Section 3: Pathogen Biology and Ecology

SUPPLEMENTAL PLANT HOSTS FOR *XYLELLA FASTIDIOSA* NEAR FOUR TEXAS HILL COUNTRY VINEYARDS

Project Leader:
Mark C. Black
Dept. of Plant Pathology & Microbiology
Uvalde, TX 78801

Project Director:
James S. Kamas
Dept. of Horticultural Sciences
Texas Cooperative Extension
Texas A & M University
Fredericksburg, TX 78624

Researchers:
Alfred M. Sánchez and James L. Davis
Dept. of Plant Pathology & Microbiology
Uvalde, TX 78801

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ABSTRACT
Floras near four Texas Hill Country vineyards were surveyed *for Xylella fastidiosa* from late 2003 through mid 2004. Two vineyards had histories of Pierce's disease (Gillespie County, Llano County) and two did not (Gillespie County; Travis County). In 2003, 526 plant samples representing 49 plant families were tested one or more times with serology (DAS ELISA) and 80 specimens were dilution plated in attempts to confirm positive serology reactions and estimate *X. fastidiosa* concentrations in plant tissue. Two perennial Asteraceae species were then surveyed in winter, spring, and early summer and serological detection was lowest in spring. Bacterial strain characterizations are underway. This study has implications for site selection, weed control in and near vineyards, rogueing of vineyards, and the need for pathogen-free planting stock.

INTRODUCTION
Pierce's disease (PD), caused by the bacterial pathogen *Xylella fastidiosa*, is the greatest limiting factor for growing *Vitis vinifera* in most of Texas. Associations of *X. fastidiosa*, known vector glassy wing sharpshooter *Homalodisca coagulata*, other xylophagous insects, and numerous host plant species in warmer climates of Texas are apparently ancient and complex. Widespread death of European grape plants has been a common occurrence in much of Texas, perhaps since the first of many plant introductions 400 years ago. There are numerous scientific advantages to studying a biological system where pathogen, vectors, and host plants are native and endemic. However, little is known about the diversity of plants and the bacterium, or potential biocontrol agents in warmer regions of Texas.

In the mid 90's, the incidence and severity of Pierce's disease escalated in the Texas Hill Country (west of Austin and north of San Antonio). While this area of Texas was once thought to be a PD risk transition zone, many established Hill Country vineyards have seen increased vine mortality due to PD. It is speculated that a series of warm winters allowed the pathogen to become more widely distributed throughout the native plant community, providing the initial inoculum for vine infections. While the disease is not known to occur in the northern Panhandle of the state, recent outbreaks at higher elevations in far-west Texas raise questions about pathogen survival and transport into commercial grape plantings.

Variation exists within and among strains of *X. fastidiosa* with some degree of specialization to be more pathogenic on certain plants and less pathogenic on others (Hopkins 1984, Purcell and Hopkins 1996). However, wine grape plants inoculated with "citrus strain," thought to be most different from "grape strain," PD-like symptoms developed on grape (Li et al., 2002). Questions abound regarding plasticity of bacterial strains in response to changes in insect vectors, climate, plant species composition near vineyards, and grape cultivars.

The greatest genetic variations within species of pathogens, vectors, and potential biocontrol agents typically occur where the species first evolved or coexisted. The *X. fastidiosa* center of origin probably includes the coastal areas of the U.S. near the Gulf of Mexico, including large areas of Texas. Various supplemental hosts may harbor diverse strains of *X. fastidiosa*, perhaps even mixed infections within a single plant. A non-native and highly susceptible species (e.g., *V. vinifera*) growing nearby may be repeatedly challenged by bacteria carried by xylem-feeding insects feeding on both weeds and the introduced plant. Numerous *X. fastidiosa* strains may have potential for some reproduction in European grape (Hopkins 1984, Li et al., 2002, Purcell and Hopkins, 1996), but the highly pathogenic populations that reproduced the most rapidly in wine grape xylem fluids and were vectored most efficiently quickly become predominant.

OBJECTIVES
Our objectives were to survey annuals, perennials, woody plants, and ornamentals near vineyards for colonization by *X. fastidiosa* using serology (ELISA) and dilution plating, and to collect isolates for European grape pathogenicity studies and other strain characterization.

RESULTS

Some plant families had no positive serology reactions and two native grape species and two other native Vitaceae species were never positive with either technique in 2003 (Table 1). Plant samples that reacted serologically for *X. fastidiosa* in 2003 were from 12 plant families, but dilution plating (Hill and Purcell, 1995) with SCP buffer (Hopkins 1988) confirmed the bacterium in specimens from only eight families (Table 2). Identification of selected colonies was confirmed with serology.

Xylella fastidiosa was detected in and cultured from weeds at three (two with PD histories, one with no PD history) of the four vineyards in 2003 (Tables 3, 4). Three weed host species were found at all four vineyards (Mexican hat, western ragweed, hierba del marrano). Two weed host species were found only at the two vineyards with PD histories (giant ragweed, common sunflower). Near one no-PD-history vineyard (Travis County), *X. fastidiosa* was in some nearby weeds, but weed control in the vineyard blocks was good and vineyard perimeters were closely and often mowed.

Supplemental hosts of particular interest were five species in Asteraceae (Table 3). Two are perennials and three are annuals. Serological detection rates for two Asteraceae perennials were higher in summer and fall 2003 (aboveground plant parts, Table 3) and winter 2004 (belowground and soil surface-level plant parts) than in spring 2004 (belowground and soil surface, Table 5). Serology was not consistent among plant parts when petiole and root (Mexican hat) and underground stem, horizontal root and vertical root (perennial [western] ragweed) were tested separately. Overwintering *X. fastidiosa* may not be highly systemic on these species through winter and spring. Spittlebug nymphs (Cercopoidea) were frequently found on these two Asteraceae species in the spring, especially in riparian habitats. Fungal and bacterial contamination of dilution plates were much more pronounced in winter and spring from plant parts belowground or near the soil surface and *X. fastidiosa* concentrations could not be estimated.

This bacterium was also detected and cultured from certain urban trees and shrubs in urban landscape situations in Fredericksburg, Uvalde and San Antonio in summer and fall (Table 1). Colonies of *X. fastidiosa* on sap dilution plates developed earlier for grape and redbud compared to sycamore and oleander in 2003. There were either too few positive samples for us to compare colony growth rates, or results were mixed among sample dates and locations for Mexican hat, western ragweed, hierba del marrano, western soapberry, cedar elm, giant ragweed, and common sunflower.

CONCLUSIONS

Knowledge of PD epidemics in Texas increases prospects for disease control in other wine grape production regions. This work focused on surveys for supplemental *X. fastidiosa* host plants at diverse vineyard sites. Future work will utilize the bacterial isolates and plant community data at PD and non-PD vineyards to explore new control strategies.

A. H. Purcell described four requirements for a plant species to be an important source for *X. fastidiosa* acquisition by xylem-feeding insects: 1) frequently inoculated with *X. fastidiosa*; 2) attractive food host for the insect carrier; 3) *X. fastidiosa* spreads beyond the inoculation site [systemic spread]; and 4) $\geq 10^4$ c.f.u./g of *X. fastidiosa* in xylem-containing plant tissue.

Education efforts related to PD risk in European wine grapes grown in the Texas Hill Country include:

A. Site selection. Avoid locating vineyards near riparian habitats because more weeds found there probably meet the four requirements listed above for important bacterial sources.
B. Plant species composition. Based only on circumstantial evidence to date, presence of common sunflower and great (giant) ragweed may indicate higher site risk. This may be because of insect behavior on these two weeds.
C. Weed control. Until Texas *X. fastidiosa* strains are characterized, broadleaf weed control within and near vineyards should remain a priority, including frequently mowed perimeters.
D. Rogueing. Infected and symptomatic *V. vinifera* vines contain *X. fastidiosa* with high c.f.u./g. Early PD detection while incidence in still low, and immediate rogueing should be considered to help reduce vine-to-vine spread.
E. Planting stock. Infected tolerant (few if any acute symptoms) cultivars grown in Texas and other southern states, including *V. aestivalis*, can be reservoirs of X. fastidiosa (L. Moreno, unpublished). Infected planting stocks of these varieties are potential sources of inoculum if planted adjacent to *V. vinifera* and in previously PD-free areas.

Results are pending from 2004 greenhouse wine grape plant inoculations with *X. fastidiosa* isolates from grape, weeds and woody ornamentals to determine pathogenicity. Work in progress includes estimating frequency of selected plant species at four vineyards to learn more about high and low risk sites, and strain characterization in this and another laboratory.

Table 1. Selected plant families negative for *Xylella fastidiosa* in one or more species with ELISA and in some cases, also with dilution plating in 2003.

Family	Number of plant specimens
Cupressaceae	2
Cyperaceae	14
Euphorbiaceae	12
Juncaceae	3
Onagraceae	12
Poaceae	43
Solanaceae	16
Taxodiaceae	7
Vitaceae[z] (excluding *Vitis vinifera, V. aestivalis*)	31

[z]*Cissus trifoliata* (L.) L., *Parthenocissus* spp., *V. mustangensis* Buckl., *V. berlandieri* Planch.

Table 2. Plant families with one or more species positive for *Xylella fastidiosa* with serology and dilution plating in 2003.

Family	Species
Apocynaceae	Oleander (*Nerium oleander* L.)
Asteraceae	[five species, see Table 3]
Fabaceae	Redbud (*Cercis canadensis* L.)
Fagaceae	Red oak (*Quercus* sp.)[y]
Platanaceae	Sycamore (*Platanus occidentalis* L.)
Sapindaceae	Western soapberry (*Sapindus saponaria* L.)
Ulmaceae	Cedar elm (Ulmus crassifolia Nutt.)
Vitaceae	European grape (*Vitis vinifera* L.)[z]

[y]D. Appel, T. Kurdyla and M.Vest, unpublished data.
[z]Also in 2004, *V. aestivalis* Michx L. Moreno, cv. Cynthiana/Norton, by serology and immuno-fluorescence; M. Black, cv. Lenoir (uncertain parentage) by serology with dilution plating pending.

Table 3. Five weed species in Asteraceae collected near four vineyards and positive for *Xylella fastidiosa* with serology and dilution plating in summer and fall 2003.

			Percent positive			
			Serology (ELISA)		Dilution plating	
Common name	Scientific name	Longevity				
Perennial (western) ragweed	*Ambrosia psilostachya* DC.	Perennial	33%	N=54[y]	65%[z]	N=17
Red-spike Mexican hat	*Ratibida colunifera* (Nutt.) Woot. & Standl.	Perennial	19%	N=48	89%	N=9
Hierba del marrano (slim aster)	*Symphyotrichum divaricatum* (Nutt.) Nesom	Annual	21%	N=14	100%	N=3
Great (giant) ragweed	*Ambrosia trifida* L.	Annual	57%	N=7	75%	N=4
Common sunflower	*Helianthus annuus* L.	Annual	25%	N=12	33%	N=3

[y]Number of specimens tested.
[z]Dilution plating usually done only with samples positive or questionable positive with serology.

Table 4. *Xylella fastidiosa* c.f.u./g[u] estimates for wine grape and five Asteraceae weed species at four locations in the Texas Hill Country in 2003.

	Vineyard location and history			
Plant species	Llano PD[v]	Gillespie PD[w]	Travis no PD[v]	Gillespie no PD[x]
Wine grape	10^6-10^8	10^6-10^7	-[y]	-
Perennial (western) ragweed	10^4-10^6	10^6-10^7	10^3-10^6	0
Mexican hat	10^6-10^7	10^3	10^3-10^6	0
Great (giant) ragweed	10^6	-	.[z]	.
Common sunflower	10^5	-	.	.
Hierba del marrano	10^7	-	10^4	0

[u]Colony forming units per gram of xylem-rich plant tissue.
[v]Near riparian habitats.
[w]Near smaller, varied, somewhat seasonal riparian habitats.
[x]Not near significant riparian habitat.
[y]Species found but not sampled, or ELISA-negative sample not dilution plated.
[z]Species not found.

Table 5. Winter, spring and summer 2004 survey of Mexican hat and perennial (western) ragweed for colonization by *Xylella fastidiosa* near four Texas Hill Country vineyards. Results of dilution plating on PWG semi-selective medium were all negative through August 2004.

Season	Location and PD history			
	Gillespie PD	Llano PD	Gillespie No PD	Travis No PD
	---------- Positive samples, % (N=total number of samples) ----------			
Winter (Feb, Mar)	17% (N=30)	20% (N=40)	.[z]	43% (N=37)
Spring (Apr, May)	9% (N=33)	5% (N=41)	.	20% (N=41)
Summer (Jun-Aug)	0% (N=6)	10% (N=10)	20% (N=4)	83% (N=5)

[z]Site not sampled.

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DEVELOPING A MICROARRAY-PCR-BASED IDENTIFICATION AND DETECTION SYSTEM FOR *XYLELLA FASTIDIOSA* STRAINS IMPORTANT TO CALIFORNIA

Project Leaders:

Jianchi Chen
USDA, ARS, PWA
San Joaquin Valley Agricultural Sciences Center
Exotic and Invasive Diseases and Pests Research
Parlier, CA 93648

Edwin L. Civerolo
USDA, ARS, PWA
San Joaquin Valley Agricultural Sciences Center
Exotic and Invasive Diseases and Pests Research
Parlier, CA 93648

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ABSTRACT

From the analysis of the 16S rDNA sequence of *Xylella fastidiosa*, we have identified four single nucleotide polymorphisms (SNPs). The combination of these four SNPs placed all of the known *X. fastidiosa* strains into four groups. With a few exceptions, the four SNP groups are very similar to those based on other genetic analyses such as RAPD analysis, whole 16S rDNA sequence analysis, and the combination of phenotypic characterization, particularly pathogenicity tests. Of particular interest is the PD group. All eight PD strain 16S rDNA sequences from different labs clustered into the same group characterized by two SNPs. Utilizing the SNP information, primer sets, Teme150fc-Teme454rg, specific to PD strain group, and Dixon454fa-Dixon1261rg, specific to non-PD almond leaf scorch group, were designed. More than 200 *X. fastidiosa* strains isolated from California have been tested for the specificity of these SNPs and the results were quite consistent. A microarray system, initially based on the characteristic SNPs from the 16S rRNA locus, is under construction. Coupled with PCR using universal 16S rDNA primers, the microarray-PCR based system has a high potential for quick and accurate *X. fastdiosa* strain identification.

INTRODUCTION

The need to accurately identify and differentiate *X. fastidiosa* strains is becoming more apparent considering the coexistence of different pathotypes in the same crop (Chen et al., 2004a, b). This prompted us to research on improvement of pathogen detection. Polymerase chain reaction (PCR) has been a common technique for *X. fastidiosa* identification. There are, however, technical problems limiting the application of PCR. False positive amplifications can occur among related organisms in the environment sharing similar genetic sequences. Specific primers may fail to amplify DNA from a particular isolate if there is a spontaneous mutation(s) in the primer-binding site, leading to a false negative result. The sensitivity and specificity of PCR amplification tend to be inversely related.

The rationale of this project is to maximize the sensitivity of PCR technology. To increase pathogen detection specificity, microarray methodology based on the principle of DNA hybridization is applied to further confirm the accuracy of the amplified DNA fragments (Chen and Civerolo, 2003). Conceptually, the development of high-density oligonucleotide arrays allows massively parallel hybridizations to occur on the same surface, permitting high levels of probe redundancy and multiple independent detections of a diagnostic DNA sequence. Because of the taxonomic value and available large genomic sequence database, single nucleotide polymorphisms in the 16S rRNA gene are particularly useful. Other genes and intergenic regions could also be the targets due to the availability of complete genome sequences from four different *X. fastidiosa* strains.

OBJECTIVES

The overall goal of this project is to develop and evaluate a microarray-PCR-based system for accurate and quick identification of *X. fastidiosa* strains. A particular emphasis is on strains currently important in California. Two specific objectives are:

1. Using the complete and annotated genome sequence of *X. fastidiosa* Temecula strain as a guide, select appropriate DNA sequences and evaluate their potential for pathotype / genotype identification. Design and construct a DNA microarray; and
2. Evaluate the effectiveness of the constructed microarray through hybridization experiment. Using the microarray as a reference, analyze genomic variation of different pathotypes with multiple strains collected from broad geographical areas and hosts.

RESULTS AND CONCLUSIONS

Selected sequences in the genome of *X. fastidiosa* Temecula were used as preliminary queries to identify diagnostic sequences. Because of the sequence availability, most comparisons were made to the four complete genome sequences including PD-Temecula, citrus variegated chlorosis-9a5c, almond leaf scorch disease-Dixon and oleander leaf scorch disease-Ann-1. In general, the tested genome DNA sequences showed high level of similarity as expected. However, single nucleotide polymrphisms were found in most cases. Yet, the number of SNPs varied from gene to gene. Genes of evolutionary importance were particularly emphasized because they could provide a more stable and, therefore, a more consistent base for strain identification. Thus, special efforts were made on DNA sequences from *rrn* operons. In addition,

16S rDNA is by far the most sequenced locus in bacteria including *X. fastidiosa* that has at least 38 sequences currently available. These 38 16S rDNA sequences from eight different sources were retrieved from the GenBank database. The sequences were aligned using CLUSTAL-W program. Nucleotide variations were examined manually. Only the variations supported by multiple sequences were considered as true SNPs. The nucleotide order in the 16S ribosomal RNA gene, PD0048, in the *X. fastidiosa* strain Temecula genome sequence was used as reference to standardize the nucleotide number (Table 1).

Currently, the microarray system is still being established. The evaluation of SNPs for strain identification was done using PCR methodology. The Primer 3 program was used to facilitate primer designs. All primers were designed with Tm = 60±3 C. The basic strategy of primer design was to arrange the SNPs at the 3' end of the oligo-primers. Two multiplex PCR formats were implemented. For the three primer format, primers Teme150fc - Teme478rg-XF16s1031r generated two dominant amplicons, a 348 bp band for the PD group, and a 700 bp band for non-PD group generated by A non-specific prime paired with Teme150fc. In the four primer format, two primer sets were used. The PD group specific primer set, Teme150fc-Teme454rg was the same as in the three primer format. The other primer set, Dixon454fa-Dixon1261rg generated an 847 bp amplicon for the non-PD almond leaf scorch disease (ALSD) group (Figure 1). For comparison purpose, primer set RST31-RST33 was also included. RST31-RST33 is the most commonly used primer set for PCR identification *X. fastidiosa* at the species level. Primer specificity was also compared to non-reduntant GenBank database through the BLAST network service.

Efforts have also been made to obtain a comprehensive collection of *X. fastidiosa* strains in California with emphasis on grape and almond strains. Over 300 isolation attempts have been made from samples of grapes, almonds and other plants. Samples were collected form San Diego, Kern, Tulare, Kings, Fresno, Stanislaus, Butte, Alemeda and Solano counties. Strains were initially confirmed by biological characters such as slow growing and opalescence colony type and then by PCR with primer RST31/33. Over 200 strains were used to evaluate the specificity of the identified SNPs. Research results obtained by far consistently indicate that SNPs in the 16S rDNA sequence have high potential for *X. fastidiosa* strain differentiations. Current design strategy for microarry experiments is to place these SNPs in the center of the oligomers. Also as shown in Table 1, a total of four SNPs can be considered for oligomer designs to cover all the known strains of *X. fastidiosa*. The advantage of such a microarray identification system becomes even more obvious when 16S rDNA primers of different specificity levels, such as universal primers, are used to generate a large amount of target DNAs from a low titer of bacterial cells.

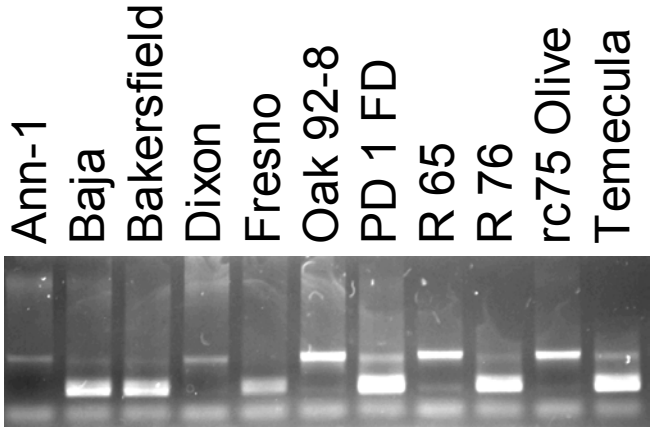

Figure 1. Representative results of multiplex PCR using the four primer format based on single nucleotide polymorphisms in the 16S rDNA sequence. The STRONG presence of the upper band (847 bp) indicates the almond leaf scorch strain group. The STRONG presence of the lower band (348 bp) indicates a grape Pierce's disease strain group.

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Table 1. List of four single nucleotide polymorphisms from 38 rDNA sequences of *Xylella fastidosa* and the related information.

Strain Name	Host	Geographic Origin	150	454	1261	1338
R116v11	Grape	Georgia	C	G	A	C
PCE-FG	Grape	Florida	C	G	A	C
PD28-5	Grape	Florida	C	G	A	C
PCE-FF	Grape	Florida	C	G	A	C
Temecula	Grape	California	C	G	A	C
GR.8935	Grape	Florida	C	G	A	C
Temecula	Grape	California	C	G	A	C
Temecula	Grape	California	C	G	A	C
Mul-2	Mulberry	Nebraska	C	G	A	C
Dixon1	Almond	California	T	A	G	C
Dixon2	Almond	California	T	A	G	C
Ann-1 1	Oleander	California	T	A	G	C
Ann-1 2	Oleander	California	T	A	G	C
PWT-22	Periwinkle	Florida	T	A	G	C
PWT-100	Periwinkle	Florida	T	A	G	C
Mul1	Mulberry	Massachusetts	T	A	G	C
Elm	Elm	Washington DC	T	A	G	C
OAK	OAK	Washington DC	T	A	G	C
PLS2-9	Plum	Georgia	T	A	G	C
PLM G83	Plum	Georgia	T	A	G	C
PP4-5	Peach	Georgia	T	A	G	C
RGW-R	Ragweed	Florida	T	A	G	C
ELM-1	Elm	Washington DC	T	A	G	C
ALS-BC	Almond	California	T	A	A	T
MUL-3	Mulberry	Massachusetts	T	A	A	T
P3	Coffee	Brazil	T	A	A	T
B14	Citrus	Brazil	T	A	A	T
SL1	Citrus	Brazil	T	A	A	T
CRS2	Coffee	Brazil	T	A	A	T
CM1	Coffee	Brazil	T	A	A	T
CI.52	Citrus	Brazil	T	A	A	T
CO.01	Coffee	Brazil	T	A	A	T
CVC93-2	Citrus	Brazil	T	A	A	T
9a5c	Citrus	Brazil	T	A	A	T
9a5c	Citrus	Brazil	T	A	A	T
PE.PLS	Pear	Taiwan	T	A	A	C
PL.788	Plum	Georgia	T	A	A	C
OSL92-3	Oak	Florida	T	A	A	C

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DNA MICROARRAY AND MUTATIONAL ANALYSIS TO IDENTIFY VIRULENCE GENES IN *XYLELLA FASTIDIOSA*

Project Leader:
Donald A. Cooksey
Dept. of Plant Pathology
University of California
Riverside, CA 92521

Collaborators:
Korsi Dumenyo
Dept. of Plant Pathology
University of California
Riverside, CA 92521

Rufina Hernandez-Martinez
Dept. of Plant Pathology
University of California
Riverside, CA 92521

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ABSTRACT
The development of successful management and control strategies of Pierces disease of grape requires the identification of virulence and pathogenicity genes and determining how they functions to control the disease development process. Based on the presumption that biofilm formation is a major pathogenicity factor of *Xylella* and that it may play a major role in the disease causing process, we have been studying the factors – genetic and environmental that affect biofilm formation by *Xylella fastidiosa.* We have identified that, Bovine serum albumen, a component of PW medium specifically inhibits biofilm formation in *X. fastidiosa* and that this inhibition is BSA concentration dependent. Because of its effect on the biofilm formation *in vitro*, we are studying the expression profiles of *X. fastidiosa* genes in the presence and absence of BSA in the media. We have also identified a global regulatory gene, *rsmA* (*rsm* = *r*egulator of *s*econdary *m*etabolism) that control biofilm. An *rsmA*-deficient strain of *X. fastidiosa* forms more biofilm *in vitro* than the wild type. In a preliminary nylon membrane DNA macroarray experiment using about a 100 select candidate pathogenicity genes, we have determined an increased expression of 15 genes in the mutant when compared to the wild type parent. We are now using full genome microarrays of *Xylella fastidiosa* to catalogue the genes whose expressions are controlled by either *rsmA* or BSA. The results from these ongoing analyses using both approaches should help us catalogue *X. fastidiosa* genes which may be involved in pathogenicity and biofilm formation. Subsequent genetic analysis of the genes to be identified should give us some understanding of not only how pathogenicity is regulated in this bacterium but also how to tackle the problems posed by Pierces disease.

INTRODUCTION
Although the exact mechanism of Piece's disease is not completely understood, infected grape plants show symptoms resembling those of water-stress. Moreover, the xylem-limited *Xylella fastidiosa* bacterium produces biofilm *in vitro* and *in planta* (4, 9, 10, 12). Putting these two observations together, it has been suggested that this biofilm clogs up the vascular tissues of the plant and occlude water and nutrient transport. Because of this assumed importance of biofilm formation in the disease mechanism of *Xylella fastidiosa*, we have been studying signals and factors affecting biofilm formation in a bit to identify the regulators of pathogenicity in *Xylella fastidiosa*. *rsmA* is a post-transcriptional regulatory gene that controls pathogenicity and secondary metabolism in a wide group of bacteria including Gram positive and negative organisms (1, 3, 11, 15). In *Erwinia* spp. and other related plant-associated bacteria, rsmA together with its regulatory noncoding RNA pair, *rsmB* control many phenotypes including pathogenicity, extracellular polysaccharide and enzyme production, and elicitation of hypersensitive response, pigment formation, motility and antibiotic biosynthesis). And in *E. coli* and related enterobacterial human pathogens, *csrA* and *csrB*, the homologues of *rsmA* and *rsmB* regulate, among others, glycogen biosynthesis and biofilm formation (6, 8, 17, 19). Because of the role of biofilm formation on the pathogenicity of many bacterial pathogens (5, 14), and the fact that *rsmA* or its homologs control both pathogenicity and biofilm formation in different bacteria, we wanted to determine the possible role of *rsmA* on biofilm formation in *Xylella*. We found that *Xylella fastidiosa* strains vary widely in their biofilm forming abilities and this is influenced by the culture medium in which the assay is carried out.

We report that BSA is the specific inhibitor of biofilm formation in PW medium and that the amount of biofilm the bacterium forms if inversely proportional to the concentration of BSA in the medium. Further, we show that biofilm formation is regulated by *rsmA* gene as *rsmA*$^-$ mutants form higher levels of biofilm than the wild type parent. We confirm this observation by showing that the heterogonous expression of *Xylella fastidiosa rsmA* in *E. coli* reduces biofilm formation in this bacterium. Put together, these suggest that *rsmA* may regulate pathogenicity in *Xylella fastidiosa* through its effects on factors such as biofilm formation in the plant.

