory level of graft success; the grafted plants are now nearly large enough to inoculate with *Xf* to test for graft transmissibility of DFS.

Figure 6. Grafted tobacco plants into which *Xf* has been inoculated. A normal SR1 tobacco scion is grafted onto transgenic DSF-producing tobacco. The graft point is noted with blue tape, and XF has been inoculated above the graft union. The plant is as yet asymptomatic

Objectives 1 and 4. Disease control with endophytic bacteria. Producing DSF in bacterial endophytes. We have been successful in producing large quantities of DSF in endophytes like *Erwinia herbicola* and also in lab strains of *E. coli* (Table 2). We recently were able to transform a putative efficient endophyte of plants, *Rizobium etili* G12 with both the *Xcc* and *Xf rpfF* (DSF biosynthetic gene) and have obtained production of DSF in this strain (Fig. 7). This DSF-producing endophyte has been inoculated into grape to determine both its ability to move and multiply within grape as well as its ability to interfere with the disease process. The *R. etli* strain G12 was found to move within grape tissue after inoculation into either the stem or the leaves. When measured 4 weeks after inoculation by puncture inoculation into one site in the stem measurable populations of *R. etli* were seen as far as 50 cm away from the point of inoculation (Fig. 8). While the population size away from the point of inoculation were relatively low in this short time interval since inoculation, this strain clearly has the ability to move within grape; we are excited about this result since no other of several bacterial strains that we have investigated for the ability to move within grape has ever exhibited any ability to move beyond the point of inoculation. Further studies are underway to determine the population sizes to which *R. etli* will grow given more time after inoculation. *R. etli* also has the ability to move within grape leaves and multiply to high population sizes. When applied as a point source to leaves using a penetrating surfactant, cells of *R. etli* could be found up to 3 cm away within 1 week, and population sizes of this strain increased 100-fold within 3 weeks after inoculation (Fig. 9). Studies are continuing to determine the maximum population size that this strain can achieve in grape leaves.

Fig.7. Endophytic strains of *R. etili* producing *Xcc and Xf* DSF. The GFP florescence produced by an *Xcc* DSF biosensor which is sprayed on the plates, is indicative of DSF production

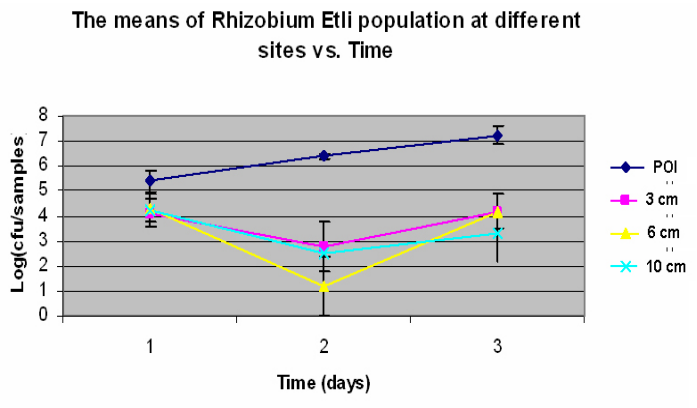

Fig. 9. Population size of *R. etli* in leaves one, two, and three weeks after inoculation at a single point.

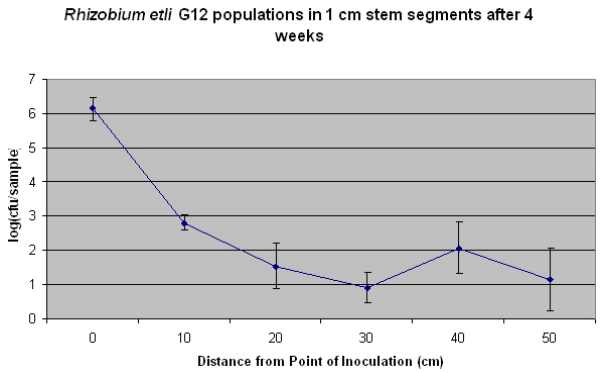

Fig. 8. Population size of *R. etli* in stems 4 weeks after inoculation at one point.