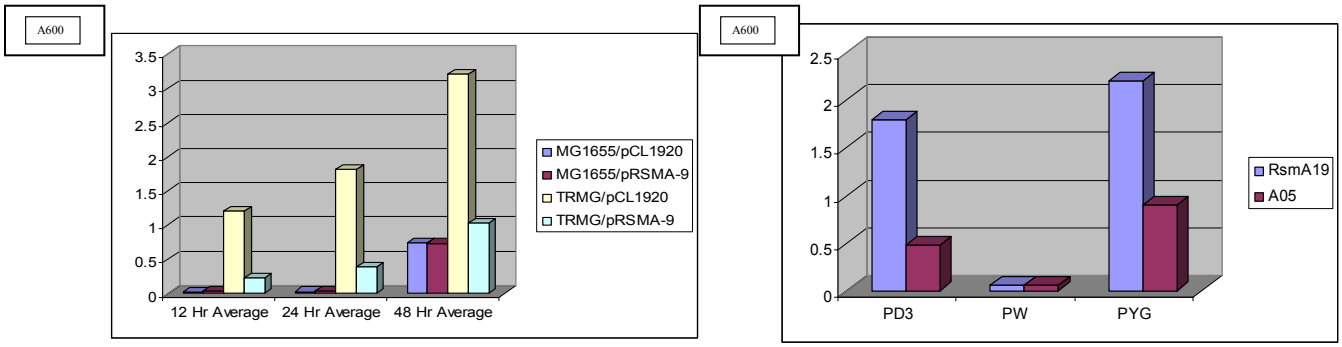
OBJECTIVES

1. Use DNA microarray analysis to identify virulence and pathogenicity genes in *Xylella fastidiosa* through coordinate regulation with a known virulence factor or expression *in planta* during infection.
2. Clone and mutate putative virulence genes and characterize virulence defects in a bid to understand the mechanism of virulence.

RESULTS

Cloning, Characterization of rsmA and the Construction of rsmA Mutant of Xylella fastidiosa

As mentioned above, three observations let us to investigate the role of *rsmA* in pathogenicity and biofilm formation in *Xylella fastidiosa*: 1, the homologues of the gene are widely distributed in the prokaryotic world; 2, the gene controls pathogenicity and virulence in many phytobacteria and 3; in *E. coli*, the gene controls biofilm formation. To determine the role of *rsmA* in *Xylella*, we the cloned the gene and characterized it. The authenticity of the cloned gene was confirmed with DNA sequencing. *Xylella fastidiosa rsmA* is a small gene that encodes a predicted product is 72-amino acid with a putative RNA-binding protein. Heterologous expression of *X. fastidiosa rsmA* in a biofilm overproducing *csrA* mutant of *E. coli* resulted in reduced biofilm formation indicating that the gene does have a role in biofilm formation (Figure 1). After confirming that the cloned gene is indeed *rsmA*, we determined the effect of the mutation on biofilm formation in *Xylella.* The mutant and wild type were assayed for their ability of form biofilm in vitro. Observation show that, the mutant formed more biofilm that the parent (Figure 1). Since the ultimate goal is to identify virulence genes, we tested whether *rsmA* mutants are pleiotropically affected in the expression of any genes. For this, we used the nylon membrane DNA macroarrays of about 100 select pathogenicity genes based on the published genomic sequences (7, 16, 18). Hybridization of ^{32}P-labelled total cDNA reveal 15 genes which were more than 10-fold induced in the mutant (Table 1).



MG1655: Wt type *E.coli*
TRMG: *csrA*$^-$ derivative
pCL1920: Spc^R pSC101 replicon plasmid
pRSMA-9: *X. fastidiosa rsmA* clone in pCL1920

A05: Wild type *X. fastidiosa*
RsmA19: *rsmA*$^-$ derivative of A05
PD3, PW, PYG: *Xylella fastidiosa* growth media

Figure 1. Left. Biofilm formation by *csrA*$^-$ *Ecoli* is suppressed by *X. fastidiosa rsmA* and (Right), *rsmA* mutant of *X. fastidiosa* form more biofilm than their wilt type *rsmA*$^+$ parents.

Identification of the PW Medium Component that Inhibits Biofilm Formation

Because of the increasing evidence of links between biofilm formation and pathogenicity in many biofilm forming bacteria (2, 13), we were interested in identifying any possible factors that control biofilm formation. We had long observed that *Xylella fastidiosa* make more biofilm when grown in PD3 medium than in PW medium. We explored this difference between the two media by adding different components of PW media to PD3 medium in order to identify the component responsible for the inhibition of biofilm formation. Our result show when Bovine serum albumen (BSA) was added to PD3 medium, biofilm formation was reduced; implying that BSA is the inhibitor. We then wanted to see of this inhibition depends on the concentration of BSA present in the medium. Different concentrations of BSA were again supplemented into PD3 basal medium and the bacterium was assayed again for biofilm formation. Our results (Figure 2) again show that the bacteria formed less biofilm with increasing concentration of BSA. These results clearly indicate that BSA is a specific inhibitor of biofilm formation. We are now utilizing this information in our full genome microarrays experiments to determine identify the genes which are coordinately regulated with biofilm as has been done for another strain of *Xylella fastidiosa* (4).

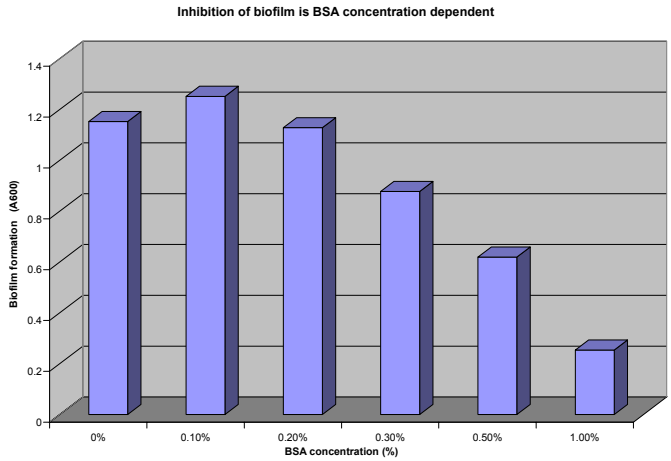


Figure 2. Inhibition of biofilm formation is BSA concentration dependent

Table 1. List of genes overexpresses at least 10-fold in RsmA19.

Gene name	Function	Volume Ratio (A19/A05)
brk	BrkB protein	**14.4**
pilE	Type IV pilin	**10.8**
chi	Chitinase	**12.0**
pcp or lpp	Peptidoglycan-associated outer membrane lipoprotein precursor	**25.7**
pilU	Twitching motility protein	**10.6**
vacB	VacB protein	**14.0**
algH	Transcriptional regulator	**18.3**
algU or algT	RNA polymerase sigma-H factor	**21.8**
ccmA	Heme ABC transporter ATP-binding protein	**14.5**
colS	Two-component system, sensor protein	**10.7**
tapB	Temperature acclimation protein B	**69.8**
fucA1	Alpha-L-fucosidase	**10.9**
pilT	Twitching motility protein	**13.3**
gcvR	Transcriptional regulator	**12.9**
clpP or lopP	ATP-dependent Clp protease proteolytic subunit	**13.2**

CONCLUSIONS

In conclusion, we have identified a genetic factor and an environmental factor, both of which control the important phenomenon of biofilm formation; a process that is tightly linked to pathogenicity of *Xylella fastidiosa*. *rsmA*$^-$ mutants of *Xylella fastidiosa* form more biofilm that the parents and the presence of BSA in the medium suppresses biofilm formation by the bacterium. We have identified 15 preliminary genes which are coordinately regulated with *rsmA* mutation and possibly, biofilm formation. We are using high density DNA microarrays to catalogue *Xylella fastidiosa* genes which are up- or down-regulated with *rsmA* mutation and reduced biofilm formation due to BSA in the medium. This work will contribute significantly to fundamental information on the genetics and pathogenicity of *Xylella fastidiosa.* This information is essential for any attempt to design a management strategy for PD based on the disease mechanism. The identification of previously unknown virulence genes can also lead to recognition of new unforeseen targets for management strategies. In addition, the construction of a DNA microarray for this pathogen, and identification of genes differentially expressed during infection, will complement work by others on differential expression of grapevine genes during infection. This will open the door to "interactive genomic" studies that will enhance our understanding of the bacterial-plant interaction that leads to Pierce's disease, and in the future, studies of interactions with its insect vectors.

Work in Progress

We have developed whole genome arrays of *Xylella fastidiosa* and are presently analyzing gene expression levels between the wild type and *rsmA* mutant, growth with and without BSA and *in vivo* versus *in vitro* conditions. We hope to catalogue the genes whose expressions are associated with biofilm formation, *rsmA* mutation and infection. Those genes which will overlap with more than one approach will be especially interesting for further analysis. Genetic analysis of these genes therefore should open a window for us into what goes on during the infection process. The *rsmA* mutant together with its parent is also being assayed for pathogenicity on grapes. In addition, we have constructed several mutants in a select candidate pathogenicity genes and are in the process of analysis these for the effects of the mutations and hence the roles of these genes in the bacterium.

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CULTURE-INDEPENDENT ANALYSIS OF ENDOPHYTIC MICROBIAL COMMUNITIES IN GRAPEVINE IN RELATION TO PIERCE'S DISEASE

Project Leaders:

Donald A. Cooksey
Dept. of Plant Pathology
University of California
Riverside, CA 92521

James Borneman
Dept. of Plant Pathology
University of California
Riverside, CA 92521

Reporting Period: The results reported here are from work conducted from October 1, 2003 to September 30, 2004.

ABSTRACT

Culture-independent, nucleic acid-based methods of assessing microbial diversity in natural environments have revealed far greater microbial diversity than previously known through traditional plating methods. If true for grapevines, then this has important consequences for Pierce's disease management strategies that involve the establishment of introduced bacteria systemically in the grapevine xylem. Such establishment will likely be influenced by the presence of yet uncharacterized microorganisms, and knowledge of endophytic communities and their dynamics will therefore be important to the successful implementation of these strategies. In addition, analysis of microbial community composition in different hosts and conditions could lead to the identification of new biological control agents. We are employing a novel method, called oligonucleotide fingerprinting of rRNA genes (OFRG), that was recently developed by the Co-PI for analyzing microbial community composition in environmental samples.

INTRODUCTION

In recent years, culture-independent, nucleic acid-based methods of assessing microbial diversity in natural environments have revealed far greater microbial diversity than previously known through traditional plating methods (Amann et al., 1995). This is true for water, soil, the plant rhizosphere, and the plant leaf surface (Yang et al. 2001). A recent culture-independent analysis of bacterial populations inside of citrus plants in relation to *Xylella fastidiosa* also suggested that bacterial endophytic populations are much more diverse than previously realized (Araújo et al., 2002). If true for grapevines, then this has important consequences for Pierce's disease management strategies. Several strategies are being investigated to biologically control *Xylella fastidiosa* in grapevines, including the use of antibiotic-producing endophytes (Kirkpatrick et al., 2001), endophytes that disrupt cell-to-cell signaling by the pathogen (Lindow, 2002), endophytes that degrade xanthan gum (Cooksey, 2002a), and the use of nonpathogenic strains of *Xylella* for competitive exclusion of pathogenic strains (Cooksey, 2002b). These strategies have in common the need to establish an introduced strain systemically in the grapevine xylem. Such establishment will likely be influenced by the presence of yet uncharacterized microorganisms, and knowledge of endophytic communities and their dynamics will therefore be important to the successful implementation of these strategies. In addition, analysis of microbial community composition in different hosts and conditions could lead to the identification of new biological control agents.

We are employing a novel method that was recently developed by the Co-PI for analyzing microbial community composition in environmental samples. This method can be used to characterize both bacterial and fungal communities (Valinsky et al., 2002a; 2002b). Previous culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE), generate only superficial descriptions of microbial community composition (Araújo et al., 2002). A far more complete view of total microbial community composition can be achieved by amplifying, cloning, and sequencing of conserved rRNA genes from the hundreds or thousands of microorganisms present in an environmental sample, but this is prohibitively expensive for any significant number of experiments. The new methodology, called oligonucleotide fingerprinting of rRNA genes (OFRG), represents a significant advance in providing a cost-effective means to extensively analyze microbial communities. The method involves the construction of clone libraries of rDNA molecules that are PCR amplified from environmental DNA, arraying of the rDNA clones onto nylon membranes or specially-coated glass slides, and subjecting the arrays to a series of hybridization experiments using 27 different end-labeled DNA oligonucleotide discriminating probes (Borneman et al., 2001). The process generates a hybridization fingerprint and identification for each clone that is essentially like sequencing the individual clones.

The state of knowledge of the relationship between *Xylella fastidiosa* and the resident endophytic flora of grapevines is at a very early stage. Work to date has been limited to the culturing of endophytes from grapevines, but even this has led to the realization that grapevine xylem sap contains a complex community of microorganisms. Bell et al. (1995) cultured over 800 bacterial strains from grapevine xylem fluid in Nova Scotia. Dr. Bruce Kirkpatrick has also isolated several hundred bacterial strains from grapevine xylem fluid in two counties of California (Kirkpatrick et al., 2001). In citrus, the culture-independent DGGE method of microbial community analysis was compared with culturing of endophytes in relation to the citrus variegated chlorosis strain of *X. fastidiosa* (Araújo et al., 2002). It was found that DGGE detected the major bacteria that were cultured from citrus xylem, but it also detected other bacterial species that had not been cultured. In addition, this method showed differences in microbial communities in different plant varieties, and most importantly, between citrus that was infected vs. non-infected with *X. fastidiosa*. This provides support to our hypothesis that there are likely to be important

interactions between *Xylella* and indigenous microflora in grapevines. With the greater resolving power of the oligonucleotide fingerprinting technique proposed in our study, we expect to make considerable advances in our knowledge of grapevine microbial communities and their interactions with *Xylella* or with other endophytes being considered for establishment as biological control agents.

OBJECTIVES

1. Characterize the diversity and community structure of endophytic microorganisms in healthy and infected grapevines.
2. Compare endophytic microbial populations in different susceptible and tolerant grapevine cultivars, in different hosts that support high or low populations of Xylella, and in plants grown under different conditions.
3. Characterize the potential interactions of endophytic populations with Xylella and introduced biological control agents through experimental manipulations.

RESULTS

Several DNA extraction and PCR amplification protocols were tested over the past year. Most procedures yielded too many clones that were of plant origin. Even extracted plant sap contained considerable plant DNA, of mitochondrial and chloroplast origin, that amplified with different versions of prokaryotic-specific ribosomal DNA primers. The use of filtration with various pore sizes to remove plant material from extracted sap also did not eliminate plant DNA from the samples. Finally, we recently succeeded in selectively extracting and amplifying bacterial DNA from grapevine sap using differential centrifugation to remove DNA of plant origin (naked or in organelles). Plant sap was extracted from grapevines with a pressure pump and centrifuged at 8,000 rpm for 1 hr. The pellet was suspended in 1 ml phosphate buffered saline and loaded onto a tube containing percoll. After centrifugation for 30 min at 22,000 rpm, fractions were collected and subjected to DNA isolation. Isolated DNA was amplified with rDNA primers and cloned (Table 1). Fractions containing bacteria yielded only one plant-derived DNA clone out of 58 in the first experiment, and similar results were obtained when the experiment was repeated. A full-scale extraction and amplification from symptomatic and asymptomatic grapevines from the field is in progress.

Table 1. Bacterial species identified from rDNA sequences amplified from grapevine sap in preliminary tests.

Acidovorax sp.
Agrobacterium sp.
Bacillus macroides
Burkholderia sp.
Caulobacter sp.
Escherichia coli
Escherichia fergusonii
Pseudomonas putida
Pseudomonas syringae
Rhizobium tropici
Shigella flexneri
Teichococcus ludipueritiae
Xylella fastidiosa
Unidentified Acinetobacter
Unidentified Proteobacterium
Unidentified Sphingomonas

CONCLUSIONS

Most of the endophytic species that we detected through cloning of bacterial rDNA sequences were not detected in previous culture-based approaches to identify endophytes in grapevine (Bell et al., 1995; Kirkpatrick, 2003). Since the 16 species that we detected were identified among just 58 clones in our recent preliminary studies, we expect that our full surveys of endophytic bacteria in grapevine this year will yield a far greater diversity than previously known. Researchers working on biological control of the pathogen, as well as disease resistance in grapevine cultivars, will benefit from the information gained in this work. The work should enhance discovery of potential biological control agents for Pierce's disease and the implementation of biological control efforts underway.

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FUNDING AGENCIES

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IMPORTANCE OF GROUND VEGETATION IN THE DISPERSAL AND OVERWINTERING OF *XYLELLA FASTIDIOSA*

Project Leaders:
Kent M. Daane
Division of Insect Biology, Dept. of Environmental Science, Policy, & Management
University of California
Berkeley, CA 94720

Alexander Purcell
Division of Insect Biology, Dept. of Environmental Science, Policy, & Management
University of California
Berkeley, CA 94720

Cooperators:
Glenn Yokota
Division of Insect Biology
University of California
Berkeley, CA 94720

Elaine Shapland
Division of Insect Biology
University of California
Berkeley, CA 94720

Christina Wistrom
Division of Insect Biology
University of California
Berkeley, CA 94720

Tarcisio Ruiz
Division of Insect Biology
University of California
Berkeley, CA 94720

Reporting Period: The results reported here are from work conducted from January 2004 to October 2004. The CDFA grant was awarded in June 2004.

ABSTRACT
The purpose of this project is to determine the ability of alternate host plants, specifically "weeds," in almonds and vineyards to serve as reservoirs for *Xylella fastidiosa* (*Xf*) and for new inoculations by the glassy-winged sharpshooter (GWSS). We collected and analyzed weed and GWSS samples in and around commercial vineyard and almond fields for the presence of *Xf* on a monthly basis. *Xf* has been recovered from weeds collected during February and March, while no collected weeds tested positive for the presence of *Xf* between April and September. Monthly ground cover sampling will continue through the winter, as this time period may prove most important in the persistence of *Xf* over consecutive growing seasons. GWSS collected from alternate host plants have also been processed for *Xf* and have shown that adults collected on many species harbor *Xf* in their mouthparts. Results from these experiments will help to identify what time of year and what ground cover species are of most concern to growers wanting to control the spread of PD with minimal environmental impact.

INTRODUCTION
The economic viability of California's vineyards and almonds has received considerable attention of late because of the expanding range of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* , which can vector the xylem-limited bacterial pathogen, *Xylella fastidiosa* (*Xf*) (Goodwin & Purcell 1992, Redak et al. 2004). *Xf* is the causal agent of Pierce's disease (PD) and almond leaf scorch (ALS) as well as other plant diseases. The arrival of GWSS has dramatically changed the epidemiology of *Xf* and its associated diseases in California (Redak et al. 2004). GWSS may not be an "efficient" vector of PD (Almeida & Purcell 2003a,b; Purcell & Saunders 1999a,b), but it presents a more serious threat, in part, because of its wide host range (Redak et al. 2004) and dispersal abilities (Blua et al. 2003). Of importance here is that the wide host range of *Xf* commonly overlaps with plant species visited by GWSS. Our proposed research will focus on the common host range of both vectors and pathogen, with an emphasis on potential annual weeds that may provide an overwintering reservoir for *Xf* and a spring feeding site for vectors of PD and ALS.

How can this work impact control decisions? An excellent example of an overlooked insect-pathogen-host triangle is stinging nettle (*Urtica urens*), a common weed throughout the Central Valley. In our 2003 survey, we found that stinging nettle was a common host for GWSS in springtime, and recent DNA extraction showed the presence *Xf* in 60% of stinging nettle collected near a Kern County PD-infected vineyard. Whether or not *Xf* titer is high enough in these weeds for GWSS acquisition and transmission is not known, and is one aspect of the proposed study. Regardless, management of common hosts may be a critical component of epidemiology and area wide management of PD and ALS (Redak et al. 2004). With over 145 natural or experimental host plants for *Xf* that can cause PD, the insect/pathogen relationship is far too diverse a subject for one study. For this reason, we are studying the common landscape and ground vegetation found near vineyards and almonds in the San Joaquin Valley.

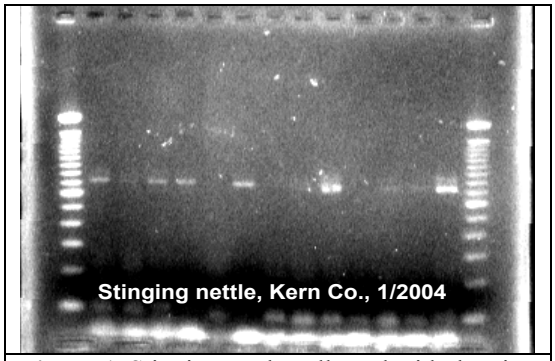

Figure 1. Stinging nettle collected with the vine rows of a PD-infected vineyard showed 9 of 12 samples positive for *Xf*.

OBJECTIVE

1. Determine the presence of *Xylella fastidiosa* in alternate host plants that are commonly visited by glassy-winged and native sharpshooters in selected ecosystems in the San Joaquin Valley; with samples representing different seasons and annual or perennial hosts.

RESULTS

Insect and Plant Samples

GWSS and native sharpshooter (Feil and Purcell 2001) visitation on common non-agricultural crops were monitored to determine the importance of the seasonal period as a component of PD epidemiology. Newly molted adult vectors need to acquire *Xf* from overwintering reservoirs in order to spread PD. GWSS displays seasonal preference for different plant hosts (Daane et al. 2003, 2004), which are often related to host plant phenology or condition (Anderson et al. 1992). We have observed that in winter and early spring, GWSS preferentially feed on perennial weeds such as stinging nettle, filaree (*Erodium* sp.) and common groundsel (*Senecio vulgaris*) in or near vineyards.

GWSS were collected in May, June, July and August from urban ornamental plants that may serve as a host for transferring *Xf* from cities to agricultural land. Insects analyzed for the presence of *Xf* in their mouthparts with the DNeasy Tissue Kit from Qiagen (Bextine 2004). Adult GWSS collected from oleander, xylosma, Chinese elm and riparian zone plants tested positive for *Xf*, while insects collected from crape myrtle tested negative for *Xf*. Nymphal GWSS testing positive for *Xf* were found only on oleander during the month of June. Nymhal GWSS testing positive for *Xf* indicate from which plant the insects are acquiring the bacteria, but will not pose a threat for long since with each successive molt, the insects loose their ability to transmit *Xf*. Adult GWSS testing positive are more of a concern, as an adult GWSS can move between many plants during its lifetime, feeding and spreading *Xf*.

Presence of Pathogen

Non-agricultural plants commonly visited by sharpshooters were screened for the presence of *Xf*. While lists of *Xf* and sharpshooter host plants are available, there are some basic questions that have not been addressed for the San Joaquin Valley: How common is *Xf* in non-agricultural plants? How often do GWSS feed on *Xf* hosts?
Vineyards with heavy infestations of PD were sampled for ground vegetation weeds in and around the crops once a month from January through September. Collections focused on the most abundant variety of weeds, and three samples were taken from each weed species on each date. Samples were processed with either the selective media scheme of PWG and PD3, or with immunocapture DNA extraction and subjected to PCR with universal primers RST-31 and RST-33 (Minsavage 1994). Some weeds collected in January and February were found to contain *Xf*, but after early March, *Xf* was not detected in any weeds collected (Table 1).

Pathogen Population Levels

For GWSS to acquire and transmit *Xf*, the titer of *Xf* within plants typically should be equal to or greater that $\log 10^{4}$ (CFU per g), the threshold population required for acquisition for most sharpshooters (Almeida & Purcell 2003a,b). For chronic PD and ALS to develop, *Xf* infections must survive the winter, which can vary depending on temperature and the degree of plant dormancy (Almeida & Purcell 2003c, Feil & Purcell 2001) and the plant species.

Table 1. Winter/spring weed samples tested for the presence of *Xylella fastidiosa.*

Date	Abundant Weeds	*Xf*
4 February 2004	stinging nettle	+
11 February 2004	stinging nettle	+
3 March 2004	chickweed	+
	bluegrass	+
	shepherd's purse	+
	filaree	-
	alfalfa	-
10 March 2004	tall grass	-
	bluegrass	-

Preliminary analysis of ground cover weeds was conducted using selective media PWG and PD3. However, due to the large amounts of naturally occurring bacteria in wild weeds, all samples were contaminated beyond our ability to count *Xf* colony growth. The same samples were then processed using immunocapture DNA extraction and PCR, which did detect *Xf* in some weeds. When we no longer detected *Xf* in weeds after mid-March, we then tested the sensitivity our extraction methods and PCR. We found that using the immunocapture DNA extraction protocol for plants, we are able to detect at least 1.43×10^{-6} CFU/g of *Xf* DNA, which was satisfactory in ruling out faulty DNA extraction methods. The sensitivity of PCR to detect *Xf*

with RST-31 and RST-33 was also examined, and found to detect 6.5×10^{-5} µg/mL of DNA. In addition, an internal set of primers was developed so that nested PCR is now possible for samples appearing negative with traditional methods.

Pathogen Strain

A simple assay was conducted to categorize *Xf* by its common strains. Recent genetic and cross-inoculation studies showed that *Xf* had genetically distinct strains in different host plants (e.g., oak, oleander, grapes) (Almeida & Purcell 2003c, Chen et al. 1995, Henderson et al. 2001). Typically, *Xf* isolates from one plant species are genetically similar, despite different geographical origins. However, *Xf* isolated from almonds can be genetically separated into three distinct strains – with one ALS strain recovered in orchards in the northern San Joaquin Valley (ALS-*Xf*SJV) that is genetically more similar to grape strains than the two other ALS strains (ALS-*Xf*1, ALS-*Xf*2).

The few weeds samples that returned positive results in the winter and spring were analyzed using restriction enzyme digestion, and have so far been found to be all of the northern San Joaquin Valley (ALS-*Xf*SJV). The lack of positive results for *Xf* in vineyard weeds after mid-March prevented us from analyzing any changes (new inoculations) of *Xf* strains. However, we were able to analyze the strain of *Xf* in the mouthparts of the GWSS tested, and found that these insects were also found to be carrying *Xf* of the PD type. These results are consistent with previous findings that strains of *Xf* tend to be host-specific (Almeida and Purcell 2003c).

CONCLUSIONS

The results of this study indicate that the winter and spring weeds may be the most important reservoirs for *Xf* in vineyards infected with Pierce's Disease. We recovered *Xf* from four species of weeds that have either not been studied in depth (*Stellaria sp*. and *Capsella sp*.) or would benefit from further investigation (*Erodium sp*. and *Poa annua*). We seem to have caught the tail end of the season where *Xf* is abundant in weeds, so the next season's sampling scheme will focus more heavily on vineyard groundcover during the winter months of December, January and February. Future research along these lines could illuminate the importance of previously overlooked alternate host plant species.

One hypothesis for the importance of winter weeds for the persistence of *Xf* is that when symptomatic leaves senesce in late fall, they land directly on the groundcover, thus greatly enhancing the likelihood that any insect feeding there will transmit the bacteria to the weeds. Conclusive evidence of this hypothesis could provide a simple and low cost method for controlling the spread of PD.

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ROLE OF TYPE I SECRETION IN PIERCE'S DISEASE

Project Leader:
Dean W. Gabriel
Dept. of Plant Pathology
University of Florida
Gainesville, FL 32611

Reporting period: The results reported here are from work conducted from November 8, 2003 to October 31, 2004.