Various DSF-producing bacteria were tested for their ability to control Pierce's disease when applied to grape in different ways. DSF-producing *R. etli* were both needle inoculated one or more times at sites near where *Xf* was subsequently inoculated, as well as co-inoculated with *Xf* into grape stems and sprayed onto leaves with 0.5% of the penetrating surfactant Breakthru 1 week before *Xf* was inoculated into stems. The co-inoculation of *R. etli* with *Xf* greatly decreased the incidence of colonization of grape petioles compared to control plants inoculated with *Xf* alone (Fig. 10) while topical application or injection elsewhere in the stem provided little control. We presume that the relatively slow movement of *R. etli* in the stems of plants (Fig. 8) explains why co-inoculation was most effective. *R. etli* was somewhat susceptible to damage from Breakthru and its population sizes were reduced during application with this detergent. We will continue to test different ways in which *R. etli* can be introduced into plants to determine its ability to control PD. We expect that introduction of *R. etli* into stems far in advance of *Xf* will provide much better disease control. RpfC- mutants of *Xf* greatly over-produce DSF so we tested them for their ability to control PD when applied in various ways as discussed above for *R. etli*. The incidence of colonization of grape petioles with *Xf* was greatly reduced when plants were needle inoculated into grape either two weeks before plants were inoculated with *Xf* or when

coinoculated with the pathogen (Fig. 11). While the RpfC mutant does not move as well within grape as the wild-type *Xf*, its presence locally in plants can suppress the movement of wild-type *Xf* and thus lead to control of PD. These studies are promising and are being repeated.

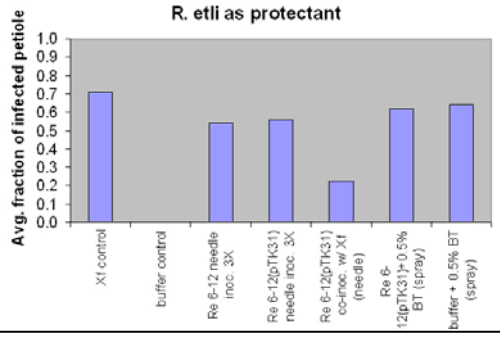

Fig. 10 Incidence of colonization of petiols of grape by *Xf* when plants were treated with DSF-producing *R. etli* in various ways.

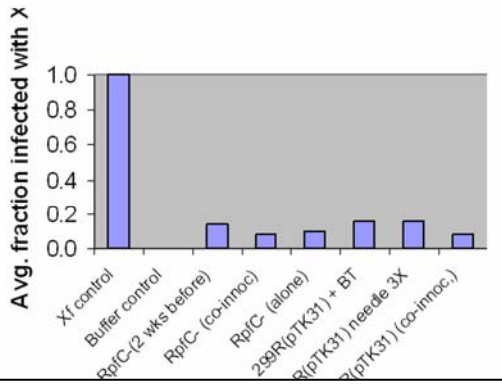

Fig. 11 Incidence of colonization of petiols of grape by *Xf* when plants were treated with RpfC mutants of *Xf* in various ways.

Detection of DSF in plants. Development of an *Xcc* biosensor efficient in detecting *Xylella* DSF. For many of the objectives of this project, in addition to the study of DSF degradation in plants an improved bioindicator for DSF would be very valuable. We are presently using an *Xcc*-based biosensor in which the endoglucanase gene is linked to a GFP reporter gene. Previous studies have shown that this biosensor is able to detect the DFS made by *Xf* but that it detects *Xf* DSF with a lower efficiency then the *Xanthomonas* DSF since the two molecules apparently differ slightly. We have devised a strategy to develop a surrogate *Xcc* biosensor system which will express all the components of DSF signal transduction of *Xf*. This should give rise to a system which is close to DSF signal transduction system in *Xcc*. We have made two different *Xanthomonas* strains in which the endogenous signal synthesis as well as signal recognition system (consisting of the hybrid two component RpfC and RpfG response regulators) has been knocked out. In one of these strains the DSF signal synthase rpfF and the DSF signal sensor RpfC has been knocked out (Fig 12). We have also made an *Xcc* strain in which the DSF synthase gene *rpfF* has been knocked out in a background of a RpfCHG deletion.
These mutants will enable us to express the *Xf* RpfC-RpfG two component system and should serve as a more sensitive surrogate host biosensor. Completion of the biosensor is expected within 3 more months. It then will be applied to the study of *Xf* DSF stability in plant extracts as originally proposed.