ABSTRACT
Xylella fastidiosa Temecula sequence information reveals no type III, but two type I secretion systems, both dependent on a single *tolC* homologue. Marker exchange mutagenesis using pGEM-T as delivery vector and *nptII* as marker was employed to generate *tolC* disruptions. PCR and Southern blot analyses confirmed marker exchange at the *tolC* locus. Grape (var. Carignane) plants inoculated with mutant (*tolC*::*nptII*) strains exhibited no symptoms of PD, indicating that pathogenic ability of PD strains may be dependant on *tolC* and type I secretion. Complementation assays using *tolC* in the mutant strains are in progress to help confirm this hypothesis.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a xylem-inhabiting Gram-negative bacterium that causes serious diseases in a wide range of plant species (Purcell & Hopkins, 1996). Two of the most serious of these are Pierce's Disease (PD) of grape and Citrus Variegated Chlorosis (CVC). The entire genomes of both PD and CVC have been sequenced (Simpson et al., 2000). Availability of the complete genomic DNA sequence of both a PD and a CVC strain of *Xf* should allow rapid determination of the roles played by genes suspected of conditioning pathogenicity of CVC and/or PD. For example, analyses of the CVC and PD genomes showed that there was no type III secretion system, but there were at least two complete type I secretion systems present, together with multiple genes encoding type I effectors in the RTX (repeats in toxin) family of protein toxins, including bacteriocins and hemolysins. RTX proteins form pores in lipid bilayers of many prokaryotic and eukaryotic species and cell types; at least one is associated with pathogenicity in plants. However, lack of useful DNA cloning vectors and/or techniques for working with either CVC or PD strains have impeded progress in functional genomics analyses.
Last year we focused on attempts to perform marker-interruption in the PD strains using various suicide vectors and techniques. Although marker-interruption using suicide vectors is normally an efficient, single crossover event in many bacteria, repeated marker-interruption attempts with *X. fastidiosa* in our lab and in others have failed (Feil et al., 2003; Gaurivaud et al., 2001; Guilhabert et al., 2001). Since marker-exchange has now been reported to be successful with *X. fastidiosa* (Feil et al., 2003), we report here the utility of marker-exchange to generate *tolC* interruption in *X. fastidiosa* PD strain and the role of *tolC* in pathogenicity.

OBJECTIVES
The primary objective of this work is to determine the effect of type I secretion gene knockouts on pathogenicity of a PD strain on grape.

RESULTS
X. fastidiosa strain Temecula (Guilhabert, 2001), was grown in PD3 (Davis et al., 1981) and confirmed to be pathogenic on Madagascar periwinkle and Grape (var. Carnignane). Symptoms appeared after 2 months. Marker-exchange mutagenesis of *tolC* was performed using pJR6.3. This plasmid carries an internal fragment of PD1964 (*tolC* of Temecula) interrupted at an internal *BamH*I site by an *nptII* gene from pKLN18 (kindly provided by K. Newman and S. Lindow). One microgram of pJR6.3 DNA was use to transform electrocompetent cells (prepared by washing 10 ml of four day old PD3 broth culture of *X. fastidiosa* Temecula, serially with 10, 5, 2 ml of ice-cold deionized water and resuspending in 100 µl the same) by electroporation (1mm gap cuvettes; 1800 volts). Electroporated cells were allowed to recover in 1 ml of PD3 broth for 24 hours at 28 ^{o}C and were spread on PD3 plates amended with kanamycin (50 µg/ml). Plates were incubated at 28 ^{o}C for 10 days and single colonies were screened for interruption of *tolC* by PCR analysis and by Southern blot hybridization. The results (Figure 1) indicate that *tolC* gene can be disrupted and marker-exchange was efficient in generating gene-disruptions in *X. fastidiosa*.

Plant inoculation assays were performed in collaboration with Dr. Don Hopkins, at the Mid-Florida Research and Education Center, Apopka, Florida. Grape plants (var. Carnignae) were inoculated with the wild-type *X. fastidiosa* Temecula strain and the mutant (*tolC*::*nptII*) strain in triplicates. The plants were maintained under green-house conditions and were evaluated for Pierce's disease symptoms at 60 and 90 days after inoculation. The results (Figure 2) showed loss of pathogenicity of *X. fastidiosa tolC::nptII* mutants on grapes. All the three plants inoculated with the wild-type Temecula strain exhibited typical PD.

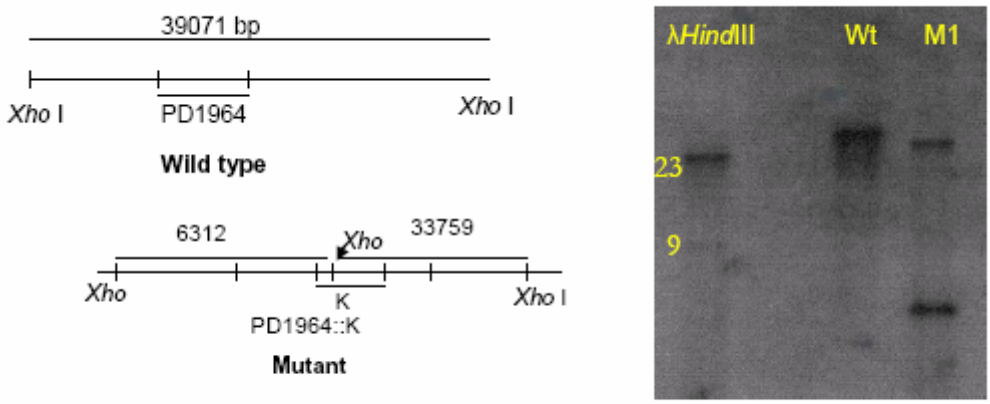

Figure 1: Southern blot of *tolC::aptII* mutant (MI) and wild type total DNA cut with *XhoI*. *XHO* I is internal to the *nptII* gene. The probe was PD1964 (wild type *tolC* from Temecula, 1459 bp).

For complementation assays, PD1964 was amplified by PCR, cloned into pGEM-T, verified by sequencing and sub-cloned into pUFR47, a wide host range replicon based on *repW* (DeFeyter et al., 1993) and pBBR1MCS-5, a wide host range replicon based on a *Bordatella* replication origin (Kovach et al., 1995). pUFR47 and pBBR1MCS-5 containing the entire *tolC* gene are referred as pJR13.2 and pJR22.2 respectively. Non-pathogenic Temecula mutant M1 was transformed with pJR13.2 and pJR22.2 independently by electroporation as described above. The cells were recovered in 1 ml of PD3 broth for 6 hours and were spread on PD3 plates amended with Gentamycin (5 µg/ml). The plates were incubated at 28 ^{o}C for 10 days and single colonies were screened for the presence of pJR13.2 /pJR22.2 and also for the integrity of *nptII* integration, by PCR assay. Grape plants (var Carnignane) were inoculated in triplicates with wild-type *X. fastidiosa* Temecula, mutant M1, M1/pJR13.2, and M1/pJR22.2 strains and are currently being monitored for Pierce's disease symptoms. Preliminary results indicate possible complementation using both vectors. These results need to be repeated and confirmed, and these tests are currently in progress.

Figure 2: Grape var. Camignane 90 days after inoculation with wild type Temecula (left) and *tolC::nptII* mutant M1 (right).

CONCLUSIONS

Type I secretion gene *tolC* (PD1964) of *X. fastidiosa* Temecula was disrupted by marker exchange mutagenesis. The mutant strains lost all pathogenicity, indicating a critical role of tolC in pathogenicity of *X. fastidiosa* on grape. Complementation assays are in progress and could result in a demonstration of a role of *tolC* in pathogenicity. If such a role can be confirmed, it would indicate several important molecular targets for potential PD control methods.

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FUNDING AGENCIES

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ISOLATION AND FUNCTIONAL TESTING OF PIERCE'S DISEASE-SPECIFIC PROMOTERS FROM GRAPE

Project Leader:
David Gilchrist
Dept. of Plant Pathology
University of California
Davis, CA 95616

Cooperators:
Douglas Cook
Dept. of Plant Pathology
University of California
Davis, CA 95616

James Lincoln
Dept. of Plant Pathology
University of California
Davis, CA 95616

Reporting Period: This two-year project was initiated on October 1, 2004. Obviously, there are few results to report at this time. Only a discussion of the justification, objectives, and timetable will be presented per request by the Pierce's Disease Symposium organizers.

ABSTRACT
Among the potential solutions to Pierce's disease in grapes are approaches based on gene transfer technology that focus on understanding the underlying biochemical and molecular mechanisms regulating PD. One of the research priorities identified by the 2003 PD/GWSS project reviews and as indicated in the 2004 RFP was the need to identify, clone and characterize unique DNA sequences that specifically regulate the expression of grape genes in tissues that are infected with *Xf*. Emphasis was placed on the urgency and practical utility of isolating promoters of PD responsive genes. One of the major bottlenecks in using transgenes, either expressed as proteins or as inhibiting RNAs in grape (or any plant) is the lack of suitable promoters to specifically drive the expression of a transgene on a specific trait (susceptibility to PD) in particular tissues (e.g., vascular tissue) or in response to particular situations (e.g., sharpshooter feeding or *Xylella* infection). In the absence of tissue or response-specific promoters, transgenic strategies to either understand or control PD one can use only so-called constitutive promoters. The basic problem associated with the use of constitutive promoters is that the transgene is expressed in all cells all the time, not just in the tissue or cells where the gene is needed. Highly controlled induction is needed if the interest is in altering gene expression to avoid a cellular change (disease) that is initiated in one or a few isolated cells. The isolation and characterization of *Xf*-responsive promoters has immediate and direct application to several current PD projects that are studying the biochemical or molecular genetic basis of PD at the cellular and tissue levels in grape. It also is of practical importance that these promoters will be useful in either the up- or down-regulation of the expression of a specific gene-of-interest. The difference in presence or absence of the target gene product is determined by whether the promoter is used to drive a sense or an anti-sense construct of the gene of interest.

INTRODUCTION
The objective of promoter analysis is to identify and characterize cis-acting DNA (adjacent) sequences that, when induced, regulate PD-associated gene expression in grapes. Although regulatory sequences frequently occur just upstream of the transcription start site, they can also be found much further upstream. Transcript abundance can also be controlled post-transcriptionally, often by cis-acting sequences in the 3' untranslated region of a gene. Thus, the challenge in our studies is to demonstrate that the cis-acting sequences have a unique functional role in PD symptom development. It is not the goal of this proposal to understand mechanisms of transcriptional regulation, but rather to isolate and confirm sequences that are active in the regulation of gene expression when *Xf* is present as an inducer of a select set of genes.

To test whether a particular DNA sequence, that lies adjacent to a gene of interest, is involved in the regulation of that gene, it is necessary to introduce such putative regulatory sequences into a cell and then determine if they are activated when the inducer (in our case, *Xf*) is introduced into the system. This is done by combining a regulatory sequence with a reporter sequence (in our case, GFP) that can be used to monitor the effect of the regulatory (promoter) sequences in the presence of *Xf*.

We have identified a set of plant genes whose expression is correlated with infection by *Xylella fastidiosa* as part of a recent study of expressed sequence tags from *Xf*-infected and healthy *V. vinifera* plants in the Napa Valley. The genes are essentially off (silent) in plants that have not been exposed to the pathogen, but strongly induced in both natural field infections and greenhouse inoculated plants. Three of these genes are induced early during disease development, prior to the occurrence of symptoms, while the fourth gene is induced in symptomatic tissues only.

In addition to their utility for engineering PD resistance in grape, the advent of *Xf*-induced reporter gene expression would provide an extremely powerful tool to examine other host responses in their intact cellular and tissue context. With such tools, it should be possible to examine the chemical and/or physical cues from the insect or pathogen that trigger host gene

expression and the deleterious effect of the disease. Moreover, the recent development of *Xf*-GFP strains by Dr. Steven Lindow at UC Berkeley offers the possibility of dual labeling to simultaneously monitor pathogen spatial distribution and host gene expression. Such dual labeling experiments are made possible by the availability of multiple forms of GFP protein engineered to fluoresce with distinct spectral characteristics. It is conceivable, for example, that host genes might be induced specifically in live cells, adjacent to sites of pathogen colonization of xylem elements, and this technology would provide the means to test such hypotheses.

OBJECTIVES

1. Identify and determine sequence of promoters driving genes specifically transcribed in grape tissue or cells of plants infected with *Xf*.
2. Construct transformation-ready vectors containing *Vitis* promoter-GFP reporter gene fusions that will be used for the functional assay of putative promoters. (GFP=green fluorescent protein) identified in (1)
3. Conduct transient functional assays of the promoter-GFP fusions in stems, leaves and roots infected with *Xf*.
4. Produce stable transgenic grape plants with promoters that functioned effectively in the transient assays and characterize the strength of the selected promoters using the GFP-reporter
5. Distribute promoters to Pierce's Disease research community to facilitate characterization of cloned grape genes suspected to be involved in PD susceptibility or resistant to *Xf*. These promoters will have application in situations where the goal is to <u>either</u> up- or down-regulate expression of a specific gene-of-interest; the latter by localized expression of anti-sense gene constructs.

RESULTS

Since this project just began October 1, 2004, there are few results to report. We have employed a postdoctoral researcher and are currently sequencing the BAC clones indicated in the objectives.

Experimental Procedures to Accomplish Objectives

I. DNA Sequencing and promoter identification:

A. Isolation and characterization BAC clones containing the Xylella-induced genes.

Bacterial Artificial Chromosome (BAC) libraries of *V. vinifera* are available as high density filters for gene identification in grapes through the UC Davis CA&ES Genomics Facility (http://cgf.ucdavis.edu/). High-density filter sets of the library were used for hybridization with ^{32}P-labeled probes corresponding to four *Xylella*-induced transcripts. A combination of restriction enzyme fingerprinting and DNA sequencing of BAC-derived PCR products was used to determine that each probe hybridized to a single genomic locus containing the gene of interest. One BAC clone was selected for each transcript and used to prepare a sheared BAC sublibrary, which is currently being subject to random shotgun sequencing.

B. Sequence the BAC clones to completion.

Although our specific interest is in sequences immediately 5' and 3' to the candidate genes (maximum 10 kbp) we will sequence regions beyond where we believe the promoters to reside. The rationale derives from efficiencies and strategies of modern sequencing techniques; it is both faster and more cost effective to use the BAC shotgun strategy described below which automatically provides additional sequence information for less cost that if we were to attempt to focus on shorter regions immediately adjacent to either end of the candidate genes.

C. Identify 5' promoter regions in the sequenced genomic clones based on comparison to cDNA sequences currently in hand for the four genes.

We have complete cDNA sequences for each of the candidate genes that will facilitate annotation of the BAC clones and identification of regions immediately upstream and downstream of the transcription units. As described below, we will use PCR to isolate and clone these 5' and 3' regulatory sequences into transformation ready vector constructs (see below). Generally, we anticipate using conventional 3' terminators, such as that from the *Agrobacterium* octopine synthase gene (*ocs*). However, one of the candidate genes (a <u>s</u>mall <u>a</u>uxin <u>up</u>regulated, *saur,* mRNA homolog) is predicted to confer post-transcriptional regulatory properties that may be involved in *Xylella*-specific RNA levels. Thus, we will clone the 3' region of this candidate gene and incorporate its structure into a subset of the transgene constructs described below.

II. Construct transformation-ready vectors systems containing *Vitis* promoters fused to GFP.

A set of plasmids has been constructed previously that allows the rapid assembly of novel binary plasmids in E. coli. One is a low copy backbone plasmid with elements from Agrobacterium; the second is a high copy E. coli plasmid containing a cassette of T-DNA elements; and the third is a high copy E. coli plasmid comprised of a linker and many unique restriction sites for ease of cloning the several classes of sequences to be recovered and tested. These plasmids will be used to construct a collection of binary vectors containing grape 5' promoters and 3' sequences for expression of GFP genes. Analysis of the sequence of the appropriate BAC clones will allow the design of PCR primers to amplify and clone the 5' promoter and 3' sequences of the transcriptionally regulated grape genes into novel binary vectors. (Details of the plasmids are available upon request.)

III. Production of transgenic plants and plant tissues of grape and application of transient assay of promoters

We will employ three different but functionally related approaches to testing and characterizing the isolated promoter regions indicated above. All three of the approaches described below will be initiated simultaneously in the interest of time. Each of the promoters of the four genes will be assembled in several different configurations with the reporter gene (GFP) and will be evaluated in conjunction with a constitutive promoter (CaMV 35S or FMV 34S) giving a total number of 40 transgene constructs. Total costs will be minimized by terminating any of the whole plant transformants bearing promoter constructs that are demonstrated by the transient or *A. rhizogenes* assays to be unresponsive to the presence of *Xf*.

A. Stable, full-plant grape transformation will be provided on a recharge basis by the Ralph M. Parsons Foundation Plant Transformation Facility at the rate of $2,000 per construct. This facility is located at UC Davis as a service oriented facility dedicated to providing cost effective plant transformation services for the University of California system and outside academic and industrial partners.

B. Transient and root-specific stable transformations will be used for rapid identification of promoter specificity and relative strength. The intent is to decrease the number of whole plant transformations that need to be conducted -- because whole plant transformation is labor intensive, time consuming and expensive. The transient assays using *Agrobacterium tumefaciens* and the root transformations by *A. rhizogenes*, bearing the test promoters and marker genes, will be conducted by techniques that have used successfully for several years in the Gilchrist Lab.

C. *A. rhizogenes*-derived root transformations will be used for initial assay of the expression of transgenes in differentiated tissue with vascular connections to *Xf*-infected stem sections. *A. rhizogenes* effects stable transformation of plant tissues by transferring genes of interest to intact plants under controlled conditions. The inoculation with A. rhizogenes bearing a gene of interest leads directly to the formation of transformed roots, which appear within 2-3 weeks and at which point the pathogen can be introduced into the assay system. Our procedure will be to introduce the putative promoter sequence, coupled to GFP, into grape roots via transformation as indicated above. Our recent data obtained with the *Xf*-GFP indicates that the bacteria can move both up and down from the site of infection. Hence, the presence of the bacteria, either directly placed in the transformed tissue with the putative promoter constructs have a chance of responding to the direct presence of *Xf* (in the roots) or to distal signals from bacteria present in the stem. Not only will these assays indicate *Xf* responsive promoters, some information on the strength of the promoters but whether they are responsive to distance signals also. These are all procedures that have been developed in our lab with grape as recipient host tissues.

IV. Characterization of GFP expression during *Xylella* infection and leafhopper feeding to identify desired promoter specificities.

Confocal Microscopy. Real time, non-destructive images of the isolated promoters driving the expression of GFP in grape plants will be obtained using a laser activated confocal microscope (BioRad MRC1024) by excitation at 488nm with a Krypton/Argon 15 mW laser. The use of the laser allows non-destructive GFP detection in intact plant leaves and roots. For stem imaging, hand sectioning will be used. Three different fluorescent emissions can be detected simultaneously depending on the filter set used. Current configuration is with the following three filters: (emission filter 578nm-618nm); (emission filter 506nm-538nm); and (emission filter 664nm-696nm).

The first characterized promoters are expected to be available beginning in February 2006 with the final characterization and methods for expression completed by May of 2006. All promoters and characterization details will be available for research purposes at the conclusion of the two-year project.

CONCLUSIONS

The research envisioned will be accomplished by combining expertise and materials from two laboratories, active in PD research, to isolate and characterize PD-responsive promoters from grape. The current project led by Dr. Cook has already identified several genes that are expressed strongly in *Xylella*-infected tissues, but not in healthy counterparts. The project led by Dr. Gilchrist has developed both a transient leaf-based and a stable root-based grape assay and has identified putative anti-PD genes from grape. We are poised to isolate the promoters of the PD-responsive genes from BAC genomic DNA libraries of Cabernet Sauvignon in the Cook lab and functionally test them by techniques used in the Gilchrist lab.

FUNDING AGENCIES

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

SCREENING OF GRAPE CDNA LIBRARIES AND FUNCTIONAL TESTING OF GENES CONFERRING RESISTANCE TO PIERCE'S DISEASE

Project Leaders:
David Gilchrist
Dept. of Plant Pathology
University of California
Davis, CA 95616

James E. Lincoln,
Dept. of Plant Pathology
University of California
Davis, CA 95616

Cooperators:
Andrew Walker
Dept. of Viticulture and Enology
University of California
Davis, CA 95616

Steven Lindow
Dept. of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Reporting Period: The results reported here are from work conducted from October 1, 2003 to October 1, 2004.

ABSTRACT
Our overall objective is to identify genes from cDNA libraries of either grape or heterologous plants that, when induced in grape, will disrupt infection, spread or symptom development by the xylem-limited bacteria, *Xylella fastidiosa* (*Xf*). We are interested in the effect of the genetic disruption of PD symptoms on the movement or establishment of the bacterium in the xylem of susceptible grape plants. Specific objectives are to: a) create cDNA libraries from several different grape backgrounds, including three with PD resistance; b) develop a functional *A. rhizogenes*-based cDNA screen in grape; and c) investigate the potential of blocking PD symptom expression and disease impact with anti-PCD (anti-apoptotic) transgenes. To these ends we have created full-length cDNA libraries from resistant and susceptible grape and developed an *Agrobacterium rhizogenes*-based transformation procedure that provides a functional screen for genes that alter the disease phenotype. Transformation of grape explants with *A. rhizogenes* results in the emergence of a transformed root containing a single new DNA insert, from which the transgene can be re-isolated for characterization. The identified genes will be those that directly affect the ability of the pathogen to cause disease and is not dependent on DNA sequence relationships. Pathogenicity tests with any isolated disease-disrupting cDNA will first involve a transient expression system using micro-propagated (MP) plants that are vegetative clones of sterile grape plants in small plastic boxes that can be infected with *Xf* under sterile conditions. This ensures that these plants will have uniform physiology without confounding by stress inductions as would likely occur in the field or greenhouse grown plants. The MP plants show foliar symptoms typical of infected plants under field and greenhouse conditions. Transient assays with test genes involve infiltration of *A. tumefaciens* containing the gene of interest into MP leaf tissue. The bacteria transfer the test gene into leaf cells that are presymptomatic will determine if the expression of the transgene in the leaf can block PD symptoms.

INTRODUCTION:
Published information from our laboratories confirms that specific transgenes from homologous or heterologous plants, that block PCD during plant disease development (4), as well as chemical inhibitors of apoptotic proteases (3), can arrest both symptom development and microbial growth *in planta* in a range of plant-microbe interactions (3, 4, 5). The conserved genetically determined PCD process can be studied by biochemical, cytological and genetic techniques and can be transgenically manipulated by techniques developed in our laboratory (3, 4). Based on previous results we tested the effect of the p35 transgene from baculovirus on viability of roots, produced on *Xf* infected chardonnay and observed protection of the roots against death in the presence of *Xf*. We believe that the effect of specifically expressing anti-apoptotic transgenes in PD infected tissues on the development of death-related symptoms in grape will contribute significant information in terms of PD biology and physiology. In a longer time frame these data will likely yield genetic or chemical-based signaling strategies for protection of grape against infection by *Xf* in years not decades, perhaps similar to the effects we reported previously in tomato (4).

OBJECTIVES
1. Construct cDNA libraries from several different grape backgrounds including from lines with PD resistance and from infected and uninfected grape tissue.
2. Conduct functional *A. rhizogenes*-based cDNA screen and clone genes that give altered phenotype in grape.
3. Evaluate specific anti-apoptotic plant genes in grape for effect on *Xf* and PD symptoms.
4. Determine the potential of blocking PD symptom expression with anti-apoptotic transgenes through chemical induction of such genes in transgenic grape tissue or by tissue-specific expression in roots or vegetative tissue of *Xf* infected grapes.
5. Use a combination of genetic and signal molecule discovery tactics to elucidation of the molecular basis of susceptibility

RESULTS

Construction of cDNA Libraries

The construction of a grape cDNA library initially proved much more difficult than we had experienced in making libraries from 4 other plant species. Isolation of mRNA was not difficult but the grape tissue contains high levels of phenolic compounds in an oxidative environment that contaminate the RNA, rendering it difficult to reverse transcribe. We now have an efficient protocol for generating full-length cDNA libraries from grape using an antioxidant cocktail during homogenization and CsCl gradient purification of RNA. The Hanes City (*V. shuttworthii*) and Chardonnay libraries are completed with 300,000 members each with an average insert size of 1000 bases . The tissue source was field grown plants provided by Dr. Walker. The susceptible Chardonnay is used as a recipient host to screen cDNA libraries. We have begun screening these libraries while continuing to develop libraries from Cowart (*M. rotundifolia*) and Dr. Walker's resistant tester line 8909-15. The inserts for all libraries are cloned into the binary vector B5 for direct transformation into the *A. rhizogenes* functional screen in Chardonnay and a transient assay. The transient assay is based on a leaf infiltration approach that we have used successfully for tomato and tobacco disease assays of putative resistance genes. For transient assays, selected cDNA inserts in the B5 vector are used to transform *Agrobacterium tumefaciens* strain GV2260. The resulting GV2260 transformed bacteria are then pressure infiltrated into attached pre-symptomatic leaves of *Xf* infected MP plants. The ability of the expressed gene to inhibit symptoms is then evaluated. As potential cloned resistance genes become available they also will be used to identify homologues from the Chardonnay cDNA library that may provide resistance by simple alteration in expression level within the homologous host in a time and tissue specific manner. These full-length cDNA libraries are available to all grape researchers in this program.

Screening of cDNA Libraries

The *Agrobacterium rhizogenes*-based transformation procedure results in the induction of transformed roots from infected (or healthy) vegetative tissue sections following co-cultivation with the transforming bacteria. Each emerging root is an independent transformation event, contains a single new DNA insert from which the transgene can be re-isolated by PCR for characterization. Figure 1 **(**below) illustrates the successful transformation of all emerging roots from a grape stem explant with the green fluorescent protein (GFP). This technique is a functional cDNA library screen (each root contains a different cDNA library member) for genes from grape libraries that block either bacterial multiplication, movement, or symptom expression. We previously determined that viable roots do not form on host tissue explants that are infected with *Xf* unless protected by transgenes. The genes that will be identified will be those that directly affect the ability of the pathogen to cause disease and are not dependent on DNA sequence relationships. The library is being screened in sets of 50,000 cDNAs to improve the efficiency in terms of handling numbers of symptom blocking cDNAs. Based on previous experience with tomato, we expect that less than 0.01% of the cDNAs will effectively protect against PCD and/or the disease development. This underscores the need for a highly effective functional screen. In order to provide sufficient *Xf*–infected tissue of similar physiological state for transformation, we developed a micro-propagation (MP) technique for producing clones of sterile grape plants in small plastic boxes that can be inoculated with *Xf* under sterile conditions illustrated in Figure 2 at the right. The MP plants show foliar symptoms typical of infected plants under field and greenhouse conditions (See leaf in foreground). Plants produced under these same conditions also are the source of *Xf* infected stem sections used for transformation in the *A. rhizogenes* functional screen.

Figure 1

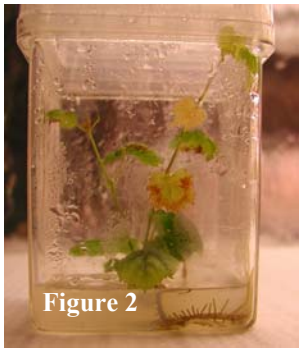


Figure 2

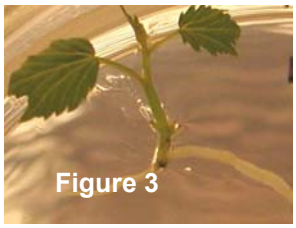
The major advantage of the MP plants is that they are much more efficiently transformed than the greenhouse-derived tissue, which tends to be more highly lignified and produces fewer transformed roots. As a means of fast tracking the cDNA screen while optimizing the grape transformation procedure, we have screened approximately 30,000 members of the Chardonnay cDNA library by *A. rhizogenes* transformation of tomato cotyledons. The resulting roots were subject to disease-dependent PCD induction by treatment with the pathogenic toxin FB1 (1, 2). PCR was used to amplify the Chardonnay cDNA insertion from the surviving tomato roots. The cDNA inserts were then cloned and sequenced. Using this analysis of the Chardonnay cDNA library, we so far have found several grape full-length cDNAs (encoding open reading frames) that protect tomato roots from disease-linked programmed cell death (PCD), a death process that is functionally equivalent to the death of cells in *Xf* infected grape. These grape genes are now being re-evaluated in the *A. rhizogenes*-grape system for protection of Chardonnay grape tissue against symptoms due to the presence of *Xf* in the xylem. Several potentially protecting cDNAs that protect roots are now in the queue to produce whole plant transgenics by the UCD Plant Transformation Facility (Table 1). The expression of these genes in the protected roots was confirmed by northern analysis (unpublished). Most of these genes share sequences homologous with animal genes known to block disease-linked PCD.

Table 1. "Short list" of plant anti-apoptotic genes, derived from functional screen of cDNA libraries, for transformation into grape

Name	ID (putative)
404	empty vector
P35	baculovirus p35
G8	glutathione-S-transferase
G71	cytokine-like protein
P14LD	pathogenesis related gene secretory form
P14	pathogenesis related gene non-secretory form
MT	metallothionine
Y376	mycorrhiza up regulated gene
Y456	nematode up regulated gene

It is important to emphasize that this screen is not dependent on the presence or role of PCD in PD but will detect any gene that affects the integrity of the bacterium in the infected tissue or the ability of the bacterium to elicit symptoms of PD, regardless of whether the step being affected is strictly dependent on the induction of PCD.

Two of the genes (P14LD and Y456) were constitutively expressed in grape by *A. rhizogenes* transformation. The transformed roots were protected against *Xf* -induced death, as were those *Xf*-infected grape explants from which the emerging grape roots transformed with the p35 gene. This indicates a role for PCD in PD and provides optimism that novel genetic determinants of resistance can be identified using this screen. Given the strategies used it is likely the genes will function in grape by altering the effect of *Xf* infection in grape through suppression of symptoms either directly on cell death or indirectly by modifying the behavior of the bacterial in the xylem. It should be emphasized that the effect of anti-apoptotic transgenes on plants is not to induce so-called systemic acquired resistance (SAR) as no markers of SAR are induced in the presence of anti-apoptotic genes such as the p35 gene (4) nor were they observed in the case of the P14LD and Y456.



Our goal is to rapidly identify resistance genes in grape genotypes that block any one of several required steps in the Infection and spread of *Xf* in the xylem, steps which logically will include genetic factors regulating PCD induced by disease stress in grape. We have begun to evaluate the effect of experimental transgenes both from tomato and from grape on grape tissue bearing GFP-*Xf* in xylem elements with various cell death markers and GFP-marked bacteria. By using the GFP-tagged *Xf*, this also is a direct functional assay for genes that block bacterial movement or accumulation in the xylem of newly differentiated grape tissue (6). Of particular interest is the possibility that PD blocking signals initiated with transgenes may move systemically through the vascular system from transformed rootstocks to upper regions of grafted cultivated grape tissue affording protection against systemic movement or activity of *Xf* without genetically engineering the cultivated grape. To this end, the MP plants provide an excellent experimental system by which transformed roots can be initiated on untransformed shoots. The fact that these transformed roots can be formed within 4-6 weeks means that any gene that protects roots can quickly be evaluated for systemic protection; protection from a transformed root stock (in the real world) to an untransformed susceptible fruit producing scion illustrated in Figure 3 above.

RNA Induced Gene Silencing (RNAi)

This same system will enable us to explore the potential for using RNA induced gene silencing (RNAi) (7,9), the expressed silencing small RNA molecules, comprised of small (21 bp) sequences derived from the gene to be silenced, are known to move systemically throughout the plant (8) and silence transgenes from roots to scions. The RNAi from RNAs expressed in the roots have the potential to silence any gene from our project or from other labs that is induced in either susceptible or resistant responses, and deemed to have a definitive role in disease. The small mobile silencing RNAs further have the potential to move systemically in the plant (8) to silence genetic determinants of susceptibility. If either signals from the transgenic roots (from cDNA library screen) or roots expressing RNAi were to provide protection against PD, the best case scenario would be to simply graft a transformed shoot onto an existing infected plant and block the disease without transforming either the roots or the scion. To this end we have developed a plant transformation vector capable of expressing a hairpin RNA. As proof of concept we have used this vector to construct a GFP RNAi expression vector and have shown it is capable of knocking out GFP expression in transient assays. We are currently using *A. rhizogenes* to produce GFP RNAi roots on GFP-expressing transgenic grape shoots to explore the ability of transgenic roots to knock out expression in the shoot.

The research discussed herein has been reported at the Pierce's Disease Symposium in San Diego and in annual reports to the CDFA Pierce's Disease/GWSS Research Program. Manuscripts are being prepared on the various screens developed for the cDNA libraries and the construction of the libraries.

CONCLUSIONS

Genetic resistance and information characterizing the bacterial-plant interaction are high priority areas in the Pierce's Disease/GWSS Research Program. The goal of this project is to identify novel genes from cDNA libraries of either grape or heterologous plants that, when expressed in grape, will disrupt infection, spread or symptom development by *Xf*. Published information from our laboratory established that specific transgenes from homologous or heterologous hosts that block programmed cell death (PCD) (1) during plant disease development (4), can arrest both symptom development and microbial growth *in planta* in a range of plant-microbe interactions (3, 4, 5). PCD is now considered as a key pathway involving many gene products in numerous diseases of animals and plants. Blockage of PCD can be achieved by expression of anti-apoptotic transgenes, RNAi suppression of endogenous genes, and by chemical inhibitors of PCD. Significantly we demonstrated that expression of the anti-apoptotic p35 gene in transgenic grape tissue blocked cell death and PD symptoms in *Xf* infected tissue. We believe that examination of the molecular basis of cell death in symptomatic tissues will be very informative in the short run in terms of PD biology and physiology. In a longer time frame these data will likely yield genetic or chemical strategies for protection of grape against infection by *Xf* in years not decades.

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FUNDING AGENCIES

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UNDERSTANDING *XYLELLA FASTIDIOSA* COLONIZATION AND COMMUNICATION IN XYLEM LUMINA

Project Leaders:
Harvey C. Hoch and Thomas J. Burr
Dept. of Plant Pathology
Cornell University
NYSAES
Geneva, New York 14456

Researcher:
Yizhi Meng
Dept. of Plant Pathology
Cornell University
NYSAES
Geneva, New York 14456

Cooperator:
James N. Turner
NY State Dept. of Health
Wadsworth Center
Albany, New York

Reporting Period: The results reported here are from work conducted from October 2003 to October 2004.

ABSTRACT
Microfluidic chambers were fabricated using photolithographic and soft-molding methods. The chambers were made to mimic the physical parameters of grape xylem vessels in which *Xylella* cells were studied temporally and spatially for colonization and biofilm development. *Xylella* bacteria were observed to migrate by 'twitching' motility against a rapid flowing medium in microfludic chambers. Twitching motility is important in explaining how this pathogen is able to migrate against the flow of the plant's transpiration stream to colonize previously non-invaded xylem vessel regions. Mutant strains with insertions in *pilB, pilQ*, and *fimA* genes established the roles of short pili, and longer type IV pili in biofilm development and long distance migration of the bacterium.

INTRODUCTION
Pierce's disease of grape is generally recognized as being caused by restricted sap flow and resultant water stress due to plugging of xylem elements by live bacterial aggregates and associated mucilage. It is not clear whether the extracellular polymeric mucilage is of bacterial and/or plant origin. Based on the analysis of the complete genome sequence of *Xylella fastidiosa*, gums produced by the *X. fastidiosa* are similar to the 'xanthan gums' produced by *Xanthomonas campestris* pv *campestris*, although they may be less viscous (Simpson et al., 2000). In addition, tylose development in xylem vessels in response to the presence of the bacterium further restricts sap flow (Mollenhauer and Hopkins, 1976). These general concepts regarding *X. fastidiosa* pathogenicity are readily recognized; although, it is not understood how the bacteria become established in the turbulent habitat of a 'fluid conduit' i.e., xylem vessels and tracheae, to form colonies. In addition, how the bacteria are disseminated throughout the xylem vessels from insect-vector feeding sites has long been a particularly puzzling and important question. Long-distance intra-plant migration of the bacteria is even more perplexing since xylem sap flow is always down the pressure gradient, viz., with the transpiration stream that flows toward the leaf. Even under nocturnal conditions when leaf stomates are mostly closed, cuticular transpiration maintains sap flow toward the leaf, albeit at slower rates. Sap flow is seldom stagnant, and rarely, if ever, moves in a reverse direction away from the leaves. Since *X. fastidiosa* is a non-flagellated bacterium, one hypothesis for its ability to migrate against the normal flow of the plant's vascular system has been through the slow and incremental expansion of the bacterial colony through repeated cell division along xylem vessel walls. Another possibility is that occasional cavitation of the water column causes momentary reversal and short distance flow of the sap, thereby carrying the bacteria down the xylem elements. Neither of these scenarios satisfactorily explains colonization of upstream xylem regions.

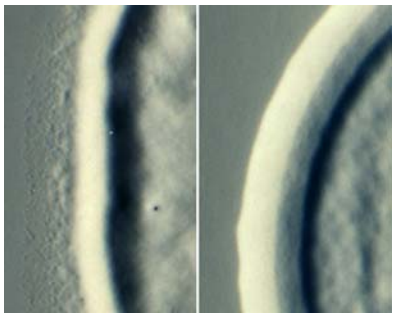
Investigations conducted during the last research period concentrated on understanding biofilm development and how *Xylella* bacteria are able to colonize regions 'upstream' from their initial site of introduction. Toward this, we generated mutant strains to help answer these queries, and we used microfluidic chambers in which we were able to examine the temporal and spatial aspects of bacterial colonization.

OBJECTIVES
To understand how the physical parameters of xylem tracheae and vessels influence *Xylella fastidiosa* colonization. Toward this, we evaluated bacterial movement, colony formation, and biofilm development. Our approach has been to use microfabricated 'artificial' vessels that mimic topologies and chemistries of xylem vessels.

RESULTS
Development of Artificial Xylem Vessels (Microfluidic Chambers)
Microfluidic chambers were fabricated from polydimethylsiloxane (PDMS), supported by a microscope slide with the channel side sealed with an air plasma treated cover glass. The mold for the PDMS device was prepared in silicon wafers using photolithographic procedures. 'In' and 'out' ports and tubing were sealed to the microfluidic chamber. Flow of media through the chambers was facilitated with a syringe pump. Chamber dimensions were as previously reported, but generally were 50-100 μm in width and depth, and up to 14 cm in length.

Mutagenesis of Xylella

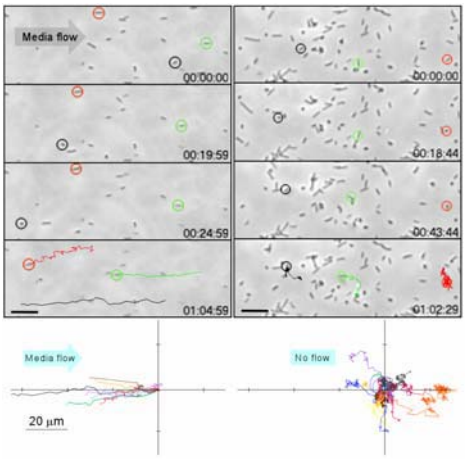
The EZ::TN Transposome system was used to generate *X. fastidiosa* mutants (Guilhabert et al., 2001). Two types of mutants were sought: biofilm modified mutants, and mutants deficient in 'twitching' (type-IV pili) movements. Ninety-six well polystyrene microtiter plates were used to screen for biofilm-modified mutants. The wild-type strain was used as a baseline control for biofilm development. Crystal violet, added to each well, served as an indicator for the presence of biofilm. Wells exhibiting either enhanced or decreased biofilm expression as compared to the wild-type strain were identified visually. Subsequently, biofilm development was assessed by dissolving similarly stained biofilms with DMSO and quantifying by absorbance (A620) in a microtiter plate reader. Screening for twitch minus mutants was performed on modified PW solid medium (Davis et al., 1981). Colonies with a peripheral fringe were designated as having a normal twitching phenotype characteristic of wild-type *X. fastidiosa*. Colonies lacking a peripheral fringe were designated as having a twitching defect.



Light micrographs of wild-type and twitch-minus mutant (1A2) colonies on agar medium with and without a peripheral "fringe."

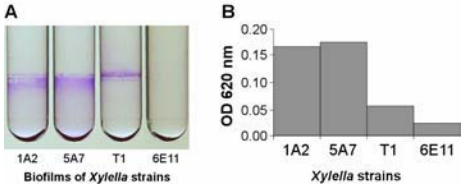
Movement and Biofilm Development of Xylella Bacteria

Wildtype *Xylella fastidiosa* (Temecula) exhibited a colony morphology, viz. fringed margin, consistent with twitching motility that is observed in other bacterial species. Time-lapse imaging of bacteria at the colony edge, revealed both individual bacteria and aggregates of cells that migrated between 0.01-0.32 µm min^{-1}, generally in a direction away from the colony periphery. When the bacteria were introduced into a microfludic chamber, twitching movements propelled migration of individual cells in various directions depending on the rate and direction of medium flow. Under stagnant no-flow conditions, the cells exhibited no directional preference for migration. However, when the medium was passed through the chamber at approximately 20,000 µm min^{-1} (volumetric flow rate = 0.20 µL min^{-1}), a rate comparable to grapevine xylem sap flow under high transpiration conditions (Braun and Schmid, 1999a; Braun and Schmid, 1999b; Lascano et al., 1992; Peuke, 2000), the bacteria migrated predominately against the direction of flow. Under both flow and no-flow conditions the cells were either prostrate on the substratum or, often they were erect and attached at one pole. Maximum twitching speed for *X. fastidiosa* cells examined under flow conditions was 4.9 ±1.1 µm min^{-1} (n = 17), a speed comparable to the observed rate of bacterial spread within grapevines assessed through destructive sampling (Newman et al., 2004).
(Also see, http://www.nysaes.cornell.edu/pp/faculty/hoch/movies/)

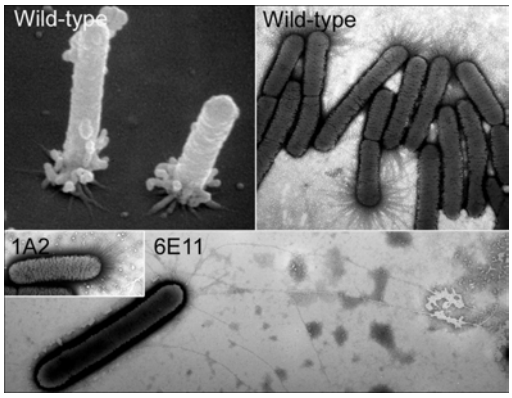


Light micrographs of time-lapse series depicting paths of three (circled red, green, black) wild-type twitching bacteria in microfluidic channels under flow (left) and no flow (right) conditions. Scale bar, 10 µm. Time (h:min:sec). Lower figure, cumulative twitching motility paths for 17 cells under corresponding conditions for 60 min, respectively.

A number of mutant strains were identified as twitching-minus mutants; two (1A2, 5A7) are reported here. Colony peripheries of 1A2 and 5A7 were well demarcated and without bacteria distinctly separated from the main colony mass (lack of peripheral fringe). Colony expansion for these two mutants occurred through repeated cell division and gradual spread as the cell mass increased. When examined in the microfluidic chambers, neither mutant strain exhibited migration, with or without medium flow. Both of these strains were biofilm enhanced. Another mutant, 6E11, was found to be biofilm deficient but still produced colonies with a peripheral fringe and exhibited active twitching, similar to that observed for the wild-type strain. Growth rates of all mutants were not significantly different from the wild-type strain. Sequence analysis of mutants 1A2, 5A7, and 6E11 indicated that transposon insertion occurred in ORFs PD1927, PD1691 and PD0062 of the Temecula genome corresponding to putative genes *pilB*, *pilQ*, and *fimA*, respectively. PilB is known to function as a nucleotide binding protein supplying energy for pilin subunit translocation and assembly, whereas PilQ is a multimeric outer membrane protein that forms gated pores, through which the pilus is extruded (Wall and Kaiser, 1999; Alm and Mattick, 1997; Strom and Lory, 1993). Mutants deficient in these proteins have smooth colony edge phenotypes, do not twitch, and are generally devoid of type IV pili (Kang et al., 2002; Huang and Whitchurch, 2003; Alm and Mattick, 1997; Strom and Lory, 1993). Disruption of *fimA* in *X. fastidiosa* (Feil et al., 2003) as well as in *E. coli* (Orndorff et al., 2004) indicates that the gene encodes for an essential protein of type-I pili that functions in surface attachment and biofilm formation.



Biofilm formation by *X. fastidiosa* wild-type (T1) and mutant strains 1A2, 5A7, and 6E11 following 7 days growth.



SEM and TEM of wild-type cells attached to the substratum at the pili-bearing polar ends. Mutant strains 1A2 and 6E11 depicting only short pili and only longer type-IV pili, respectively.

Electron microscopy substantiated the presence of polar pili on the wild-type and many of the mutant strains.
Negative staining of TEM preparations of the wild-type strain revealed an abundance of pili, the majority of which were 0.4-1.0 μm in length with many additional filaments 1.0-5.8 μm in length. Mutant strains 1A2 and 5A7 had only the shorter class of pili, whereas strain 6E11 had predominantly long pili. The correlation between the presence of long and short pili on the wild-type *X. fastidiosa* strain, the occurrence of essentially only long pili on the twitching, biofilm-deficient strain (6E11), and the absence of long pili on the twitching-minus, biofilm-enhanced mutants (1A2 and 5A7), clearly relates to distinct functional roles for two length classes of pili.

CONCLUSIONS

Microfabricated fluidic chambers were created to mimic plant xylem vessels, in which we studied the non-flagellated *Xylella fastidiosa* bacterium. We discovered that the bacteria migrate 'upstream' by twitching motility, which explains, in part, how they are able to travel against the flow direction of xylem sap to invade non-colonized plant regions.

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FUNDING AGENCIES

Funding for this project was provided by the University of California Pierce's Disease Grant Program.

ISOLATION OF BACTERIOPHAGES SPECIFIC FOR *XYLELLA FASTIDIOSA*

Project Leader:
Michele M. Igo
Section of Microbiology
Division of Biological Sciences and
California Agricultural Experimental Station
University of California
Davis, CA 95616

Cooperators:

Carol Lauzon	Bruce Kirkpatrick
Dept. of Biological Sciences	Dept. of Plant Pathology
California State University	University of California
Hayward, CA 94542	Davis, CA 95616

Reporting Period: Funding for this project was received in September 2004.

ABSTRACT
This report gives an overview of the project. The goal of this project is to isolate a collection of viruses (phages) that can infect and replicate in *X. fastidiosa* (*Xf*). This collection will then be screened to identify phage exhibiting useful biological properties.

INTRODUCTION
The causative agent of Pierce's disease (PD) is the Gram-negative bacterium *Xylella fastidiosa* (*Xf*). *Xf* is highly specialized and is capable of multiplying in both the foregut of xylem-feeding insects, such as the glassy-winged sharpshooter and in the xylem system of the host plant (for recent reviews, see 4, 6, 7). The complex nature of the bacterial-host interactions that take place during the PD infectious cycle and the fastidious growth properties of *Xf* in the laboratory present a formidable challenge to researchers working with this bacterium. At present, there are only a few methods available to perform such basic operations as genetic exchange, mutant isolation, strain construction, and complementation. Further complications of working with *Xf* arise because of its slow generation time, its tendency to form aggregates, and its poor plating efficiency. Finally, few methods are available for disrupting the interaction between *Xf* and its hosts, which is a key component of the PD infectious cycle. As a result, there are currently no effective treatments to cure infected vines.

In other Gram-negative bacteria, bacteriophages, phage derivatives and phage components have played a major role in overcoming these issues (1, 3, 8). For example, phages have been used to move genetic markers between strains, for complementation, and as cloning vectors. In addition, phages have been used as diagnostic reagents to detect pathogenic bacteria, and as therapeutic agents in bacterial infections. Unfortunately, since not all phages possess exploitable properties, it is usually necessary to isolate a collection of phages that infect the bacteria of interest and then to screen the individual phages for desirable properties.

Based on studies of environmental samples, it has been estimated that there are $>10^{30}$ tailed phages in the biosphere and that phage typically outnumber bacterial cells 10 to 1 (2). These studies also revealed that phages could be found anywhere that their bacterial hosts are present. This observation has already proven true for *Xf*. Carol Lauzon and her colleagues have reported the presence of two phages associated with *Xf* from infected grapevines (5). The goal of this project is to isolate a collection of phages that are capable of infecting and replicating in *Xf* (Aim 1). These phages will then be screened individually to identify specific phages that have the potential to be used as genetic tools and for killing *Xf en planta* (Aim 2). Phages capable of moving genetic markers between *Xf* strains would give researchers in the field a powerful tool for investigating the properties of this unusual bacterium and establishing which parts of its genetic material make it such a deadly pathogen for certain varieties of grapes. Furthermore, phage or mixtures of phages capable of killing *Xf* would provide the tools necessary to determine the feasibility of using phage therapy to control the spread of PD.

OBJECTIVES
The primary goal of this project is to isolate a collection of phages as pure stocks and to screen this collection for phages that exhibit useful biological properties for studying and controlling the growth of *Xf*.

Specific Aim 1: Generate a collection of pure phage stocks that infect *Xf*.
- 1A) Collect environmental samples that potentially contain *Xf* specific phages.
- 1B) Isolate and obtain pure stocks of phages from the samples.

Specific Aim 2: Identify specific phage with potentially useful properties within our collection.

2A) Screen the collection to identify virulent phages.

2B) Screen the collection to identify generalizing transducing phages.

RESULTS AND CONCLUSIONS

The first goal of this project is to generate a collection of *Xf*-specific phages that exhibit different biological properties. To increase our chances of obtaining a diverse set of phages, we have collected samples from PD-infected grapevines growing in different vineyards in Northern California. Using infected grapevines as a source seems particularly promising based on the work of Dr. Lauzon and her colleagues (5). Our strategy has been to collect sap from infected vines and samples from the tissue of symptomatic plants. We have also collected soil samples from around infected grapevines to determine if the soil is a good source of *Xf*-specific phage. The next step in our analysis will be to determine if any of these samples contain phage that can infect *Xf*. As a starting point, we will use previously published protocols that have successfully been used to isolate phages from environmental samples for other Gram-negative bacteria.

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THE *XYLELLA FASTIDIOSA* CELL SURFACE

Project Leader:
Michele M. Igo
Section of Microbiology
Division of Biological Sciences and
California Agricultural Experimental Station
University of California
Davis, CA 95616

Cooperators:

Andrew Walker	Bruce Kirkpatrick	Linda Bisson
Dept. of Viticulture and Enology	Dept. of Plant Pathology	Dept. of Viticulture and Enology
University of California	University of California	University of California
Davis, CA 95616	Davis, CA 95616	Davis, CA 95616

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ABSTRACT
A common response of Gram-negative bacteria to environmental stress is to change the composition of their cell surface, particularly the protein composition of their outer membrane. These changes are known to have a profound effect on the sensitivity of Gram-negative bacteria to detergents, antibiotics, and bacteriophages. The goal of this project is to determine how environmental changes influence the protein composition of the *Xylella fastidiosa* (*Xf*) outer membrane. Our strategy has been to isolate the outer membrane fraction from *Xf* cells grown under different environmental conditions. The proteins in this fraction are then separated by one- or two-dimensional gel electrophoresis and their identity established by peptide mass fingerprinting. In this report, I have focused on experiments that examine the *Xf* outer membrane protein profile using one-dimensional gel electrophoresis. This analysis has allowed us to assign three outer membrane proteins to specific genes on the *Xf* chromosome. These gels have also allowed us to examine how the composition of the *Xf* outer membrane changes in response to environmental signals and the physiological state of the bacterial cell.

INTRODUCTION
Pierce's disease (PD) is a devastating disease of grapevines that is caused by the Gram-negative, endophytic bacterium *Xylella fastidiosa* (*Xf*). Although the specific details of the disease process are not fully understood, an important feature is the ability of this pathogen to colonize the xylem tissue of plants and the foregut of insect vectors (for a recent review, see 5). As with most pathogenic bacteria, successful colonization is dependent on the ability of planktonic *Xf* cells to adhere to the host cell surface and to form a microcolony (3, 4, 7). This surface-associated growth commonly leads to the formation of a biofilm. Biofilm-associated *Xf* bacteria constitute a major component of the bacterial biomass in the host tissue. In contrast, planktonic bacteria are less prevalent and are seen primarily as a mechanism for the bacteria to translocate from one surface to another.

The transition of bacteria from the planktonic to the biofilm-associated state involves profound physiological changes (3). The most obvious change is the production of an exopolysaccharide matrix, one of the distinguishing characteristics of a bacterial biofilm. However, the matrix-enclosed mode of bacterial growth requires many other changes, including changes in the protein composition of the bacterial cell envelope. In Gram-negative bacteria, these changes include differences in both the relative abundance of some major outer membrane proteins and the appearance or disappearance of specific high-affinity receptor proteins. This differential expression allows the bacteria to cope with the new environmental condition and with alterations in the nutrient supply.

Changes in the protein composition of the outer membrane are known to have a profound effect on the sensitivity of Gram-negative bacteria to detergents, antibiotics, and bacteriophages (8). As a result, strategies designed to attack planktonic cells are usually not effective against biofilm-associated cells (3). Therefore, in order to develop effective methods for controlling the spread of *Xf*, it is important to obtain information concerning the protein composition of the *Xf* outer membrane and how the composition of this membrane changes in response to environmental signals and the physiological state of the bacterial cell.

OBJECTIVES
The goal of this project is to analyze the outer membrane proteome of *Xf* and to determine how the outer membrane protein profile changes in response to various physiological and environmental conditions. Our experiments are designed to address two objectives:
1. Identify the major outer membrane proteins of *Xf* and assign them to a specific gene on the *Xf* chromosome.
2. Determine how the protein composition of the *Xf* outer membrane is influenced by environmental signals and signals from the infected grapevine.

RESULTS

The primary focus of our research during this reporting period has been to analyze the outer membrane proteome of *Xf* and to assign the outer membrane proteins to specific genes on the *Xf* chromosome. In last year's Symposium Proceedings (6), we described our protocol for analyzing the protein profile of the *Xf* outer membrane. This protocol involves rupturing the *Xf* cells with a French pressure cell and isolating the outer membrane fractions by sucrose density gradient centrifugation. The proteins in this fraction are then analyzed using SDS-polyacrylamide (PAGE) gel electrophoresis. These gels have allowed us to quantitate the amount of the different proteins in the *Xf* outer membrane and to predict the sizes of the proteins based on their migration in the gels. Figure 1 shows a series of SDS-polyacrylamide gels, which reveal the outer membrane profile of *Xylella fastidiosa* strain Temecula 1. These Coomassie-stained gels indicate that there are at least 14-16 major proteins in the *Xf* outer membrane. The sizes of the outer membrane proteins range from 130K to 18K. (Proteins smaller than 18K would not have been detected in this series of experiments.).