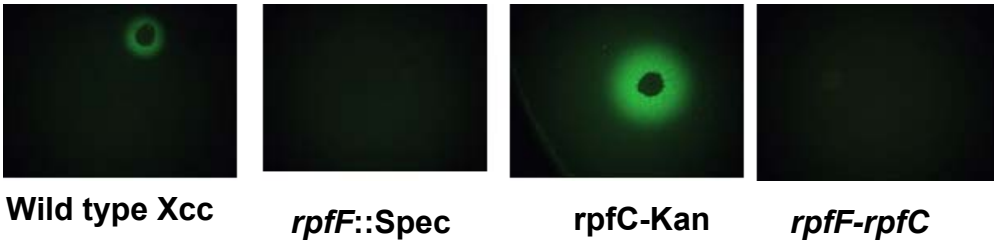

Fig. 12 Different *Xcc* mutants constructed to serve as surrogate host for expressing the *Xf* RpfC-RpfG two component DSF signal transduction system. The presently used *Xcc* biosensor 8523/pKLN55 is sprayed over the colonies. Presence of DSF is detected by the GFP fluorescence of the biosensor

We also are investigating the use of *Xf* itself to detect DSF**.** Among the several genes that we know to be regulated by DSF, those genes most strongly regulated include *pil* genes involved in twitching motility, several genes such as *fimA* and *hxfA* and *HxfB* which are involved in cell-surface adhesion, and gum genes involved in production of EPS. We thus have examined the phenotypes of an *rpfF*- mutant of Xf exposed to different amounts of DSF to determine if it can be used to bioassay for the presence of DSF. Initial results are encouraging. Suppression of twitching motility of the *rpfF*- mutant was observed when DSF was added at concentrations greater than about 10 uM (Fig. 13). Likewise, cells of the *rpfF*- mutant which were not adherent, and thus which did not form cell-cell aggregations became much more adherent to each other when DSF was added at concentrations greater than about 10 uM (Fig. 14). Thus it appears that we can assess the concentration of DSF in samples using either a cell twitching assay or a cell adhesion assay using *Xf* cells, although both assays are time consuming and somewhat qualitative.

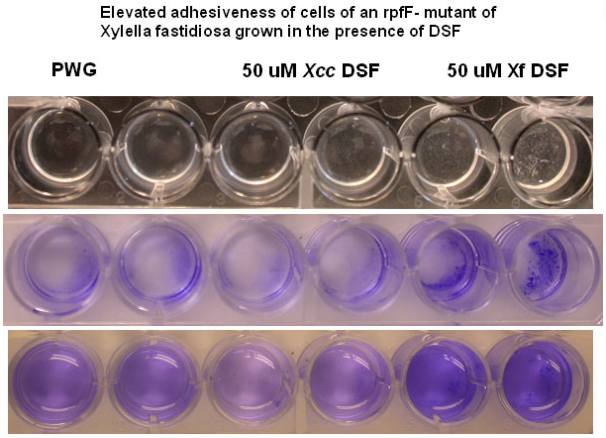

Fig 13

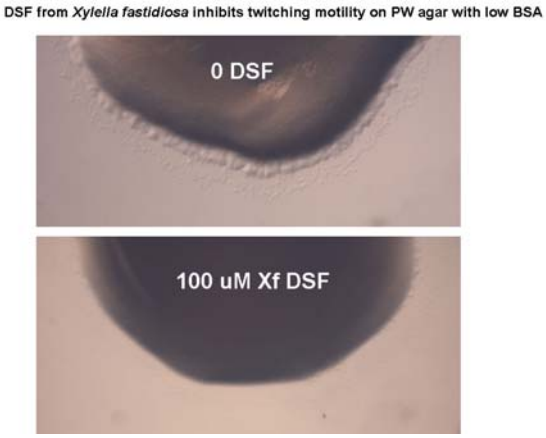

Fig 14

Initial results have shown relatively little induction of EPS production in an *rpfF*- mutant of *Xf* by the addition of DSF; little EPS was observed whether DSF was added to culture medium or not. We are investigating in cooperation with Rodrigo Almeida other medium contents which might be needed for EPS production and have very preliminary evidence that EPS production can be stimulated by DSF under the correct culture conditions. EPS abundance will then be measured both chemically and immunologically as an estimator of DSF abundance.