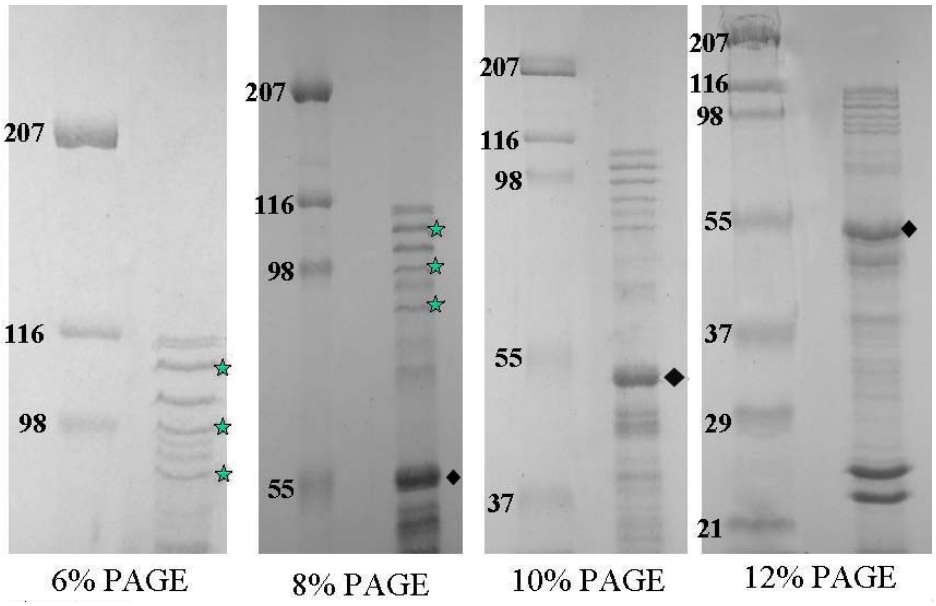

Figure 1: The outer membrane profile of *Xylella fastidiosa* strain Temecula 1.
Proteins in these gels were identified using Coomassie blue stain. The numbers indicate the size of molecular weight standards and their migration on the different percentage gels (left lane). On each gel, the outer membrane proteins from *Xf* Temecula 1 are present in the right lane. The diamonds indicate the location of the MopB protein on the different percentage gels. The stars indicate the locations of the three excised bands, which contained a unique protein based on the MALDI-TOF spectra.

The most abundant outer membrane protein is the MopB protein, which has been characterized by George Bruening and his colleagues (2). Using their purified MopB protein, we have been able to determine the location of the MopB protein relative to other proteins in our outer membrane profiles. (MopB is indicated by the diamonds in Figure 1). The next step in our analysis was to assign additional proteins to specific genes on the *Xf* genome. For these experiments, we separated the proteins in the outer membrane fractions on preparative SDS-PAGE gels and excised five distinct bands from the gels. The proteins in each band were then subjected to trypsin digestion and the resulting fragments were analyzed by MALDI-TOF-MS at the UC Davis Molecular Structure Facility. The resulting information was analyzed using MS-Fit at Protein Prospector (UCSF; http://prospector.ucsf.edu). Analysis of the bands at ~114K and ~104K indicated that more than one protein was present in the excised gel fragment. In contrast, the other three bands contained unique proteins. This allowed us to assign these three outer membrane proteins to specific genes on the *Xf* chromosome (10). The locations of the bands containing these proteins are indicated by the stars in Figure 1.

The largest of the three proteins is ~108K and corresponds to PD1283. PD1283 is predicted to encode a 958 amino acid protein and has been classified as a TonB-dependent receptor protein. The second protein is ~98K and corresponds to PD0326. PD0326 is predicted to encode a 784 amino acid protein and shows homology to the outer membrane protein/protective antigen OMA87. Based on this homology, PD0326 is also called the *oma* gene in some databases. The third protein is ~90K and corresponds to PD0528. Interestingly, this gene is classified in many databases as an inner membrane. However, our analysis of this protein using relatively new computer algorithms suggests that PD0528 encodes a beta barrel outer membrane protein (1). This assignment is more consistent with our fractionation results, which indicate that the PD0528 protein is a major component of our *Xf* outer membrane fraction.

Our analysis of the outer membrane fractions using one-dimensional (1-D) gels illustrates the validity and power of our approach for assigning outer membrane proteins to specific genes on the *Xf* chromosome. However, it was not possible to completely separate all of the outer membrane proteins using 1-D gels. To overcome this problem, we are analyzing our

outer membrane fractions using two-dimensional (2-D) gel electrophoresis with the assistance of our cooperator Linda Bisson and a graduate student in her laboratory, Paula Mara. This technique separates proteins based on their isoelectric points (p*I*) and their apparent molecular weights. In our initial experiments, we identified over 40 well-separated spots and have analyzed these gels using Phoretix proteome analysis software. This software has allowed us to make a tentative assignment of molecular weights and isoelectric points to many of the predominant proteins. To confirm the identification of some of the ambiguous spots, we plan to cut out these spots and identify the proteins using MALDI-TOF-MS as described above. Although we are still working out some technical details, using 2-D gels will allow us to determine the relative abundance of each of the outer membrane proteins under different environmental conditions (the focus of Objective 2). These gels will also provide us with a proteome map for *Xf* Temecula 1 outer membrane, which we can then compare to the published whole-cell protein map for *Xf* CVC (9).

CONCLUSIONS

Proteins on the bacterial cell surface play an important role in the ability of pathogenic bacteria, such as *Xf*, to induce the disease state. During the past year, we have used one-dimensional gel electrophoresis to examine the *Xf* outer membrane profile and have assigned three proteins to specific genes on the *Xf* chromosome. We have also been developing a protocol for analyzing the *Xf* outer membrane proteome using two-dimensional gels. Once these technical details have been worked out, we will be in the position to examine how different physiological and environmental signals affect the relative abundance of specific *Xf* outer membrane proteins. This information should provide valuable insights into the role of the outer membrane proteins in *Xf* virulence and identify potential new targets that may help in the development of effective strategies for controlling the spread of PD.

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ANALYSIS OF *XYLELLA FASTIDIOSA* TRANSPOSON MUTANTS AND DEVELOPMENT OF PLASMID TRANSFORMATION VECTORS

Project Leader:
Bruce Kirkpatrick
Dept. of Plant Pathology
University of California
Davis, CA 95616

Cooperator:
Magalie Guilhabert
Dept. of Plant Pathology
University of California
Davis, CA 95616

Reporting Period:

ABSTRACT
We screened over 1,000 random Tn5 *Xylella fastidiosa (Xf)* mutants in Chardonnay grapevines growing in the greenhouse in 2003. Approximately 10 of the mutants exhibited a hypervirulent phenotype, i.e. vines inoculated with these mutants developed symptoms sooner and died sooner than vines inoculated with the wild type *Xf* parental strain. The identity of the Tn5 insertion sites in these mutants was reported at 2003 PD Symposium. In 2004 we re-inoculated these hypervirulent mutants into another set of Chardonnay, Chenin blanc and Thompson seedless vines and the hypervirulent phenotype was reproduced in all 3 varieties. Movement and populations assays showed that the hypervirulent mutants moved faster and reached higher populations than wild type *Xf*. In the first Chardonnay screen, we identified an unexpectedly high number of avirulent mutants. Because some of these may have been the result of poor inoculation we sequenced the DNA that flanked the Tn5 insertion in all the mutants. Those mutants with Tn5 insertions in genes other than "house keeping" genes were re-inoculated into a new set of vines and their pathogenic phenotype is being determined. Additional small (1.3kb) native *Xf* plasmids were engineered as potential *Xf/E. coli* shuttle vectors. However, like our other similar constructs, these plasmids were not stably maintained without antibiotic selection, and not useful tools for *in planta* gene complementation studies.

INTRODUCTION
During the past 4 years one of the objectives of our research on Pierce's disease (PD) has involved the development of transformation and transposon mutagenesis systems for the bacterium that causes Pierce's disease (PD), *Xylella fastidiosa* (*Xf*). We developed a random transposon based mutagenesis system for *Xf* in 2001 (Guilhabert et. al., 2001). Recently, we developed two *E.coli/Xf* plasmid shuttle vectors, one based on the plasmid RSF1010 and the other based on a small cryptic plasmid found in one of the grapevine *Xf* strains, UCLA. Both those plasmid shuttle vectors replicate autonomously in *Xf* (Guilhabert and Kirkpatrick, 2003; Guilhabert and Kirkpatrick, manuscript submitted for publication). However these plasmids are only stably maintained in *Xf* cells that are kept under selection using the antibiotic, kanamycin. Therefore, these vectors will be useful for *in vitro* studies of *Xf* gene function; however they cannot be used to study the function of *Xf* genes in the plant host. We evaluated other plasmids that can be stably maintained in *Xf* cells inoculated into plant hosts.

The complete genome sequence of a citrus (Simpson et al., 2000) and a grape (Van Sluys et al., 2002) strain of *Xf* have been determined. Analysis of their genomes revealed important information on potential plant pathogenicity and insect transmission genes. However, approximately one-half of the putative ORFs that were identified in *Xf* encode proteins with no assignable function. In addition, some of the putative gene functions assigned on the basis of sequence homology with other prokaryotes may be incorrect. For these reasons we felt that it was important to develop and assess the pathogenicity of a library of random Tn5 mutants in order to identify <u>any</u> gene that may influence or mediate *Xf* pathogenicity. Our group, as well as other PD researchers, is evaluating specific mutants in *Xf* genes that are speculated, based on homology with other gene sequences in the database, to be involved with pathogenicity. However, screening a random transposon (Tn) library of *Xf,* a strategy that has led to the identification of important pathogenicity genes in other plant pathogenic bacteria, may identify other novel genes, especially those that regulate the expression of pathogenicity/attachment genes that will be important in the disease process. Using Tn5 mutagenesis, there is a high probability that we can knock out and subsequently identify *Xf* genes that mediate plant pathogenesis. Proof that a particular gene is indeed mediating pathogenicity and/or insect transmission would be established by re-introducing a cloned wild type gene back into the *Xf* genome by homologous recombination, or more ideally, introduce the wild type gene back into *Xf* on the plant stable shuttle vector.

OBJECTIVES
1. Screen a library of *Xf* transposon mutants for *Xf* mutants with altered pathogenicity, movement or attachment properties.
2. Identify and characterize anti-virulence *Xf* genes.
3. Identify and characterize virulence *Xf* genes.
4. Develop a *Xf/E. coli* transformation plasmid that is stable *in planta*

RESULTS AND CONCLUSION
Objective 1
Using the transposome technology previously described (Guilhabert et al., 2001) we obtained 2000+ *Xf* Tn5 mutants, which should represent fairly random mutagenesis events throughout the *Xf* genome. During the spring and summer 2002, we inoculated 1,000 chardonnay plants with individual *Xf* Tn5 mutants using a pinprick inoculation procedure (Hill and Purcell,

1995; Purcell and Saunders, 1999). The vines were grown in pots in a greenhouse using a nutrient-supplemented de-ionized drip irrigation system. The parental, Temecula strain served as a positive control and a water inoculation served as a negative control. Two months after inoculation, the vines were observed for symptom development approximately every two weeks for 6 more months (32 weeks total after inoculation). The symptoms were rated on a visual scale from 0 to 5, 0 being healthy and five being dead. Rating of 1 showed only one or two leaves with the scorching symptom starting on the margins of the leaves. Rating of 2, showed two to three leaves with more developed scorching. Rating of 3 showed all the leaves with some scorching and a few attached petioles whose leaf blades had abscised (match sticks). Rating of 4 showed all the leaves with heavy scorching and/or numerous match sticks.

We successfully identified *Xf* mutants with altered virulence, confirming for the first time, that screening a library of Tn5 *Xf* mutants in susceptible hosts can identify genes mediating *Xf* pathogenicity. We also developed a two-step procedure, direct PCR on *Xf* colony and direct sequencing of the PCR product that can rapidly identify *Xf* Tn5 insertion sites.

Objective 2

Six months after inoculation (see objective 1), 10 of the inoculated Chardonnay vines showed hyper-virulence, i.e. more severe symptoms compared to the vines inoculated with wild type *Xf* cells. This phenotype was further confirmed in Chenin Blanc and Thompson Seedless grapevines. Further analysis demonstrated that all the hypervirulent *Xf* mutants tested showed i) earlier symptom development, ii) higher disease scores over a period of 32 weeks and iii) earlier death of inoculated grapevines than vines inoculated with wild type; thus demonstrating that the hypervirulence phenotype is correlated with earlier symptom development and earlier vine death in multiple *Vitis vinifera* cultivars. The hypervirulent mutants also moved faster than wild type in grapevines. These results suggest that i) wild type *Xf* attenuates its virulence *in planta* and ii) movement is important in *Xf* virulence. The mutated genes were sequenced and their insertion sites confirmed by PCR amplification and sequencing of PCR products. None of the mutated genes had been previously described as anti-virulence genes, although six of them showed similarity with genes of known functions in other organisms. The hypervirulent mutants were further characterized for *in vitro* and *in planta* attachment. One of the hypervirulent mutants was altered in its microcolony formation and biofilm maturation within the xylem vessels (Figure 1). We are in the process of further characterizing the protein involved in *Xf* biofilm maturation.

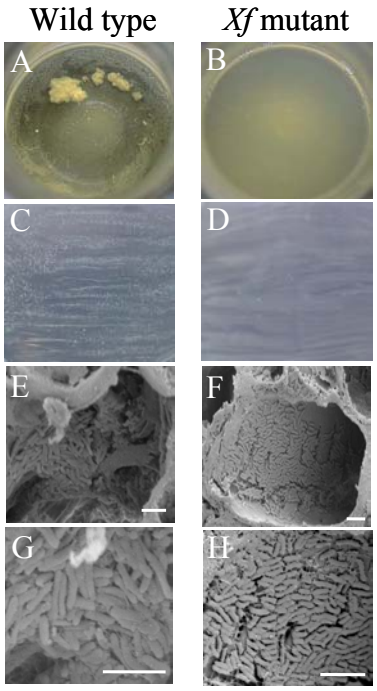

Figure 1: A hypervirulent *Xf* mutant showns a lack of microcolony formation and biofilm formation. Panels A-G are *Xf* wild type cells; Panels B-H are *Xf* mutant cells. Panels A and B wild type and mutant cells, respectively, inoculated into PD3 medium in a 125 mL flask and placed on a shaker. The degree of self-aggregation was visualized after 10 days of incubation. Panels C and D wild type and mutant cells, respectively, plated onto PD3 medium plates. The colony morphology was examined after 10 days of incubation. Panels E and F, wild type and mutant cells in xylem vessels. Note the lack of a three dimension array in the mutant compare to wild type. Panels G and H, close up of wild type and mutant cells in a biofilm. Note the wild type cells typically aggregated together side to side while the mutant cells did not aggregate in this manner. Scale bar equivalent to 5 microns in every panel.

Table 1: Function categories of *Xf* DNA flanking Tn5 transposon insertion in putatively avirulent *Xf* mutants

Putative Gene function	% of Mutants Affected
Hypothetical protein	29
House-keeping	26
Phage-related protein	20
Pathogenicity/virulence	10
Intergenic region	6
Surface protein	2
Transporter	2
Regulator of transcription	1
Mobility	1
Transposon elements	1
Cell-Structure	1
Undefined category	1

Objective 3

Six months after inoculation (see objective 1), we also noticed an unexpectedly high percentage (35%) of inoculated vines that did not develop typical PD symptoms. One might have expected no more than 5% or so of the mutants to be non pathogenic. We sequenced the *Xf* DNA, flanking the Tn5 element in order to determine the specific location of the Tn5 insertion in each putatively "avirulent" mutant. Table 1 summarizes the categories of the genes that were knocked out in the avirulent *Xf* mutants. We then chose to further characterize insertions in open reading frames (ORFs) that code for proteins that have possible roles in *Xf* virulence/colonization or ORFs with no known function. Tn5 insertions in known "house-keeping" genes were not screened further. Three new Chardonnay grapevines growing in pots in the greenhouse were inoculated with each *Xf* mutant of interest as well as the appropriate controls. The experiment was done in duplicate. The rate of symptom development or lack there of, is being monitored as we described in objective 1. After 14 weeks, petiole samples at the point of inoculation (poi) and 12 inches above the poi will be taken from each mutant and control vines. *Xf* cells will be cultured from those samples in order to assess bacterial population and colonization. The insertion sites will be further confirmed by PCR.

Objective 4: Develop a Xf/E. coli Shuttle that is Stable in planta.

A plasmid DNA fraction was isolated from the UCLA strain of *Xf* and subjected to *in vitro* mutagenesis using the transposome technology that was previously used to create our Tn5 *Xf* library. This DNA was electroporated in the UCLA strain and 4 kan^R colonies were obtained. These were sequenced and found to be insertions in the small 1.3kb plasmid that we previously attempted to develop as a *Xf/E. coli* shuttle vector. These Tn5 insertions were in different areas of the native plasmid so we tested the relative stability of these plasmids by culturing the transformants on PD3 medium with and without kanamycin. After 3 passages on non-selective media the colonies were transferred to PD3 media containing kanamycin and no colonies were observed on the plates. This indicates that the plasmids containing the Tn5 insertions were lost upon culture in non-selective medium, results that were the same as our previous attempts to engineer these small native plasmids as shuttle vectors. Future work will focus on a similar strategy to construct a shuttle vector from the 5.8kb plasmid in the UCLA strain, with the hope that this construct might be stably maintained in *Xf* without antibiotic selection.

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DEVELOPMENT OF SSR MARKERS FOR GENOTYPING AND ASSESSING THE GENETIC DIVERSITY OF *XYLELLA FASTIDIOSA* IN CALIFORNIA

Project Leaders:
Hong Lin
Crop Diseases, Pests & Genetics
SJVAC, USDA-ARS
Parlier, CA 93648

Andrew Walker
Dept. of Viticulture & Enology
University of California
Davis, CA 95616

Collaborator:
Edwin Civerolo
SJVAC, USDA-ARS
Parlier, CA 93648

Reporting period: The results reported here are from work conducted from March 2004 to September 2004.

ABSTRACT
Recently available genomic sequences of four *Xylella fastidiosa* strains (PD, CVCD, ALSD and OLSD) facilitate genome wide searches for identifying Simple Sequence Repeat (SSR) loci. Sixty SSR loci were selected for SSR marker development. We designed and validated 34 SSR primers with good reliability and specificity. These SSR primers showed various levels of polymorphism with average 11.3 alleles per locus among 43 *Xylella fastidiosa* isolates. These multi-locus SSR markers, distributed across the entire genome, are a useful tool for pathogen genotyping, population genetics and molecular epidemiology studies.

INTRODUCTION
Xylella fastidiosa (*Xf*) causes economically important diseases that results in significant losses in several agricultural, horticultural and landscape crops, including grape Pierce's disease (PD), almond leaf scorch disease (ALSD), citrus variegated chlorosis disease (CVCD) and oleander leaf scorch disease (OLSD). Recent introduction and establishment of the invasive and more effective vector, the Glassy-winged Sharpshooter (*Homalodisca coagulata,* GWSS) has had a great impact on the California grape industry. Host plant resistance is a critical component of integrated crop management. If this insect becomes widely established, the use of resistant varieties may become the most reliable and effective way to control PD. However, the durability of resistant grape plants depends upon the variability and adaptability of the pathogen population and its interaction with the resistance genes of plants. Most resistance studies are performed by screening against a subpopulation of a given pathogen, and neglect that fact that changes in pathogen population structure that may lead to resistance breakdown.

It is clear that pathogen populations with a high evolutionary potential are more likely to overcome host genetic resistance than pathogen populations with a low evolutionary potential (MacDonald and Linde, 2002). The risk becomes even greater with the recent establishment of a more effective vector, the GWSS, which dramatically increases the dispersal of *Xf* genes/genotypes. In California, information regarding the population structure and genetic diversity, as well as the genetic evolutionary and epidemiological relationships, among *Xf* strains in agricultural populations is not clear. In order to develop effective management strategies, it is critical to understand pathogen population structure and genetic diversity in the agricultural ecosystem. A tool is needed that is capable of precisely, powerfully, easily analyzing *Xf* diversity and genotyping strains. We developed multi-locus DNA markers to fill this need.

OBJECTIVES
1. Perform genome-wide sequence analysis to identify Simple Sequence Repeat (SSR) loci from four *Xf* genomic sequencing databases (PD, CVCD, ALSD and OLSD). Design and develop multi-locus SSR markers.
2. Analyze genetic diversity and population structures of PD *Xf* statewide. Compile a large *Xf* allele frequency database for strain identification.
3. Use the SSR Marker system to examine interactions between hosts and *Xf* including adaptation, host selection and pathogenecity of *Xf* strains

RESULTS
SSR Locus Identification and Primer Design
1. A genome wide search was performed to identify SSR loci among all four *Xf* strains (CVC 9a5c 2.68Mbp, PD Temecula 2.52Mbp, ALS Dixon 2.67Mbp, and OLS Ann-1 2.63Mbp). Figure 1 shows the distributions of SSR loci among four strains of *Xf*.
2. We used the following criteria to select SSR loci for primer design; a) each locus has single hit per genome and b) each selected locus contains at least 5 or more of repeat unit lengths.

3. Sequence alignment was then performed to remove redundant loci and to identify conserved flanking sequence regions across four strains for priming sites between 100-200 bp up/down stream of each repeat locus. This step ensures that primers designed will work for all *Xf* strains.
4. BLAST analysis was performed to examine each selected locus against more than 300 microbial genomes in GeneBank to ensure selected loci are unique. No significant hits were found (E value $<e^{-30}$).
5. All SSR primers were designed using the same parameters (50% GC, T_m=60°C, primer length ≈ 20bp, and self dimer/cross dimer ΔG = -5 kcal/mol). This facilitated SSR primer validation and should facilitate scaling up to multiplex PCR formats in future.
6. Based on the criteria and conditions above, 50 primers passed the *in silico* validation test.
7. We further evaluated 50 SSR primers using 43 *Xf* isolates collected from grape, citrus, almond and oleander hosts (see Table 1). In this study, we used thirty-four primers. The results of 34 SSR markers analyses are illustrated in Figures 2 and 3.

CONCLUSION

Repetitive DNA is ubiquitous in microbial genomes. It has been shown to be a useful tool for genetic study in prokaryotes (Belkum, et al 1998). Data from our preliminary study demonstrates that this technique works well for discriminating *Xf* strains. This project will provide an accurate and reliable marker system for genotyping, quarantine purposes, genetic diversity analyses, epidemiological analyses and risk assessment studies.

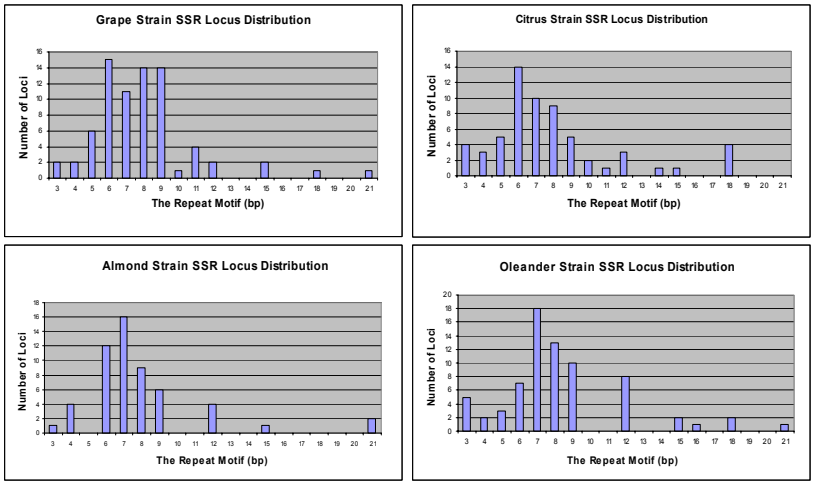

Figure 1. Summaries of SSR loci distributions in each strain of *Xylella fastidiosa*. No mono- and di-repeats occur among these four strains. The above illustrates perfect and imperfect simple repeats with repeat unit length = or > 5.

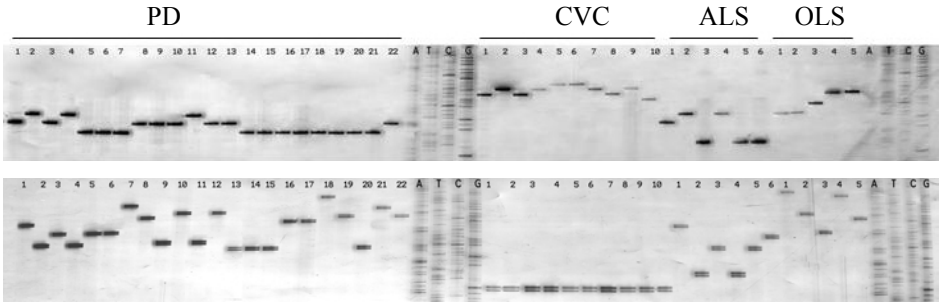

Figure 2. Examples of SSR markers with primers CSSR6 (above) and OSSR9 (below) among 43 *Xylella fastidiosa* isolates separated by 5% of polyacrylamide gel. A, T, C and G are molecular size markers.

Strain Name	Host of Origin	County or state from which strain was collected
PD-1	Grape	Kern, CA
PD-2	Grape	Kern, CA
PD-3	Grape	Kern, CA
PD-4	Grape	Kern, CA
PD-5	Grape	Temecula, CA
PD-6	Grape	Temecula, CA
PD-7	Grape	Temecula, CA
PD-8	Grape	Kern, CA
PD-9	Grape	Kern, CA
PD-10	Grape	Kern, CA
PD-11	Grape	Kern, CA
PD-12	Grape	Baja, CA
PD-13	Grape	Kern, CA
PD-14	Grape	Kern, CA
PD-15	Grape	Napa, CA
PD-16	Grape	Napa, CA
PD-17	Grape	Napa, CA
PD-18	Grape	Napa, CA
PD-19	Grape	Napa, CA
PD-20	Grape	Napa, CA
PD-21	Grape	Napa, CA
PD-22	**Grape (Temecula)***	Temecula, CA
CVC-1	Citrus	São Paulo, Brazil
CVC-2	Citrus	São Paulo, Brazil
CVC-3	Citrus	São Paulo, Brazil
CVC-4	Citrus	São Paulo, Brazil
CVC-5	Citrus	São Paulo, Brazil
CVC-6	Citrus	São Paulo, Brazil
CVC-7	Citrus	São Paulo, Brazil
CVC-8	Citrus	São Paulo, Brazil
CVC-9	Citrus	São Paulo, Brazil
CVC-10	**Citrus (9a5c)***	Brazil
ALS-1	Almond	Tulare, CA
ALS-2	Almond	Contra Costa, CA
ALS-3	Almond	San Joaquin, CA
ALS-4	Almond	San Joaquin, CA
ALS-5	Almond	San Joaquin, CA
ALS-6	**Almond (Dixon)***	Solano, CA
OLS-1	Oleander	Riverside, CA
OLS-2	Oleander	CA
OLS-3	Oleander	CA
OLS-4	**Oleander (Ann-1)***	Riverside, CA
OLS-5	Oleander	CA

Table 1. 43 *X fastidiosa* isolates were used for this study. *Labels in bold are the strains used for genome sequence.

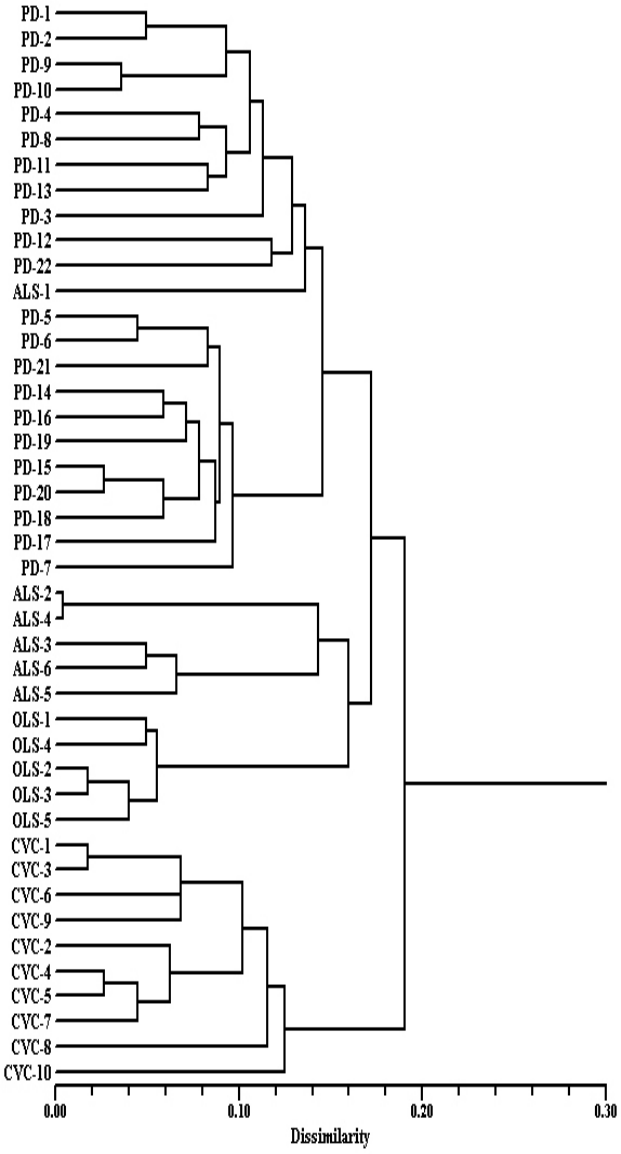

Figure 3. Dendrogram shows genetic distance among the 43 isolates in table 1. Data was compiled from 356 alleles generated by 34 SSR loci.

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FUNDING AGENCIES

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ROLE OF ATTACHMENT OF *XYLELLA FASTIDIOSA* TO GRAPE AND INSECTS IN ITS VIRULENCE AND TRANSMISSIBILITY

Project Leaders:
Steven E. Lindow
University of California
Division of Plant and Molecular Biology
Berkeley, CA 94720-3112
icelab@socrates.berkeley.edu

Alexander H. Purcell
University of California
Division of Insect Biology
Berkeley, CA 94720-3112

Cooperator:
Helene Feil
University of California
Division of Plant and Molecular Biology
Berkeley, CA 94720-3112
icelab@socrates.berkeley.edu

Reporting period: The results reported here are from work that commenced on June 1, 2004.

ABSTRACT
Attachment of *Xylella fastidiosa* to xylem vessels and insect vectors may be required for virulence and transmission; therefore we have individually disrupted *fimA*, *fimF*, *xadA*, and *hecA* to assess their role in adhesion to plants and in the disease process. We performed adhesion assays using each mutant and wild-type separately as well as combination of two of the mutants and observation of the phenotypes of these mutants under a scanning electron microscope is underway. Patterns of cell adhesion and aggregation of mutants on surfaces lead us to hypothesize that *fimA* and *fimF* are important in cell-to-cell aggregation while *xadA* and *hecA* are involved in the first steps of adhesion of bacteria to the plant host. Rooted grapevine cuttings were inoculated with FimA-, FimF-, XadA-, HecA-, and wild-type *X. fastidiosa* 'Temecula' or 'STL'. A higher incidence and severity of disease was observed in vines inoculated with the wild-type *X. fastidiosa* strain compared with FimA-, FimF-, XadA- or HecA- mutant strains. Similarly, wild-type strain STL strain of *X. fastidiosa* resulted in more vines with symptoms than FimA-, FimF- or XadA- mutants of this strain indicating that the process of attachment appears to involve similar genes in both the Temecula and STL strains. It thus appears that successful colonization of plants by *X. fastidiosa* requires both cell-to-cell and cell-to-surface attachment. To distinguish the various mutants from each other in mixed inoculations and to determine what factors affect attachment of the mutants we have constructed disrupted *fimA* vectors for use in a *gfp* marked *Xylella fastidiosa*. This will allow us to distinguish the FimA- cells from other cells in a mixture adhesion assay using fluorescence microsopy and to follow these cells in grape following inoculation with these mutants. Because *hecA* is a large gene, we are also disrupting various locations within the HecA gene. We will test these different HecA- mutants in inoculation experiments to determine the role of HecA in virulence of *X. fastidiosa* to grape.

INTRODUCTION
Adhesion is a well-known strategy used by phytopathogenic bacteria to initiate colonization of their plant hosts and a precursor step to invasion (Romantschuk et al. 1994). *Xylella fastidiosa* possesses many genes involved in attachment or adhesion. Simpson et al. (2000) identified 26 genes encoding proteins involved in the biogenesis and function of Type 4 fimbriae filaments (*pilA*, *B*, *C*…). We have focused on the fimbrial operon, which is composed of 6 genes (*fimA*, *ecdD*, *fimC*, *D*, *E*, and *F*). Even though the fimbrial mutant cells had less fimbriae than the wild type cells as seen in scanning electron micrographs, the cells seemed to still be able to attach to surfaces by another mechanism (Feil et al. 2003) (Figure 1A). This suggested that fimbriae are more important in cell-to-cell adhesion than in cell-to-surface adhesion. While FimA and FimF were found to be important in cell-to-cell aggregation (Feil et al. 2003) the initial attachment of *X. fastidiosa* to plants must involve other factors. The goal of this research was thus to assess the relative role of different fimbrial and non-fimbrial adhesins in the attachment process and to determine their role in the disease process. Among the afimbrial adhesins of *X. fastidiosa* we chose XadA and HecA to study because genes homologous to these in other bacteria were found to be virulence determinants.

OBJECTIVES
1. Determine the role of adhesins other than those found in the fimbrial operon, in particular of the adhesin XadA and hemagglutinin HecA in the attachment and virulence of *X. fastidiosa* in grape.
2. Characterize the behavior of the fimbrial and adhesion mutants of *Xylella fastidiosa* in grape and to compare this behavior over time via expression analysis.
3. Determine what factors affect attachment of wild-type or mutant cells to grape
4. Determine if these mutants can attach to the insect vector and be transmitted to grape.

RESULTS

XadA and HecA mutants of the 'Temecula' strain of *X. fastidiosa* were produced using the method described previously (Feil et al. 2003). Characterization of HecA mutants was done by PCR and sequencing. To confirm that HecA was disrupted at the HecA site, 3 kb fragments of DNA from HecA- mutant cells containing the kan insert were sequenced. Using Blast search, we found that the sequences of the mutant were identical to those of HecA on one side and to N-manoacetyltransferase on the other, indicating that the kan gene was inserted in the HecA region we wanted to disrupt. There are four large HecA homologs in the *X. fastidiosa* genome. The HecA we mutated is the third from the origin of replication of the genome. Dr. Tom Burr group at Cornell University has mutated the 3' HecA homolog using transposon mutagenesis and is characterizing this mutant. We compared wild-type to FimA-, FimF-, XadA- , and HecA- cells using the adhesion assay on silicon surfaces and SEM. We have performed adhesion assays using each mutant and wild-type separately as well as combination of two of the mutants.

We have found that XadA appears to play a major role in the early steps of bacterial adhesion to host surfaces. We observed phenotypic difference between XadA- mutant and wild-type cells of *X. fastidiosa* in culture. In particular, no rings on the sides of the flask were formed when XadA- mutant cells were grown in fructose-based medium whereas a thick ring appeared around the flask when wild-type cells were grown in the same medium. In the adhesion assay using xylem sap, more than 100-fold fewer XadA- cells adhered to a glass surface than of the wild-type cells when observed under SEM, indicating that the XadA- cells are surface adhesion-deficient (Figure 1, B and C).

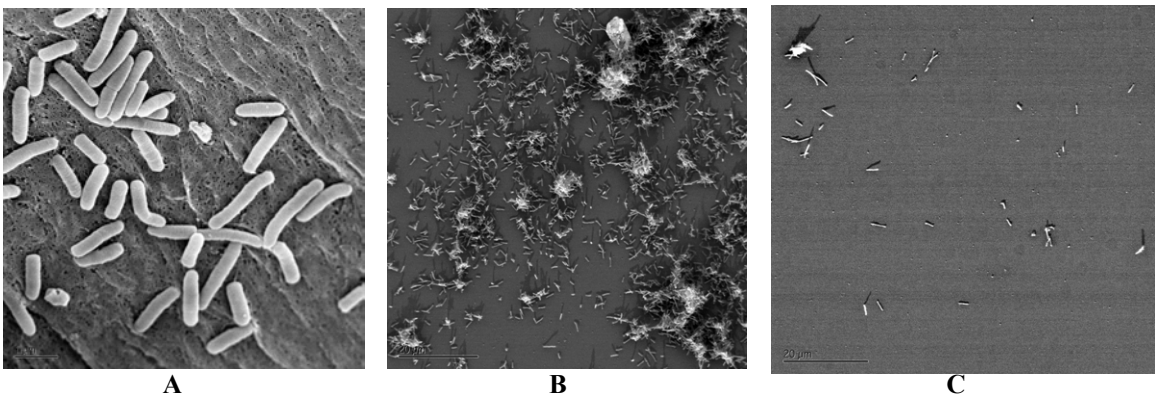
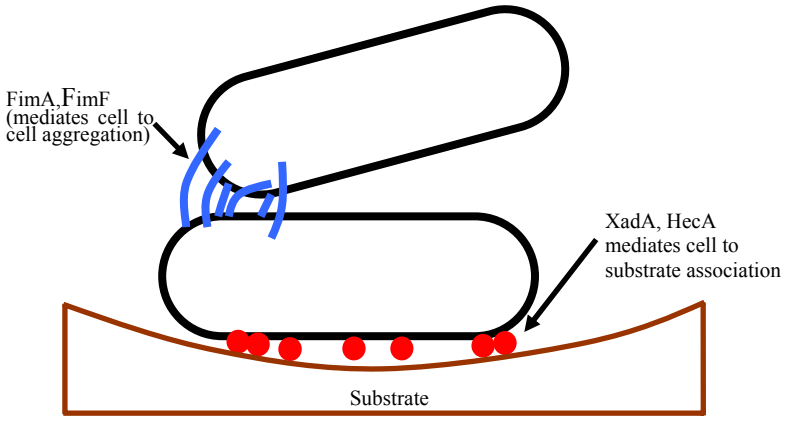


Figure 1. SEM micrographs of FimA- *X. fastidiosa* (A), wild-type (B), and XadA- .

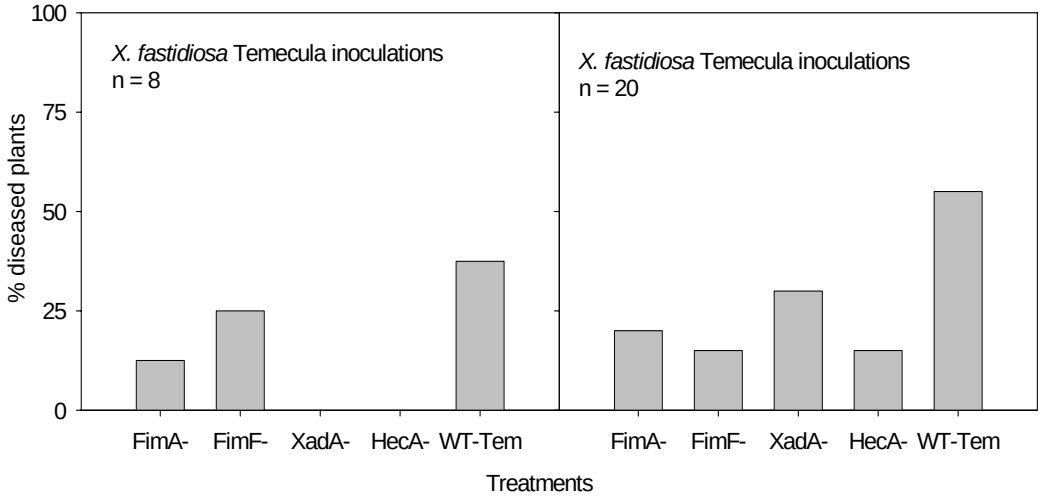
We thus have hypothesized that the afimbrial adhesins are responsible for initial attachment of *X. fastidiosa* to grape xylem vessels. Below is a cartoon depicting a summary of the hypothetical role for each mutant.



Since we have infected grape with each of these mutants (FimA, FimF, XadA, and HecA) and wild-type cells of the 'Temecula' grape strain we will soon be able to assess the pattern of colonization of the plant with the various mutants. Microscopic observation of these tissue sections will be done to visualize *X. fastidiosa* in plants and to compare the extent of colonization between mutant and wild *X. fastidiosa* strains. With a similar approach, we are determining the role of the *fimA*, *fimF*, and *xadA* genes in attachment to insects (BGSS and GWSS). We have fed BGSS in plants infected with these mutant strains and are preparing to visualize the bacterial cells in the insects to determine if different patterns of colonization of the insect have resulted form the adhesion mutation. We will also determine if the insects remain competent to transmit the various mutant strains as well. An initial experiment on acquisition/transmission using FimA, FimF and XadA mutants and

wild-type cells was not conclusive (only two plants out of 100 tested positive following transmission assays using the blue-green sharpshooter as insect vectors). We will repeat these experiments. Insects will be placed on grapes infected with the various mutants (FimA, FimF, XadA, HecA, and wild-type), and acquisition-transmission experiments will be performed. We will keep the insects for further microscopy to determine variation in attachment of the various cells to the insect.
To further test our model of the multifunctional adhesion process we will make FimA-, FimF-, XadA-, and HecA- mutants in a gfp marked *X. fastidiosa* strain (Newman et al. 2003). This will allow us to distinguish each gfp mutant from other cells in mixture experiments during adhesion assays using fluorescence microscopy. This will also enable us to use confocal microscopy to determine the three-dimensional structure of cell aggregates formed by various mixtures of *X. fastidiosa* mutants. This mixture study should enable us to verify, for example, that FimA- mutants will be found attached to the glass or plant surface, while XadA- mutants (but not FimA- mutants) will be attached to each other (and to the FimA- mutants). We will use the FimA mutants in gfp marked *X. fastidiosa* to compare attachment of these cells and wild-type cells in fructose broth. We will observe putative differences in attachment to glass and grape tissue. Difference in ring formation will also be evaluated to determine phenotypic difference.

To assess the virulence of adhesion mutants we have infected grape with each of these mutants (FimA, FimF, XadA, and HecA) and wild-type cells of the 'Temecula' grape strain and recorded the number of diseased plants over time. At a given sample time wild-type *X. fastidiosa* incited a higher incidence of disease in grapevines than either FimA- , FimF-, XadA-, or HecA- mutants (Figure 1). HecA- inoculations generally resulted in the least number of diseased vines.



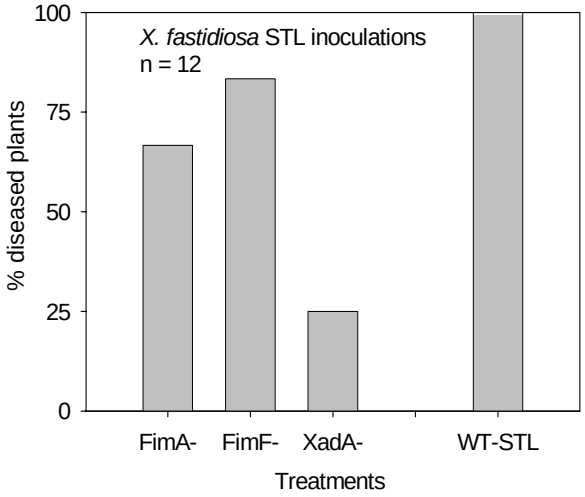


Figure 1. Percent diseased grapevines Following inoculation with either FimA-, FimF-, XadA-, HecA-, and wild-type Temecula or STL *X. fastidiosa*.

CONCLUSIONS

Since disease development was reduced in grapevines inoculated with FimA-, FimF-, XadA- or HecA- mutants compared to wild type *X. fastidiosa* strains we have shown that attachment is important for disease development. Targeting the FimA, FimF, XadA, or HecA genes could be one way to reduce disease incidence in grapevine-growing regions affected by Pierce's disease. We have now observed substantially differential attachment phenotypes for the various attachment mutants under various experimental conditions. The results clearly show that attachment is a complex process, probably involving the sequential contribution of non-fimbrial and fimbrial adhesion factors. These results should help enable an understanding of the over-all process of formation of cell aggregates in xylem vessels, which presumably are major determinants of disease

symptoms. Attachment is also affected by chemical components and now that we know the relative role of different attachment factors we will assess the role of different media components and other compounds that might be feasible for introduction into plants to determine their effects on attachment.

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FUNDING AGENCIES

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

DETERMINATION OF GENES CONFERRING HOST SPECIFICITY IN GRAPE STRAINS OF *XYLELLA FASTIDIOSA* USING WHOLE-GENOMIC DNA MICROARRAYS

Project Leaders:

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

Paul Richardson
Genomic Technologies
DOE, Joint Genome Institute
Walnut Creek, CA 94598
PMRichardson@lbl.gov

Reporting period: The results reported here are from work conducted from December 2003 to October 2004.

ABSTRACT

Xylella fastidiosa (*Xf*) has many plant hosts and causes serious diseases of several crops and ornamentals. Strains of *Xf* can be classified by the hosts that may be infected. For example, grape strains do not infect oleander and the oleander strains do not infect grape. We are using a DNA Oligo-Microarray based on the genomic sequence of the *Xf* grape strain 'Temecula' as the reference strain for a genome-wide comparison with DNA from non-virulent strains. Our approach will determine genes unique to grape strains and thus presumably important in growth and virulence of *Xf* in grape. We hypothesized that the grape strain possesses several unique genes in comparison to other strains that do not infect grape. Initially 2526 of the 2574 predicted ORFs of *Xf* 'Temecula' were designed using the "pick70" software. We manually designed 70-mers oligos for 23 additional ORFs using the same criteria as the program. The remaining ORFs for which oligos were not designed had paralogs elsewhere in the genome with up to 100% identity. Test arrays have been made to determine optimal concentrations of spotted oligos (probes) using a subset of either four or eight probes. Optimal signal intensity was found for a probe concentration of 15-25 nM/ml. All eight probes tested hybridized with labeled DNA from both the *Xf* grape strain 'Temecula' and oleander strain 'Ann'. This indicated that the 8 hypothetical small genes used for the test array were conserved amongst these two genomes. Several quality control tests are underway before we use the full array. The full array includes 2551 70-mer oligos representing the full genome of the *Xf* grape strain 'Temecula'. These oligos were generated with a 5' amino linker that allows for covalent binding to aldehyde or epoxy coated slides, therefore minimizing the background.

INTRODUCTION

Some strains of *Xf* isolated from host plants other than grape do not sustain viable populations or are not virulent in grape. In particular, many of the almond strains of *Xf* do not infect grape (Almeida and Purcell 2003). Other studies provide evidence for host specificity among the *Xf* strains. On a whole genome level, grape strains of *Xf* were found to cluster together away from oak, plum, mulberry, and periwinkle strains using RFLP data (Chen et al. 1992, Chen et al. 1995). Pooler and Hartung (1995) divided the *Xf* in 5 groups (citrus, plum, grape-ragweed, almond, and mulberry) based on RAPD-PCR data. Most almond strains are genetically distinct from the grape strains but a few clustered within the grape-strain group whereas oleander, peach, and oak strains were distinct from other strains using RAPD-PCR, CHEF gel electrophoresis, and 16S-23S rRNA sequence analysis (Hendson et al. 2001). Reciprocal inoculation studies in the greenhouse showed that the OLS and PD strains of *Xf* were not pathogenic to citrus and that the ALS strain was not pathogenic to oleander (Feil et al. *unpublished*).

Based on previous analysis, we estimate that ~4% of the whole genome of the oleander strain is unique to that strain. We hypothesized that the grape strain also possesses ~4% of unique genes in comparison to other strains that do not infect grape. To identify these genes, we will use the grape strain 'Temecula' as a reference to perform pairwise comparison experiments via DNA hybridization using each *Xf* strain that is non-pathogenic to grape. By comparing a large number of strains that both colonize and cause symptoms in grape as well as strains that do not colonize grape we should be able to identify a relatively small number of unique genes that contribute to the virulence of grape by *Xf*.

OBJECTIVES

1. Identify host-specific virulence determinants of the *Xf* grape strain 'Temecula1a'.
2. Investigate the role of these specific genes in virulence.

RESULTS

Strains and Strategy of Screening

70-mer oligodeoxynucleotides were designed using 'ArrayOligoSelector' ('Pick70') software (http://arrayoligosel.sourceforge.net) based on the coding sequence of 2526 of the 2574 predicted ORFs of *Xf* 'Temecula1'. An additional 23 oligos were manually designed from the remaining unrepresented ORFs using the same criteria as 'Pick70', except that sequence 5' or 3' of ORFs smaller than 70 bases was added to obtain an oligo of the correct size. The remaining 25 ORFs are represented by paralogs with 100% identity found elsewhere in the genome. The designed oligos were generated with a 5' amino linker that has allowed for covalent binding to aldehyde or epoxy coated slides. The Final number of ORFs represented by gene-specific oligodeoxynucleotides on the arrays is 2551 not including negative and positive

controls. Recently we have optimized our hybridization process. A probe concentration between 15 – 25 nM/ml gave the highest signal following hybridization with labeled DNA. We have the oligos to print no fewer then 5,000 slides depending on the final concentration of the oligos and the number of slides printed during each printing. These slides represent the whole genome of a grape strain of *Xf* and we will compare this genome to the genome of about 15 other *Xf* strains non-pathogenic to grape as well as to at least 15 strains pathogenic to grape.

The host range of many strains of *Xf* has been studied and we will use this information in this study. We will use well-characterized strains of *Xf* that were found to not sustain viable populations in grape or to be non-pathogenic to grape. Some strains will be chosen based on their placement in phylogenetic trees after molecular analyses (i.e several almond, oleander, oak, peach strains, etc) These strains are listed in Table 1.

Table 1. Isolates of *Xf* that will be used in the study.

Name	Host	Origin	**Log CFU/g (±SE) in grapes**	Reference
Temecula	Grape	Riverside, CA	8.4 ± 0.1	Almeida et al. 2003
STL	Grape	Napa	8.3 ± 0.1	Almeida et al. 2003
Medeiros	Grape	Fresno	8.4 ± 0.1	Almeida et al. 2003
Dixon	Almond	Solano Co., CA	3.8 ± 0.1	Almeida et al. 2003
ALS7	Almond	San Joaquin, CA	4.5	Almeida et al. 2003
Manteca	Almond	San Joaquin, CA	3.9	Almeida et al. 2003
Ann1	Oleander	Riverside, CA	None	Almeida et al. 2003
Plum 2#4	Plum	Georgia	--	Hendson et al. 2001
Oak 88-9	Oak	Florida	--	Hendson et al. 2001
Oak 92-3	Oak	Florida	--	Hendson et al. 2001
OLS#2	Oak	Georgia	--	Hendson et al. 2001
5S2	Peach	Georgia	--	Hendson et al. 2001
5R1	Peach	Georgia	--	Hendson et al. 2001
4S3	Peach	Georgia	--	Hendson et al. 2001
ML1	Mulberry	Georgia	--	Chen et al. 1992
ML2	Mulberry	Georgia	--	Chen et al. 1992

Initial DNA hybridizations was done using microarray. The DNA microarray for the Temecula strain of *Xf* is now complete. We have purchased and spotted the oligonucleotides corresponding to each open reading frame of this strain on glass slides. We can readily produce as many DNA microarrays as we and other researchers will need. As noted above, the conditions for hybridization of DNA to this microarray has now been optimized. A probe concentration of 20 nM/µl gave the highest signal following hybridization with labeled DNA. We have collected all of the *Xf* strains noted in Table 1 that will be used in initial genome comparisons using the DNA microarray. We are in the process of extracting genomic DNA from these strains as well as many other grape strains of *Xf* and will hybridize to the DNA microarray very soon. The DNA is being sheared by sonication and being reciprocally labeled with Cy3 and Cy5 fluorescent dyes. Test hybridizations are being performed to enable us to determine threshold differences for use in genomic comparisons. Images of array spots were collected as 16 bit Tiff files by scanning washed slides using the GenePix 4000B laser Scanner (Axon Instruments, Union City, CA). The GenePix Pro 4.1 software program will be used for data collection to analyze the 16 bit Tiff files and for measuring signal intensities for each. The value for spot intensity will be normalized by subtracting the respective background intensity for each spot from the initial intensity.

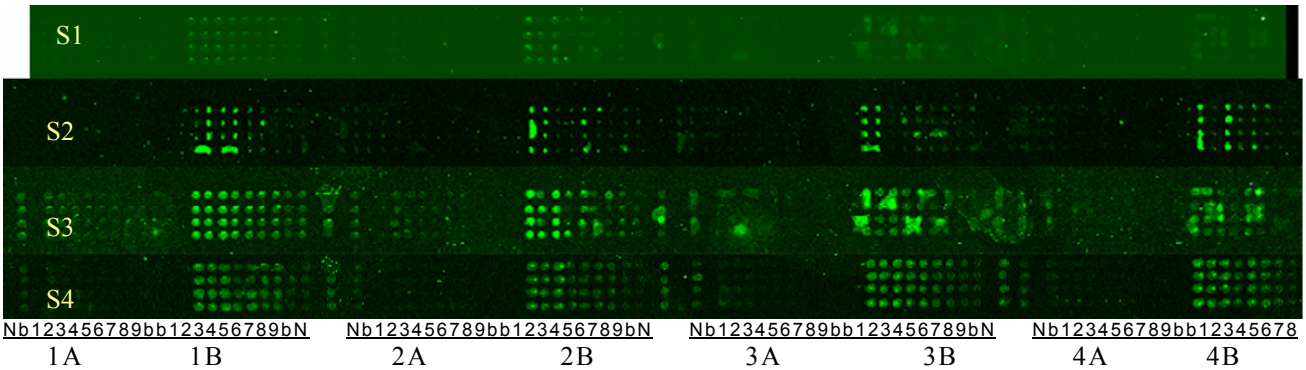

Figure 1. Combined images from four 70mer-oligo test arrays representing 8 ORFs. Each Slide (S1 – S4) was hybridized separately with cy3-labelled sheared DNA and a representative section of the resulting image was used for this figure. Oligos were spotted as in Table 1. N, negative control; b, buffer; 1, oligo concentration is 40 nM/ml; 2, 35 nM/ml; 3, 30 nM/ml; 4, 25 nM/ml; 5, 25 nM/ml; 6, 20 nM/ml; 6, 15 nM/ml; 7, 10 nM/ml; 8, 5 nM/ml; 9, 5 nM/ml. S1 and S2, epoxy-silane slides by Schott (Elmsford, NY; S3 and S4, by Telechem (ArrayIt™ Division, Sunnyvale CA). S1 and S3, hybridized with *Xf* 'Temecula' DNA; S2 and S4, hybridized with *Xf* 'Ann1' DNA.

Table 2: List of ORFs used in the Test Array in Fig 1.

Block	ORF	Function
1A	282	Hypothetical
1B	595	Hypothetical
2A	818	Hypothetical
2B	1812	Hypothetical
3A	2159	Hypothetical
3B	2255	Hypothetical
4A	2461	Hypothetical
4B	2696	Hypothetical

Upon completion of objective 1 putative grape-specific virulence genes will be identified for the mutagenicity experiment. To test the pathogenicity of the mutants, we will needle-inoculate grapes with the mutants and wild type *Xf* strains and check for pathogenicity. We will also examine the mutant cells (i.e. deficient in the unique genes to the grape strain) under scanning electron microscope (SEM) to determine their morphology in vitro and their behavior in planta. Future research to characterize virulence of these genes in various hosts has been proposed.