Objectives 2 and 3: DSF degradation as a means of disease control

Several bacterial strains were identified that had the ability to interfere with DSF-mediated signaling in Xanthomonas campestris. These bacteria represented several different taxa. Table 2. In most cases, it appeared that the ability of these bacteria to block signaling was due to enzymatic degradation of DSF.

Table 2. Characteristics of diffusible signal factor (DSF)-interfering bacterial strains isolated from various plants and laboratory strains

Strain name	Identity[a]	% Identity[b]	Origin	DSF degradation[c]	Mechanism of DSF inhibition
A	*Paenibacillus paduli*	96	Grapevine	+++	Unknown
B	*Paenibacillus paduli*	97	Grapevine	+++	Unknown
C	*Pseudomonas* sp. Bsi20664	99	Cabbage	+++	Enzymatic degradation
D	*Staphylococcus pasteur*	99	Grapevine	+++	Unknown
E	*Bacillus cereus*	99	Broccoli	+++	Enzymatic degradation
G	*Pseudomonas* sp. strainBsi20664	99	Cabbage	+++	Enzymatic degradation
H	*Pseudomonas jessenii Ps06*	99	Cabbage	+++	Enzymatic degradation
J	*Pseudomonas sp. Fa2*	99	Tomato	+++	Enzymatic degradation
L	*Staphylococcus sp. es1*	99	Grapevine	++	Unknown
	Escherichia coli (DH5α)		Lab collection	+	Unknown

[a] Identity of taxa with closest BLAST score to the sequence of the 16S RNA gene of test strain.
[b] Percent identity of the 16S RNA gene in the RDB database to the closest match to test strain.
[c] Semiquantitative estimates of DSF degradation ability based on plate inhibition assay.

The incidence of infection of grape with *X. fastidiosa* was greatly lower when the pathogen was co-inoculated with one of several different DSF-degrading bacteria (Figure 15).

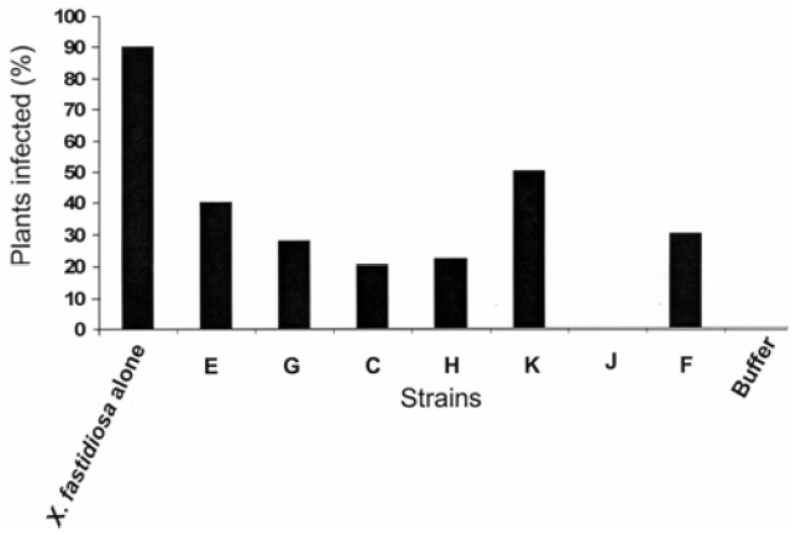

Fig. 15 Incidence of Pierce's disease of grape coinoculated with *Xylella fastidiosa* Temecula alone or with one of several different diffusible signal factor-degrading bacterial strains. Disease incidence was quantified 112 days after inoculation.

To determine the mechanism by which bacteria could degrade DSF, this process was investigated in Pseudomonas strain G which was found to be highly effective in its ability to degrade DSF. Random Tn5 mutants of strain G were selected in which its ability to degrade DSF was greatly reduced. These strains were much less effective in reducing the incidence of piece's disease when co-inoculated with *X. fastidiosa* into grape than the parental stain G (Figure 16).