CONCLUSIONS

We have now completed the extensive process of identifying unique oligonucleotides suitable for use in the DNA microarray as well as determining the conditions for hybridization. The actual process of DNA-DNA hybridization on the oligonucleotide arrays should proceed quickly and we should soon have a list of genes unique to grape strains of *Xf*. Since we have already observed differences between strains of *Xylella fastidiosa* using amplified fragment length polymorphism (Feil et al, unpublished) and via cross-inoculation experiments we expect that such unique genes will be found and be predictive of host range and/or virulence. We expect that our analyses using this method comparing the grape strain to many other strains non-virulent to grape will provide a robust and complete set of unique genes to the grape strain of *Xf*. We have the oligos to print no fewer then 5,000 slides depending on the final concentration of the oligos and the number of slides printed during each printing. These slides represent the whole genome of *Xf* and should be invaluable to other scientists also interested in strain comparisons or gene expression analysis studies. The information gathered by this study can also be used to produce specific DNA markers for differential detection of *Xf* strains such as by PCR.

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FUNDING AGENCIES

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MULTILOCUS SEQUENCE TYPING TO IDENTIFY RESERVOIRS OF *XYLELLA FASTIDIOSA* DIVERSITY IN NATURAL HOSTS IN CALIFORNIA

Project Leader:
Robert Luck
Dept. of Entomology
University of California
Riverside, CA 92521

Cooperators:

Richard Stouthamer	Len Nunney	Don Cooksey	Danel Vickerman,
Dept of Entomology	Dept. of Biology	Dept of Plant Pathology	Dept. of Entomology
University of California	University of California	University of California	University of California
Riverside, CA 92521	Riverside, CA 92521	Riverside, CA 92521	Riverside, CA 92521

Reporting period: The results reported here are from work conducted from July 2004 to October 2004.

ABSTRACT

INTRODUCTION

The ability to identify accurately and track the strains of an important infectious agent causing a plant disease is fundamental to its surveillance and management. It is also fundamental to the recognition of future changes in strains of the disease that result from 1) the invasion of exotic strains or 2) the recombination and evolution of known strains, including recombination with native strains that are as yet unrecognized. Unambiguous identification of *Xylella fastidiosa* (Wells) (*Xf*) strains and clones is of vital importance in understanding 1) the epidemiology of this bacterium, 2) the relationships between the different *Xf* strains and their host plant species, and 3) the geographic distribution of the "ancestral" strains in California. In the case of *Xf*, this is all the more critical because the introduction of the Glassy winged Sharpshooter, *Homalodisca coagulata* (Say) (GWSS), has changed the population dynamics, epidemiology, and the potential virulence trajectory of these bacterial pathogens. GWSS allows for frequent transmission between hosts not normally or as frequently visited by the native *Xf* vectors. GWSS adults feed on a wide variety of plants, and they are known to acquire multiple strains of the *Xf* (Costa *et al*. 2003). This observation takes on added significance when it is combined with the recent research findings of several recombination events between different host strains (Nunney et al. 2003, Scally et al. In Prep). Thus, the emergence of new strains that can infect new hosts or become more virulent on their traditional hosts is to be expected. To this, we can add two additional concerns. First, the identified strains in California consist of only those that are associated with a syndrome in an agricultural or ornamental host plant. We do not know how many asymptomatic indigenous strains exist in California, especially in native or naturalized alien plants because they have not, as yet, given rise to a recognizable syndrome. Second, the possibility of invasions by novel strains from other parts of the Americas cannot be ignored.

Therefore, it is critically important that we characterize the diversity of *X. fastidiosa* strains present in California especially those presumed to be the ancestral strains, i.e., those in native and naturalized alien plant hosts as a benchmark. This information is essential for fully understanding the potential for recombination and the generation of new strains.

In both central and northern California, the incidence of *Xf* in commercial vineyards is associated with the occurrence of the blue green sharpshooter (BGSS), *Graphocephala atropunctata* (Signoret) (Freitag 1951, Purcell 1975, 1976). BGSS inhabits riparian areas and has been documented as feeding on at least 16 riparian host species sequentially through the season (Purcell 1976). However, the principal species on which it feeds are the native grape, *Vitis* spp., blackberry, *Rubus* spp., Elderberry, *Sambucus* spp., stinging nettle, *Urtica* spp., Mugwort, *Artemesia douglasiana*, and cocklebur, *Xanthium strumarium* (Purcell 1976).

These species occur in riparian habitats both in northern (Purcell 1975, 1976, Purcell and Saunders 1999) and southern California (Hickman 1993, B. Boyd and M. Hoddle pers. comm.). Inoculations of these species with PD *Xf*-infected BGSS in a controlled experiment showed that the inoculated plants maintained populations of *Xf* (Purcell and Saunders 1999). A similar inoculation experiment showed that *Xf* overwintered in a subset of these plants (Purcell and Saunders 1999) but they mostly manifested asymptomatic infections that were only detectible by culturing. It is highly likely that other nonculturable, asymptomatic forms exist in these and other plants as well (Cooksey and Costa 2003, Costa et al. In Prep).

These riparian habitats harbor *Xf* which is spread from them to cultivated grapes by infected BGSS as they move from the riparian vegetation in late spring - early summer into the vineyards and plant communities adjacent to the riparian areas (Purcell 1975). Presumably GWSS acquires the inoculum from the infected plants in these areas, yet we know precious little of the variety of strains that reside in these riparian habitats. It is these ancestral strains that we seek to characterize and to associate with their host plant species and geographic locations. This information underpins the work on strain diversity and

the likely evolution of new, perhaps more virulent strains. It also is important in cataloging the strains in California so that the invasion of new stains can be detected.

OBJECTIVES

1. Collect *Xylella fastidiosa* samples from a diversity of native and naturalized alien plants in and around the riparian zones in southern and central California.
2. Collect *Xylella fastidiosa* samples from a diversity of adult sharpshooters: *Homalodisca coagulata* (Say) and *Homalodisca liturata* Fowler,
3. Characterize the *Xylella* strains that are recovered using multilocus sequence typing (MLST) and,
4. Determine the associations between specific *X. fastidiosa* strains, their plant hosts, and their geographic distributions.

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FUNDING AGENCIES

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

GENOME-WIDE IDENTIFICATION OF RAPIDLY EVOLVING GENES IN *XYLELLA FASTIDIOSA*: KEY ELEMENTS IN THE SYSTEMATIC IDENTIFICATION OF HOST STRAINS, AND IN THE SEARCH FOR PLANT-HOST PATHOGENICITY CANDIDATE GENES

Project Leader:
Leonard Nunney
Dept. of Biology
University of California
Riverside, CA 92521

Cooperators:

Richard Stouthamer	Robert Luck
Dept. of Entomology	Dept. of Entomology
University of California	University of California
Riverside, CA 92521	Riverside, CA 92521

Reporting Period: The results reported here are from work conducted from October 15, 2003 to September 31, 2004.

ABSTRACT
We have developed a robust phylogeny of the North American isolates of *Xylella fastidiosa* based on 10 genes (9288 base pairs). This supports the recent division of *X. fastidiosa* into subspecies (piercei and multiplex in N. America), however, we found 1 additional distinct taxon. The oleander isolates form a distinct group (provisionally named sandyi) that separated from the Pierce's disease group (piercei) long before European settlement of N. America, probably substantially more than 20,000 years ago. We used the phylogenetic tree to confirm the effectiveness of multilocus sequence typing (MLST) in identifying the subspecies and (within subspecies multiplex) plant-host isolates. MLST involves sequencing at least 7 genes from pure cultures. We have also developed a simpler method that distinguishes the major groups using restriction enzymes. This method has the advantage of working on mixed cultures and requiring only 3 PCR reactions. Our sequencing has confirmed that *X. fastidiosa* is largely clonal, and that within the piercei and sandyi groups there is very little genetic variability or geographical substructure. This pattern is particularly notable given the age of these groups and suggests the action of strong natural selection favoring specific clones. Finally, we found 4 (1.6%) examples of interstrain recombination, and the clustering of 3 in each of 2 isolates suggests that recombination may drive the rapid evolution of new pathotypes.

INTRODUCTION
We are utilizing the extraordinary power of genomic research to investigate aspects of *Xylella fastidiosa*'s evolutionary history. This history provides information essential for controlling and solving the problem of Pierce's disease. At a minimum, it provides an understanding of the origin of the Pierce's disease (PD) strain of *X. fastidiosa*, and the relationship of the PD strain to other isolates of *X. fastidiosa*. Knowing the level of variability within the PD strain provides important information regarding the nature of these bacteria. Low variability would suggest that the PD strain is subject to significant constraints that may make controlling the pathogen simpler. On the other hand, evidence of high variability and high levels of recombination would suggest that the rapid evolution of resistance to control measures could be a severe problem.

A high priority is to place the PD strain within a robust phylogeny, extending earlier work defining the interrelationships of the plant-host strains of *Xylella fastidiosa* (e.g. see Hendson *et al.* 2001). Schaad *et al.* (2004) have recently named the PD strain as subspecies piercei, based on DNA hybridization. They identified two N. American subspecies (piercei and multiplex). It is important to determine if that taxonomy is sufficient to describe all N. American isolates.

Given a robust phylogeny, genomic data can be used to develop effective methods for identifying host strains, using either simple assays (e.g. restriction enzymes) or more sophisticated methods. MLST (multiple locus sequence typing) (Maiden *et al* 1998) is a valuable technique for identifying bacterial strains. Unambiguous identification of strains is of considerable importance for understanding the epidemiology of Pierce's disease and the other plant diseases caused by this bacterium. Previously, this has been approached using a variety of DNA based methods (Banks et al. 1999; Hendson *et al*. 2001; Rodrigues et al. 2003; Meinhardt et al. 2003;); however, an effective methodology for identifying the plant-host strains, including when they are mixed together, has yet to be developed.

The bacterium *X. fastidiosa* is generally assumed to be clonal. However, virally-mediated horizontal transfer of genes must occur given the presence of unique regions of DNA in the different host strains (Van Sluys *et al*. 2003). The possibility of direct inter-strain genetic transfer is more difficult to detect, but needs to be investigated. If such transfer does occur, it could lead to the very rapid evolution of novel pathogenic forms. Studying the details of sequence evolution across many genes provides information on the past occurrence of such events and hence their future likelihood.

OBJECTIVES

During the last year we have focussed on the following objectives:

1. Develop a systematic multigenic method for identifying host strains of *X. fastidiosa*. Our objective is to develop a method that unambiguously identifies the known host strains, and that allows an efficient recognition of the invasion of new strains.
2. Measurement of clonal variation within host strains. Our objective is to assess within-strain genetic variability and geographical substructure at our target gene loci. From this we can infer the probable importance of plant-host adaptation.
3. Estimate the frequency of recombination. Our objective is to look for evidence of both within- and between-strain genetic transfer. Genetic transfer can dramatically increase the rate of evolution, and potentially can increase the rate at which new –more virulent- host strains arise.

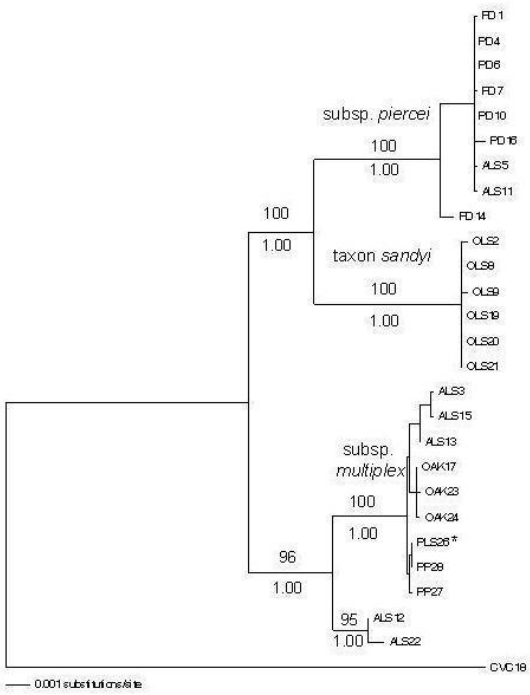

Figure 1. Phylogenetic relationships among 26 N. American isolates of X. fastidiosa from 6 species of host plant, using CVC (from S. America) as the outgroup. The maximum likelihood tree is based on 10 genes except PLS26, which was positioned in the tree based on the sequence of 7 genes. Isolates were from grapevine (PD), almond (ALS), oleander (OLS), oak (OAK), peach (PP), and plum (PLS).

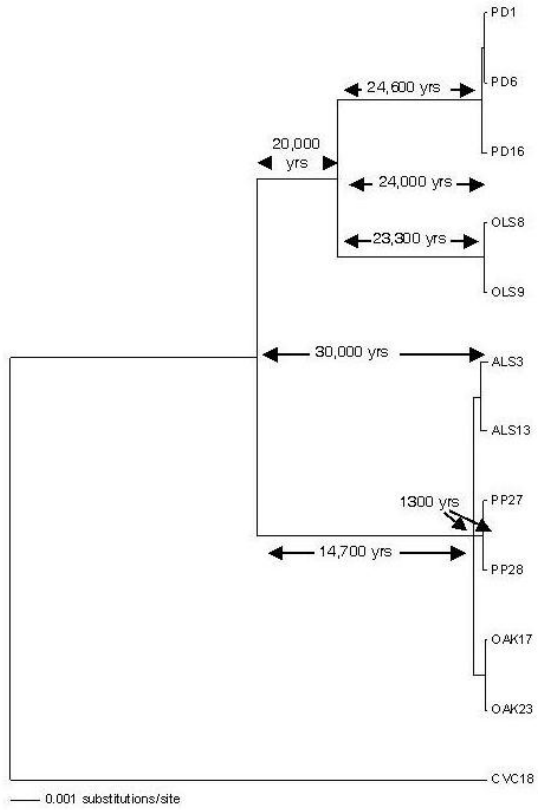

Figure 2. Phylogenetic estimates of the divergence times of the groups of X. fastidiosa based on the rate of synonymous substitution within each branch of the maximum likelihood tree.

RESULTS

Objective 1: Develop a Systematic Multigenic Method for Identifying Host Strains of X. fastidiosa.

To create a statistically robust phylogeny of the host-plant strains of *X. fastidiosa*, we sequenced 10 genes (9288 bp) from each of 25 isolates, and 7 genes from 1 additional isolate. The results are shown in Figure 1 using the S. American CVC strain as the outgroup. The tree shows three well-defined clades that are supported 100% by bootstrap procedures. Two of these clades correspond to the recently named subspecies piercei and multiplex (Schaad et al 2004). Subsp. piercei includes all Pierce's disease isolates. Subsp. multiplex includes a set of isolates from almond plus isolates from a range of host plants from the eastern US (oak, peach, and plum). The third clade contains only isolates from oleander. It is most closely related to subsp. piercei, but shows a high degree of differentiation from that subspecies (2.6% at synonymous sites). In addition, bacteria from these two groups cannot infect each other's major host plant (oleander vs. grapevine) and based on the lack of intermediates, we conclude that the oleander clade constitutes a third N. American subspecies that we have tentatively named sandyi (Scheunzel *et al* 2004).

To begin to understand the evolution of the pathogenicity of the plant-host strains of *X. fastidiosa*, it is important that we have a good estimate of the age of these clades. In particular, since this species of bacteria appears to be restricted to the

Americas and since most of the plant hosts exhibiting disease symptoms are introduced species, we need to know if these three N. American clades pre-date European colonization. We estimated divergence dates based on the rate of synonymous substitution. Assuming that such substitutions are generally neutral and driven by genetic drift, then we have that the time of origin *T* (in years) of a given clade is $T = K/(nu)$, where *K* is the number of synonymous substitutions per site in a given branch, *u* is the mutation rate per generation, and *n* is the number of generations per year. We used $u=5.4x10^{-10}$ (the E. coli rate, see Drake *et al* 1998) and n=1000, corresponding to a long-term division rate of once every 9hrs. The generation time of *X. fastidiosa* has been estimated at between 9 and 60 hours (Wells *et al* 1987), so our assumption is conservative (reducing T). The resulting estimates are shown in Figure 2. These estimates suggest that the three clades, piercei, multiplex, and sandyi, have been distinct for at least 15,000 years, and possibly much longer.

It is notable that the estimated age of the multiplex clade is 3x less than the estimated age of the parallel piercei/sandyi group. Since they are exactly the same age, the most likely explanation is that the generation time (in nature) of members of the multiplex clade is about 3x longer (i.e. *n* is smaller in eqn 1). Note that this effect is apparent both before and after the split of piercei and sandyi, (20,000 yrs plus 24,000 yrs compared to the multiplex total of 14,700 yrs), and that the rate within the piercei and sandyi clades is extremely similar (24,600 vs. 23,300).

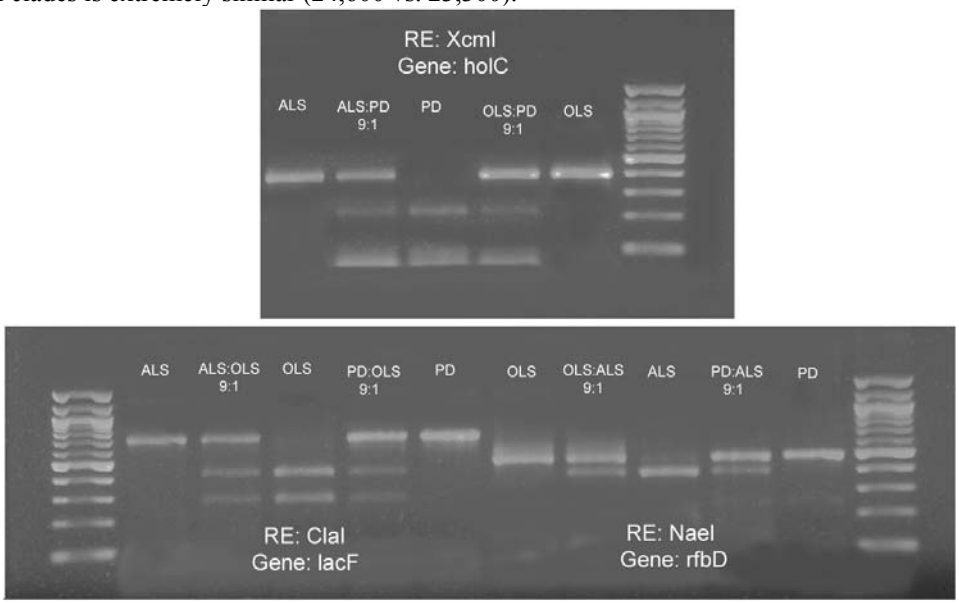

Figure 3. Restriction digests following amplification of single genes from pure-strain DNA, or from a 9:1 mix of the DNA of two strains.

We have shown that the MLST approach of Maiden *et al* (1998) can be used to document both the differences among the three major groups, and the differences among the plant-host isolates of subsp. multiplex (data not shown). The strength of this approach is that MLST data are unambiguous, can be held on a central database, and can be queried through the Web.

Using three of the target genes, we developed a PCR/restriction enzyme essay that separates the major groups of *X. fastidiosa*. We have shown that this method can be used to identify strains from mixtures of DNA (figure 3).

Objective 2: Measurement of Clonal Variation Within Host Strains

It is clear from Figure 1 that there is very little variability within the three clades. Furthermore, we found no evidence of geographical substructure. Using Kst (which measures genetic differentiation between populations relative to within populations) we found no differentiation between 2 northern California isolates of piercei (PD4,6; see fig. 1) vs. 6 southern California isolates (PD1,7,10,14, ALS5,11) (Kst = 0.00 ns), or between three northern California almond (non-piercei) isolates (ALS3,15,22) and 2 southern California isolates (ALS 12,13) (Kst = -0.26 ns). Over a longer distance, the piercei isolate from Florida (PD16) and the sandyi isolate from Texas (OLS8) showed no marked difference from the remaining isolates in their respective clades (all from California). The lack of intra-clade variability results in a phylogeny with long basal branches leading to very short terminal branches. This pattern suggests that the strains experience strong selective pressures from their host plants, eliminating all but the best-adapted clones.

Objective 3: Estimate the Frequency of Recombination

Given the low level of clade variability, the isolates exhibiting inter-strain recombination at one or more of the 10 sequenced loci can be seen quite clearly from fig. 1. They are PD14 (1 recombination), and ALS 12, 22 (recombination in 3 genes). The sites of the recombination can be seen clearly by aligning the sequences. Thus from 257 gene sequences we found 4 independent recombination events, i.e. 1.6%. It is notable that ALS 12 and ALS 22 were isolated in California from almond

trees more than 200 miles apart (Temecula and San Joaquin), but they exhibit the same 3 recombinant events. These isolates may represent the evolution of a new pathotype through recombination.

The source of the recombinant DNA could be determined by its sequence identity with the gene from a different strain. This identity suggests that these genetic transfers occurred relatively recently. Thus PD14 incorporated DNA from a multiplex ALS-type bacterium in its cysG gene.

CONCLUSIONS.

1. There are 3 clades of *X. fastidiosa* within N. America, corresponding to subsp. piercei and multiplex, and the newly named taxon sandyi that causes oleander leaf scorch.
2. The 3 clades originated at least 15,000 years ago. This guarantees that the clades could not have developed in response to host plants introduced by Europeans, e.g. oleander.
3. Isolates from the same clade showed very few genetic differences, and we found no evidence of geographical genetic structure within the piercei or sandyi clades. This limited variability within very old taxa suggests strong selection, possibly driven by host-plant adaptation.
4. Multi-locus sequence typing (MLST) is effective at identifying the three clades, and the plant-host strains within the multiplex group.
5. We can detect mixtures of the 3 main types of *X. fastidiosa* using 3 genes subject to restriction digests.
6. We observed 4 examples of recombination in a sample of 257 genes. Three of these recombinations were found replicated in two isolates. This highly non-random distribution is consistent with the possibility that new recombinant forms can rapidly generate novel pathotypes.

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FUNDING AGENCIES

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EFFECTS OF CHEMICAL MILIEU ON ATTACHMENT, AGGREGATION, BIOFILM FORMATION, AND VECTOR TRANSMISSION OF *XYLELLA FASTIDIOSA* STRAINS

Project Leader:
Alexander H. Purcell
ESPM-Division of Insect Biology
University of California
Berkeley, CA 94720-3112
purcell@nature.berkeley.edu

Researcher:
Clytia Montllor Curley
ESPM-Division of Insect Biology
University of California
Berkeley, CA 94720-3112
montllor@nature.berkeley.edu

Cooperators:
Steven E. Lindow
Dept. of Plant and Microbial Biology
Berkeley, CA 94720-3112
icelab@socrates.berkeley.edu

Harvey Hoch and Thomas Burr
Dept. of Plant Pathology, NYSAES
Barton Laboratory
Cornell University
Geneva, NY 14456-0462
hch1@nysaes.cornell.edu

Reporting Period: The results reported here are from work conducted from July 2004 to October 2004.

ABSTRACT
We have begun work on the effects of chemical and physical factors, including type of media, pH, media volume, and vessel on the *in vitro* survival, growth and substrate-attachment of a wild-type and mutant strain of *Xylella fastidiosa* (*Xf*). The volume of media in which *Xf* is incubated appears to override the importance of other variables, including any strain differences. *Xf* populations incubated in small (200uL) volumes died within 24 h in 50% of assays, but fared better as volumes increased. Preliminary results suggest that attachment to the incubation vessel is greater for wild-type compared to an *rpfF* mutant that does not produce a cell-cell signaling factor.

INTRODUCTION
Under natural conditions, *Xf* attaches to and colonizes the foregut of its leafhopper vectors and the xylem vessels of its plant hosts, creating aggregations of cells attached to their host substrates and surrounded by a polysaccharide matrix, forming a biofilm. Some progress has been made in identifying *Xf* genes responsible for particular colonizing behaviors, and the use of mutants that disable particular functions (e.g. Newman et al. 2004, Feil et al. 2003) is an invaluable aid to studies of transmission and disease. However, much remains to be learned about what environmental factors (of plant or insect origin) affect colonization; and about how such environmental factors interact with bacterial genetic factors to promote or prevent acquisition, retention and delivery of *Xf* by the vector.

The uptake of *Xf* cells by the insect and subsequent detachment of *Xf* as insects probe xylem tissue are essential for vector transmission. These simple requirements, however, belie the more complicated picture that emerges from experimental data. For example, *Xf* added to xylem sap in artificial diets were taken up but not subsequently transmitted to plants by the vector (Davis et al. 1978, Almeida and Purcell, unpublished). In addition, *Xf rpf*F mutants, which were unable to produce a cell-cell signaling factor (DSF, diffusible signal factor), were acquired by vectors; but they were not retained and were not transmitted to plants (Newman et al. 2004). Although other studies have shown that *Xf* could be transmitted within an hour of vector acquisition from plants (Severin 1949, Purcell and Finlay 1979), before anything like a biofilm could form in the foregut, the foregoing data suggest that some rudimentary level of attachment may be necessary for short-term transmission; and that retention, and by implication, colonization and biofilm formation, may be necessary for longer-term ability to transmit. However, the actual role of aggregation/attachment/colonization in the transmission of *Xf* is still largely unknown.

It is clear that both genetic and environmental factors affect colonization of *Xf in vitro*, as well as in insects and plants. Experiments with site-specific mutants of *Xf* have yielded insights into the control of aggregation/attachment/colonization phenomena, though not always in completely unambiguous ways. For example, the *Xf* DSF-deficient mutant formed biofilms and caused severe disease in mechanically inoculated plants, in spite of its inability to colonize the insect foregut (Newman et al. 2004). Cell-cell signaling, therefore, apparently plays different roles in *Xf* colonization behaviors in insects and plants. In the plant pathogen *Xanthamonas campestris,* DSF triggered dispersion of cell aggregates *in vitro*, and was suggested to promote virulence to plants (Dow et al. 2003). Mutants in two other *Xf* genes involved in formation of bacterial fimbriae that aid in attachment, *fimA* and *fimF,* showed reduced aggregation *in vitro*, but were insect transmissible, and caused disease in grapevines (Feil et al. 2003, Feil and Purcell, unpublished).

In both the plant and the vector, environmental factors that putatively affect attachment or detachment would include chemical makeup of sap from which *Xf* cells are acquired; the substrate colonized (insect foregut, xylem vessels); and movement of sap through the xylem or foregut. Media composition has a reportedly major effect on aggregation and biofilm formation of *Xf* (Leite et al. 2004). It is likely that substrate surface characteristics are also important, by analogy with

colonization and biofilm formation of other bacteria living in fluid environments (e.g., Arnold 1999, Korber et al. 1997), and attachment of *Xf* cells to inert surfaces was, in fact, dependent on surface chemistry (Hoch and Burr 2003).

Both the genetic and environmental factors that affect attachment or detachment of *Xf* are amenable to experimentation. Availability of the mutants discussed above has been and will continue to be important in allowing researchers, to expand our understanding of the role of particular colonization behaviors in transmission and virulence by using new mutants. Relevant environmental factors can be experimentally manipulated by the use of artificial diets for *Xf* acquisition by vectors; excised native and artificial substrates for *Xf* colonization; and fluidic chambers to regulate flow of medium over those substrates.