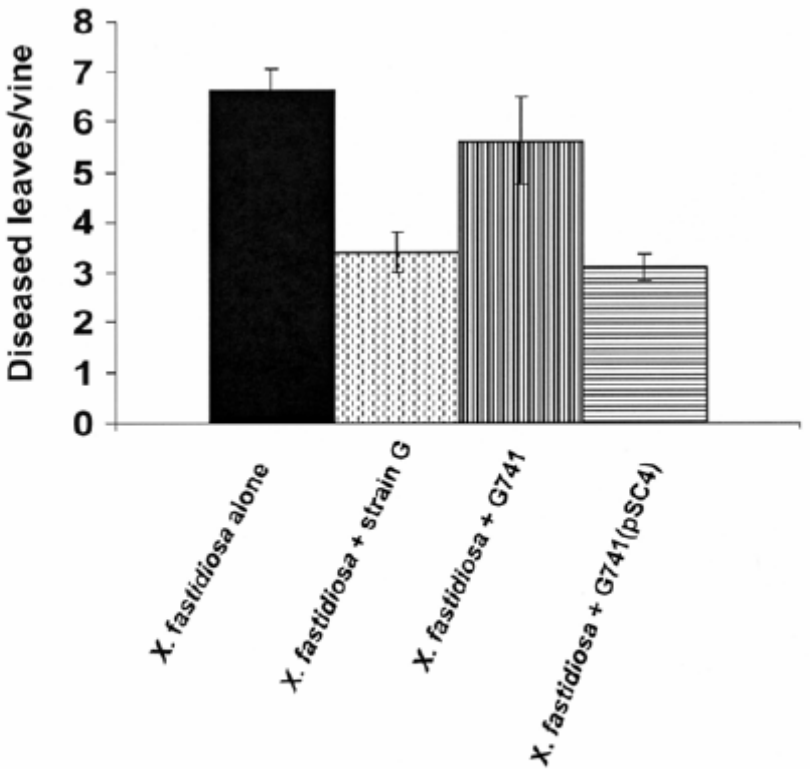

Fig. 16 Severity of Pierce's disease of grape coinoculated with *Xylella fastidiosa* STL and diffusible signal factor–degrading *Pseudomonas* spp. strain G, *CarAB* mutant G741 of *Pseudomonas* spp. strain G, or with mutant G741 complemented with pSC4, as measured 3 months after inoculation. The vertical bars represent the standard error of the mean of the number of symptomatic leaves per vine.

The identity of the genes disrupted in these mutants that were blocked in their ability to degrade DSF was identified by sequencing, and found to be *carA* and *carB*, encoding carbamoyl phosphate synthase (Fig. 17). The re-introduction of these genes into a mutant such as G741 which is incapable of degrading DSF restored its ability to degrade DSF and also restored its ability to inhibit infection by *X. fastidiosa* (Fig. 16).

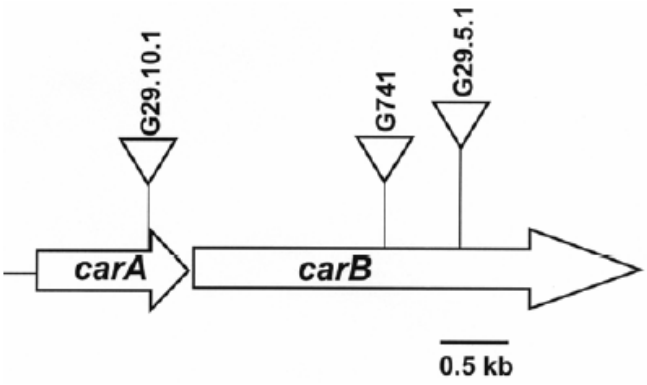

Fig17. Genomic organization of the *carAB* operon in *Pseudomonas* spp. strain G. The orientation of the direction transcription is shown with the arrow. Open triangles indicate the transposon insertion sites in mutants G741, G29101, and G2951.

Objective 6. Topical application of DSF-producing bacteria for disease control.

We have found that it is possible to establish large populations of bacteria within grape leaves, stems and petioles by simple topical applications of bacterial suspensions to plants in solutions of organosilicon surfactants having very low surface tensions. A variety of bacteria were found to colonize grape for at very high population sizes (> 10^6 cells/petiole) for extended periods of time following topical application (Fig. 18). While these bacteria apparently do not spread throughout the plant after inoculation as does *Xf*, by introducing it into the intercellular spaces and perhaps even the xylem of the plant by use of the surfactants that stimulate spontaneous infiltration of the plant, we can inoculate the bacteria into all sites within the plant. Studies have shown that topical applications of an *Erwinia herbicola* strain harboring the Xf *rfpF* gene can provide some control of PD (Fig. 19).

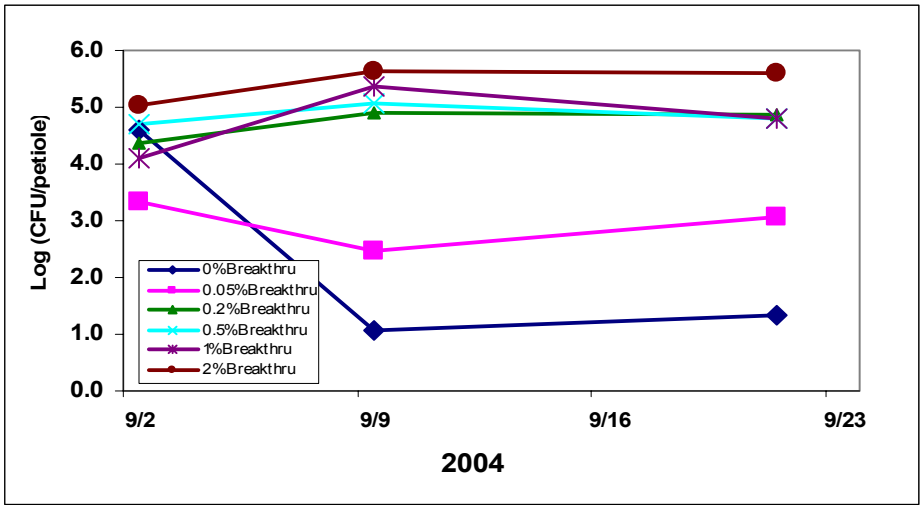

Figure 18
Population size of *E. herbicola* strain 299R within petioles at different times after spray inoculation with different concentraiotns of the organo silicon surfactant Breakthru.

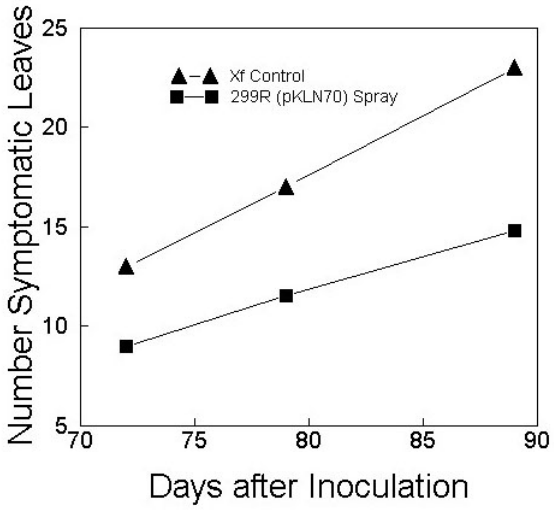

Figure 19. Severity of Pierce's Disease in grape sprayed with DFS-producing *E. herbicola* 299R harboring the *rpfF* of *Xf* Temecula compared to plants inoculated only with Temecula.

Regulation of *Xf* virulence factors We have further examined the complex pattern of gene regulation in *Xf* that are DSF dependent to better understand better the process of disease and thus strategies to alter the disease process. Analysis of the genome sequence of *Xf* revealed that several genes encoding proteins potentially involved in intracellular signaling are present. Gene expression of several genes was thus examined in both an *rpfF* and *rpfC* mutant background as well as a double mutant (Table 3). The results have enabled the production of a more complete model of DSF-dependent gene expression in *Xf* (Fig. 15). The several genes identified in Table 3 will be examined by RT-PCR in cultures of *Xf* to which plant extracts have been applied as proposed.

Fig. 20. A proposed model for DFS-mediated cell-cell signaling regulation in *Xylella fastidiosa*.

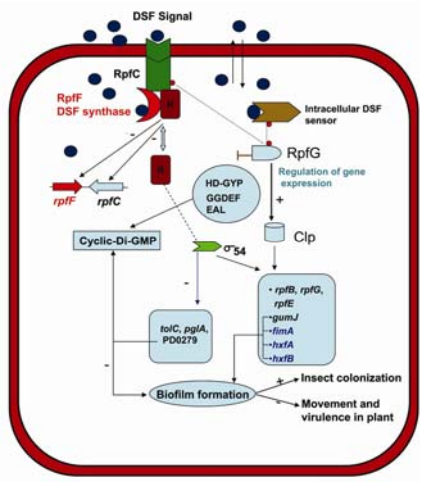

Table 3 Relative quantification of gene expression regulated by *rpfF* and *rpfC* by real-time RT-PCR

Gene name	Fold change ± SE*		
	rpfF⁻	*rpfC*⁻	*rpfF*⁻–*rpfC*⁻
fimA	0.4 ± 0.04	2.15 ± 0.18	0.73 ± 0.19
hxfA (*xadA*)	0.56 + 0.07	3.2 ± 0.1	0.7 ± 0.17
hxfB	0.15 ± 0.05	5.2 ± 0.52	0.49 ± 0.3
gumJ	0.56 ± 0.02	2.6 ± 0.2	0.4 ± 0.04
rpfF	n.d.	6.6 ± 0.71	n.d.
rpfC	4.9 ± 0.4	n.d.	n.d.
rpfE	0.73 ± 0.06	2.2 ± 0.17	0.7 ± 0.12
rpfB	0.6 ± 0.09	2.13 ± 0.07	0.50 ± 0.3
rpfG	0.7 ± 0.06	1.8 ± 0.04	1.13 ± 0.45
(PD0279)	5.3 ± 0.3	3.5 ± 0.23	0.62 ± 0.06
tolC	5.5 ± 0.7	3.8 ± 0.6	0.6 ± 0.09
pglA	1.9 ± 0.17	1.8 ± 0.04	0.7 ± 0.07

*Amount of RNA relative to that in the wild-type *X. fastidiosa* cells is equal to 1.0 and is normalized for cellular abundance by using 16S ribosomal RNA as an endogenous control. n.d. indicates not determined. Standard errors were calculated based on at least two independent experiments.

INTELLECTUAL PROPERTY ISSUES:

Based on promising initial results, an application for a patent entitled "Biological control of pathogenicity in microbes that use alpha, beta unsaturated fatty acid signal molecules" was submitted by the UC Berkeley campus in April, 2005. While much of the patent addresses disease control in *Xanthomonas* it is also applicable to *Xylella* and hopefully will facilitate the commercial adoption of disease control methods to be developed. Information regarding UC-Berkeley policies can be found at: http://otl.berkeley.edu/. The patent was initially denied, but an appeal has been submitted by the UC Berkeley campus. The transgenic plants produced to date have several aspects that may be burdened by intellectual property rights of other organization. This has been examined by PIPRA and a report is available form them on this aspect. Enhanced DSF-producing plants in the future will be made using a plant transformation vector with maximal freedom-to-operate being developed by PIPRA and we are working with PIPRA staff to ensure that technologies used in our future work should yield plants or bacterial strains immediately useable.

PUBLICATIONS FROM THIS RESEARCH:

Newman, K.L., Chatterjee, S., Ho, K.A., and Lindow, S.E. 2008. Virulence of plant pathogenic bacteria attenuated by degradation of fatty acid cell-cell signaling factors. MPMI 21: 326-334.

Chatterjee, S., R.P.P. Almeida, and S.E. Lindow. 2008. Living in two worlds: The plant and insect lifestyles of *Xylella fastidosa.* Ann. Rev. Phytopathology 46:243-271.

Chatterjee, S., C. Wistrom, and S.E. Lindow. 2008. A cell-cell signaling sensor is required for virulence and insect transmission of *Xylella fastidiosa.* PNAS 105: 2670-2675.

Chatterjee, S., K.L. Newman, and S.E. Lindow. 2008. Cell-cell signaling in *Xylella fastidiosa* suppresses movement and xylem vessel colonization in grape. Molecular Plant-Microbe Interactions 21: 1309-1315.

RESEARCH RELEVANCE STATEMENT:

Since we have shown that DSF accumulation within plants is a major signal used by *Xf* to change its gene expression patterns and since DFS-mediated changes all lead to a reduction in virulence in this pathogen we have shown proof of principle that disease control can be achieved by a process of "pathogen confusion". Several methods of altering DSF levels in plants, including direct introduction of DSF producing bacteria into plants, topical application of such bacteria to plants with surfactants, and direct application of DSF itself to plants appear promising as means to reduce Pierce's disease. Transgenic DSF-producing plants appear particularly promising and studies should soon indicate whether they could serve as a rootstock instead of a scion. While the principle of disease control by altering DSF levels has been demonstrated more work will be needed to further test the practical feasibility of how achieve this goal, and what are the most practical means to achieve disease control by pathogen confusion.