OBJECTIVES

1. Determine whether vector retention (and subsequent delivery) of *Xylella fastidiosa* is related to the chemical and physical environment from which the bacteria are grown or acquired.
2. Investigate how *X. fastidiosa* cells attach (and detach) to specific foregut regions of sharpshooter vectors. *NB: this objective is similar to one proposed from the Hoch/Burr labs with which we propose to collaborate.*

RESULTS

We have begun to address our first objective by measuring *in vitro* survival and growth of wild type *Xf* (Temecula strain) in a variety of media, at different pHs, and in different volumes of media. The media we have used to date are: xylem sap; *Xf*D2, a defined minimal medium developed in this lab (Almeida et al. 2004); and two standard media used for growing *Xf*, PW (Davis et al. 1981a) and PD3 (Davis et al. 1981b). Media pH ranged from 5.2 to 8.0, and volumes varied from 100uL to 30 mL. In all cases, media were inoculated with a 10% by volume of *Xf* suspension of approximately 10^6-10^7 cfu/mL, and samples from each were plated 6-8, 24, 48 and up to 172 h after inoculation. In one assay, media were incubated under lowered oxygen tension. We have also begun to look at a second *Xf* strain, the *rpfF* mutant KLN 61 (Newman et al. 2004).

To date, clear effects of most variables have been undetectable due to inconsistent results even in our controls. The volume of media in which *Xf* are incubated during the assays appears to override the importance of other variables, including any strain differences. For example, control *Xf* in only four out of 12 assays using media volumes of 100 to 200uL survived to 24 h; in 2 mL volumes, three of six control populations survived to 24 h; and in 30mL volumes, all (6/6) control populations survived to 24 h and beyond.

Even in assays in which *Xf* survived, most populations did not grow over 48 hours or more. In all assays so far we have used *Xf* grown from stock on solid media for 1- to 2-weeks, to inoculate the various test media. We have begun to inoculate liquid broth as well, which we will use to subsequently inoculate test media after 5 days of incubation to utilize log-phase cells already growing in liquid (Campanharo et al. 2003).

Preliminary results comparing attachment of two *Xf* strains grown in three media are shown in Table 1. Using a crystal violet assay adapted from Espinosa-Urgel (2000), we compared the relative amounts of the wild-type strain Temecula and the *rpfF* mutant KLN 61 adhering to vessels in which they had been incubated (live *Xf* were not recovered from these media after 24 h, except for strain Temecula in PW, which survived to 172 hours). These results are not yet conclusive and have not been replicated, but show an interesting trend for reduced attachment of the mutant strain, and maximum attachment of the wild-type strain in xylem compared to artificial media.

Table 1. OD_{600} of crystal violet solution eluted from rinsed wells containing *Xf* of wild type Temecula or *rpfF* mutant KLN 61grown in indicated media. n=4 for each strain in each medium. (Calculated by subtracting mean absorbance in each medium from OD of control medium without *Xf*).

	Mean OD_{600}	
Media	Temecula	KLN 61
xylem	0.031	0.010
XfD2	0.021	0.018
PW	0.015	0.008

For our second objective, our plan is to collaborate with the Hoch/Burr labs at Cornell to develop a method for assessing bacterial attachment to vector mouthparts. Together we will examine temporal aspects of cell attachment and colonization under these more realistic conditions of moving fluids through/over sharpshooter mouthparts, using dissected foregut regions placed in microfluidic (flow chamber) devices. In addition, artificial channels that mimic the relevant internal portions of vector mouthparts in flow devices (to be designed at Cornell) will be used to evaluate the effects of high velocity flow conditions on *Xf* cell attachment. We can provide bacteria-free insects and dissected mouthparts to the Cornell labs and test at Berkeley flow devices developed at Cornell. We have previously found that *Xf* colonizes specific regions of the precibarium of insect vectors after bacterial acquisition from infected grapes. This objective addresses our interest in developing an *in vitro* assay to better understand the mechanisms for such site-specific attachment and colonization.

CONCLUSIONS

Our overall objective is to understand the role of "colonization" phenomena in acquisition, retention and delivery of *Xf* by vectors. By manipulating the *in vitro* environment in which wild type *Xf* is cultured, and subsequently presented for acquisition by leafhopper vectors, we hope to understand what factors promote colonization of insect foreguts, and delivery to plants. The use of *Xf* mutants with impaired or enhanced ability to perform some part of the colonizing behavior will be important to understanding the interaction between environment and bacterial behavior affecting vector retention and delivery. Interfering with vector acquisition and inoculation (reducing or avoiding vector populations) are currently the major control methods for Pierce's disease in California. Our findings may reveal currently unanticipated ways of interfering with vector transmission and elucidate features of *Xf* biofilms applicable to this bacterium in plants.

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ROLE OF BACTERIAL ATTACHMENT IN TRANSMISSION OF *XYLELLA FASTIDIOSA* BY THE GLASSY-WINGED SHARPSHOOTER, AND OTHER FACTORS AFFECTING TRANSMISSION EFFICIENCY

Project Leaders:
Alexander H. Purcell
Dept. of Environmental Science, Policy, and Management
University of California
Berkeley, CA 94720

Rodrigo P.P. Almeida
Dept. of Plant and Environmental Protection Sciences
University of Hawaii at Manoa
Honolulu, HI 96822

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ABSTRACT
Blue-green sharpshooters (BGSS) that had long acquisition access periods (4 days) feeding on grapes with Pierce's disease symptoms, followed by a week on test plants consistently had monolayers of cells of *Xylella fastidiosa* (*Xf*) in the precibarium, the narrow channel leading from the junction of the stylet mouthparts with the head to the entrance of the cibarium (sucking pump). BGSS given short acquisition and inoculation periods that transmitted *Xf* to test plants also had small colonies or isolated attached cells of the bacterium in the precibarium. Our findings are consistent with the hypothesis that *Xf* must be present in this small area of the sharpshooter foregut and also consistent with reports that small numbers of *Xf* cells in this area are adequate for efficient transmission. These results also suggest that the back-flow of ingested sap from sharpshooters does not have to be a large volume to enable vector transmission.

INTRODUCTION
Xylella fastidiosa (*Xf*) occurs on the foregut ("inner mouth") surfaces of vectors; but the importance of precisely what part or parts of the cibarium are critical for vector transmission of *Xf* is not clear (Purcell et al. 1979). The foregut is formed as an in-folding of the outer body wall. As such, the foregut is lined with cuticle that is shed when the insect molts. Because molting interrupts vector transmission and there is no delay between acquisition and inoculation of *Xf* by vectors (Purcell and Finlay 1979), the foregut is considered to be the site from which *Xf* is transmitted by vectors. The needle-like mouthparts (formed by modified mandibles and maxillae) of sharpshooters transport plant sap to the pharynx, which is formed by the "upper" (epi-) and "lower" (hypo-) parts of the anterior head. The epipharynx and hypopharynx contain narrow grooves that come together to form the precibarium, a circular canal leading to a pump chamber (cibarium or cibarial pump) within the head. A muscle-powered, flexible diaphragm pumps ingested fluid to the gut via a tubular, flexible esophagus. A muscle-powered valve in the precibarium (the precibarial valve) can prevent the backflow of fluid from the pump to the mouthparts while the pump chamber is contracting to move fluid to the gut. Considering the function and position of the precibarial valve, *Xf* cells in the pump chamber would have to detach and move through the precibarium and the food canal of the stylets to be inoculated into plants. The correlation between the occurrences of *Xf* at the entrance of the cibarial sucking pump with its transmission to plants was not consistent, as some insects that transmitted did not have visible bacteria in this location (Purcell et al. 1979). The numbers of viable *Xf* cells was not well correlated to transmission efficiency, as many transmitting sharpshooters had few or no detectable (cultivable on artificial medium) *Xf* within their heads (Hill and Purcell 1995). Later, it was demonstrated that *Xf* also occurs on the precibarium of other sharpshooters (Brlansky et al. 1983), where *Xf* occurs distally and proximally to the valve in the precibarium but did not correlate the abundance or presence of *Xf* or its location in the insect foregut with transmission to plants. We investigated the correlation between the presence of *Xf* attached to the precibarium and transmission of the bacterium to grape by an efficient sharpshooter vector.

The blue-green sharpshooter (BGSS, *Graphocephala atropunctata* [Signoret]) is the most important vector of *X. fastidiosa* in Coastal California (Redak et al. 2004) and is an efficient vector when compared to other sharpshooters (Almeida and Purcell 2003, Purcell and Finlay 1979, Severin 1949). It is so far the most studied vector of *X. fastidiosa* in relation to transmission biology. For these reasons, we used *G. atropunctata* to study the spatial distribution of *X. fastidiosa* on the precibarium of infective sharpshooter vectors and its transmission to plants after short and long incubation periods using scanning electron microscopy (SEM). We previously reported that *Xf* had colonized the precibaria of all BGSS after by 10 or more days after acquiring *Xf* from plants. Because BGSS can efficiently transmit *Xf* even after a short period following acquisition (Hill and Purcell 1995), we used SEM to inspect the precibaria with of transmitting BGSS for *Xf* after short (1 day) acquisition and inoculation feeding periods.

OBJECTIVES
1. Determine the association of *X. fastidiosa* transmission and its location in the vector's precibarium and cibarium.
2. Determine the effects of within-plant location on vector transmission efficiency.

RESULTS

Objective 1. We conducted transmission experiments, labeled 'A' through 'C', as shown in Table 1. In 'A' we used long acquisition access periods (AAP) and inoculation access periods (IAP) to increase *Xf* transmission efficiency. We also used a long incubation period to allow bacterial colonization of the precibarium of vectors. 'B' was similar to 'A' when the incubation period is considered, but we reduced the AAP to 8 hours to determine if that had an effect on *Xf* distribution patterns. We also used 1 day AAP followed by a 1 day IAP without an incubation period (experiment 'C'). The objective was to determine regions of initial bacterial attachment in the precibarium before thorough colonization of the canal occurred. Table 1 summarizes these experiments, including results for insects with adequate head dissections but excluding other individuals from the experiment. After plant access periods, heads were prepared for microscopy and the test grape plants kept for later diagnosis. We tested grapes for *Xf* presence by visual symptoms and the culture method (Hill and Purcell 1995). Standard SEM protocols were used for preparation of samples. All individuals not adequately dissected for SEM analysis were eliminated from the experiment.

We obtained very good correlation between presence of *Xf* cells in the precibarium of *G. atropunctata* and its transmission to grape. Only one insect identified as negative, in experiment 'B', transmitted to plants. All other infected plants were associated with insects in which *Xf* was observed. When short incubation and acquisition access periods were used some positive insects did not transmit *Xf* to plants, most likely due to the short IAP used. This is consistent with the many observations that not every infective sharpshooter will transmit at every opportunity. The distribution of *Xf* in the precibarium of vectors in experiments 'A' and 'B' was the same as described in a previous report (2003 PD/GWSS Research Symposium). The length of the AAP did not affect colonization, and 2 weeks seems to be enough time for cells to colonize available surfaces of the precibarium.

Experiment 'C', with short AAP and IAP, provided information on the sites of initial bacterial attachment after acquisition. In all cases *Xf* had not fully colonized the precibarium. Most of the heads were colonized by few clusters of cells. These colonies were assumed to be located at sites of initial attachment on the precibarium by *Xf*. Figure 1 depicts representative photomicrographs of small colonies of *Xf* attached to the precibarium; Figure 2 diagrams examples of *Xf* site observed on the precibaria of 12 insects. All insects that transmitted to plants had micro-colonies on the precibarium. In those cases, cells were found both nearby the valve as well as proximally to it, immediately before the cibarium. In one case cells were only observed below (distally to) the valve entering the valve's pit.

Objective 2. Objective two was completed last year.

Table 1. Summary of transmission experiments and their respective acquisition, incubation and inoculation periods.

	Insect transfer sequence					
Exp	AAP	Incubation	IAP	No. insects [1]	Positive heads	PD plants
A	4 days	7 days	4 days	10	7	7
B	8 hours	13 days	1 days	9	3	4
C	1 days	0 days	1 days	22	12	7

[1] Includes only the number of insect heads that were adequately dissected for SEM analysis.

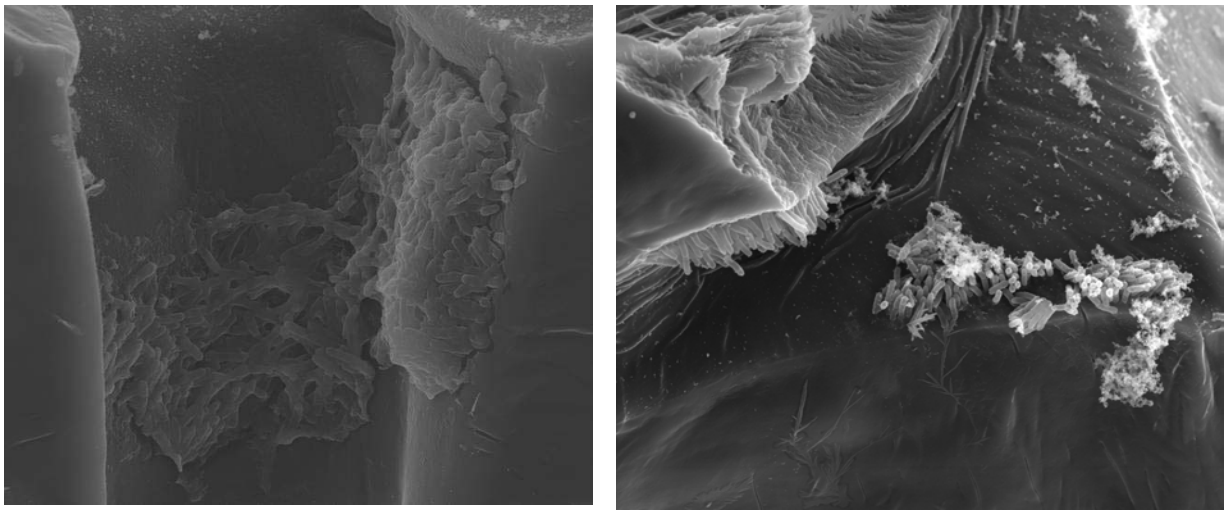

Figure 1. Clusters of *Xf* cells on the hypo- (left) and epi-(right) pharynx of two blue-green sharpshooters after 1 day acquisition feeding and 1 day inoculation feeding (different individuals). On both pharynges the colonies are limited to the proximal section of the precibarium. The clusters formed one micro-colony in the hypopharyngial precibarium (right); there are two clusters of cells on the epipharynx. Note matrix covering some of the cells on the left picture.

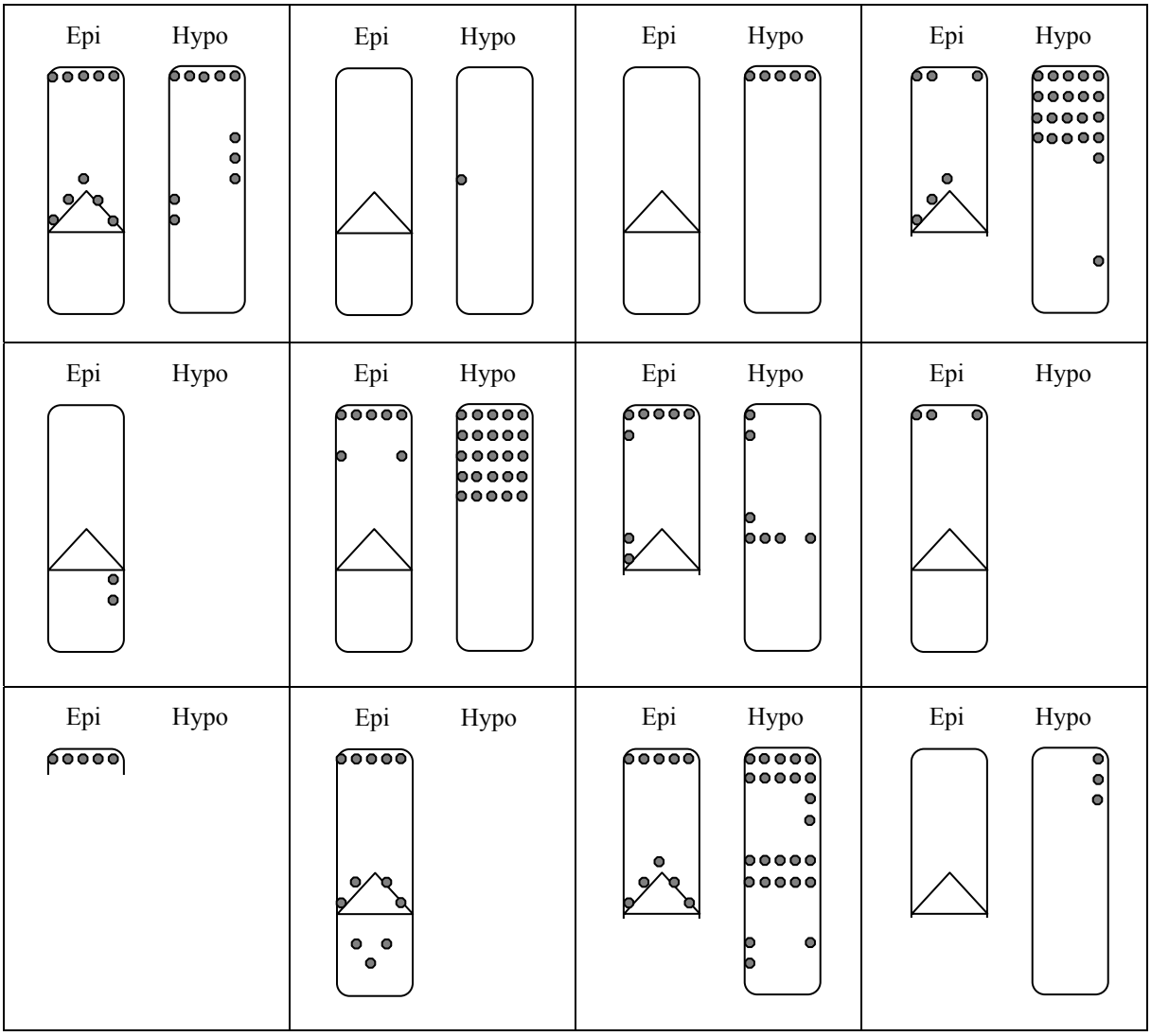

Figure 2. Diagrammatic illustration exemplifying areas with *X. fastidiosa* attached after 1 day AAP and 1 day IAP in the precibarium of 12 *Graphocephala atropunctata*. Epipharynx (Epi) and hypopharynx (Hypo) are represented, the stylets would be below and the cibarium above each figure. Precibarial valve shown as a triangle; filled circles indicate regions colonized by the bacterium. Figures not drawn to scale, sections of cuticle not available for visualization were not included in diagrams.

CONCLUSIONS

Our findings are consistent with the hypothesis that *Xf* must be present in the precibarium, the narrow channel leading from the junction of the mouthparts (needle-like stylets) with the head to the entrance of the cibarium (sucking pump), for successful inoculation to occur. It is also consistent with reports that small numbers of *Xf* cells are adequate for efficient transmission. This suggests that the back-flow of ingested sap from sharpshooters does not have to be a large volume to enable vector transmission.

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A SCREEN FOR *XYLELLA FASTIDIOSA* GENES INVOLVED IN TRANSMISSION BY INSECT VECTORS

Project Leaders:
Alexander Purcell
ESPM-Division of Insect Biology
University of California
Berkeley, CA 94720

Steve Lindow
Plant and Molecular Biology
University of California
Berkeley, CA 94720

Researcher:
Clelia Baccari
ESPM-Division of Insect Biology
University of California
Berkeley, CA 94720

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ABSTRACT
The sharpshooter vector transmission of *Xylella fastidiosa* (*Xf*) to grape causes Pierce's disease (PD). Identification of genes in *Xf* which are responsible for transmission is an essential step in understanding bacteria-vector interactions and may shed light on biofilm formation by *Xf*.

The aim of this work is to understand the role of the genetic regulon of the rpf (regulation of pathogenicity factors) system in *Xf* and its role in disease transmission. In *Xf*, the rpf system likely regulates genes important for colonization of and transmission by insect vectors. The *rpfF* gene is one of the essential genes of the rpf cell-cell signaling system. Transcriptional control regulates genes by cell-cell signaling. The rpfF gene codes for the enzyme that synthesises the signaling molecule, DSF (diffusible signal factor). This system regulates the expression of a host of genes that are as yet unidentified in *Xf*. The rpf gene cluster of *Xanthomonas campestris* pathovar *campestris* is required for pathogenesis of this bacterium to plants (Dow et al. 2000).

In a transmission experiment with the sharpshooter leafhopper *Graphocephala atropunctata* (BGSS), the *Xf* strain KLN61 (an rpfF knockout mutant) could not perform cell-cell signaling. It was not retained by the insect vector and consequently not transmitted to the plants (Newman, 2004). When the *Xf* rpfF mutant strain was compared with *Xf* wild type, it showed to be hypervirulent, non-transmissible, and lacked biofilm formation. Because the spread of Pierce's disease requires the transmission by insects, this indicates that blocking bacterial transmission by insect vectors may be a strategy for controlling PD. However, this requires a better understanding the role of cell-cell signaling by *Xf* and its importance for transmission.

INTRODUCTION
This research study, during its first year, will focus on constructing mutant libraries. By screening for mutations that suppress the non-transmissible phenotype on the rpfF mutant, we will identify the genes involved in transmission using two approaches. The first approach is to restore transmissibility through mutagenesis by disrupting genes normally down-regulated by DSF with a "disrupting transposon" (Figure 1).

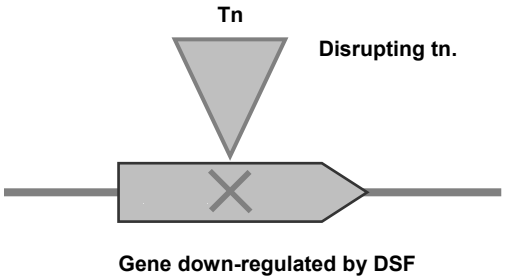

Figure 1: Disrupting transposon mutagenesis to block gene function.

In parallel, an "activating transposon" will be designed to activate transcription of genes normally up-regulated by DSF (Figure 2).

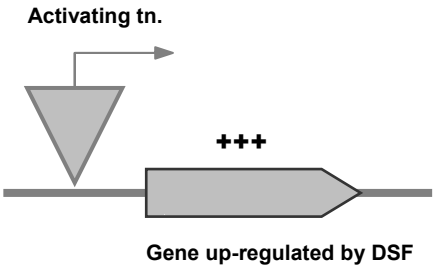

Figure 2: Activating transposon mutagenesis to enhance gene function.

The activating transposon will contain a constitutive promoter that will activate transposition of genes downstream of its insertion site (Newman, 2003). This dual approach will increase the likelihood that we can obtain mutants with restored transmission, and will give us information about those processes that are required for transmission, as well as those processes that must be "turned off" for colonization and transmission to occur. The library will be screened for disrupted gene mutants and then for activated gene mutants.

The insect vectors used for the screen in this study will be GWSS and BGSS. To screen for those mutations that restore transmissibility to the *rpfF* mutant, the gene libraries will be injected into 10 healthy plants of *Vitis vinifera* cultivar Cabernet Sauvignon. The mutant library will be mechanically inoculated into the grape plants. The plants will be kept in the greenhouse and will be monitored periodically for the presence of PD symptoms. Five plants will contain the disrupting transposon mutagenesis libraries and the other five will contain the activating transposon mutagenesis libraries. The source plants will be kept in the greenhouse to allow the strain to reproduce and grow. Group of 100 BGSS, non infective for *Xf* will be placed on the source plants to permit acquisition. The insect vectors BGSS and *Homalodisca coagulata* (GWSS) will feed on the plants containing the mutant collections.

Half of the vectors will be analyzed by bacterial culturing for the presence of *Xf* mutants 14 days after removal from infested plants. The bacteria recovered from these insects will represent mutants that have regained the ability to colonize insect foreguts. Strain KLN61 was only rarely recovered from insects at 7 days, and at 14 days it is expected that that number will be reduced to zero. This will be tested prior to the screen.

The other half of the vectors will be transferred to new healthy plants, and after 6 to 8 weeks, the plants will be cultured for the presence of bacteria. The bacteria recovered from those plants represent those mutants that have regained transmissibility.

OBJECTIVES

1. Create a library of *Xf* mutants in the *rpfF* mutant background using a disrupting transposon mutagenesis to block gene function.
2. Create a library of *Xf* mutants in the *rpfF* mutant background using an activating transposon mutagenesis to enhance gene function.
3. Design and carry out a screen for mutations in *Xf* that restore transmissibility in the non-transmissible *rpfF* mutant.
4. Identify the genes affected in the screen. These will be genes that are important for transmission of Pierce's disease (PD) by insect vectors.

RESULTS AND CONCLUSIONS

Generating the mutant libraries is the main focus of the research during this first year. We have constructed an *rpfF* knockout by allelic exchange mutagenesis using a $Strep^R$marker carried on pKLN121 plasmid. A total of 200 cfu were yield after the transformation and transferred on new media plates containing a concentration of 100ug/ml spectinomycin and 50ug/ml streptomycin as selective markers. This new $Strep^R$ strain allows compatibility with the transposome system, which confers Kan^R allowing us to proceed with the transposome-mediate mutagenesis technique soon. The transposome approach would permit us to rapidly construct a library of mutants in the *rpfF* background. It has been shown that transposome-mediated mutagenesis was successful in Kirkpatrick's laboratory when applied on *Xf* (Guilhabert et al, 2001).

To construct a mutant library in the *rpfF* mutant background gives an important advantage to this project. A secondary mutation on *rpfF* could short-circuit the need for *rpfF* in transmission, using other important genes involved in the process and restore transmissibility of the mutant strain.

To determine what genes were affected that resulted in restored transmission, we will clone and sequence the DNA flanking the transposon using standard protocols for determining genomic DNA sequence flanking insertion DNA. The identity of these genes may enable us to grasp key features of the bacterial mechanism driving transmission. For example, we may find that certain adhesins are required for attachment to the foregut if activating transposons near adhesin genes restore transmissibility.

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PATTERNS OF *XYLELLA FASTIDIOSA* INFECTION IN PLANTS AND EFFECTS ON ACQUISITION BY INSECT VECTORS

Project Leaders:
Alexander Purcell
ESPM-Division of Insect Biology
University of California
Berkeley, CA 94720

Steve Lindow
Plant and Molecular Biology
University of California
Berkeley, CA 94720

Researcher:
Christina Wistrom
ESPM – Division of Insect Biology
University of California
Berkeley, CA 94720-3112

Reporting Period: The results reported here are from work conducted from July 2003 to September 2004.

ABSTRACT
We are studying the effect of host plant tolerance on insect vector acquisition of *Xylella fastidiosa* (*Xf)* from plants tolerant, moderately susceptible, and highly susceptible to *Xf* infection. We are observing *Xf* population and distribution in tolerant and susceptible plants, and its relationship to xylem anatomy, symptom development, and bacterial acquisition by sharpshooters. Since host plant resistance is an important component in the long-term goal of curing PD, it is important to know how resistant plants affect PD spread in areas permanently infested with sharpshooter vectors. We also address the short-term goal of controlling PD spread by comparing grape cultivars in their ability to provide inoculum for vine-to-vine spread of Pierce's disease. Anatomical comparisons of three cultivars, Sylvaner', 'Cabernet Sauvignon' and 'Pinot Noir' showed that all three varieties had similar numbers, lengths and distributions of vessels. The only significant difference was that tolerant 'Sylvaner' had ~ 20 % more rays than the more susceptible 'Cabernet Sauvignon' or 'Pinot Noir' (n = 25, P = 0.01) in canes of similar age, length and diameter. In all four alternate hosts, morning glory (*Ipomoea purpurea*), mugwort *(Artemisia douglasiana)*, sunflower (*Helianthus annuus)* and annual bur-sage (*Ambrosia acanthicarpa*), the longest vessels measured were less than 13 cm long, while in grapes the longest vessels averaged 62 cm. Though alternate hosts had various vascular morphologies and stem lengths, all had shorter vessels than grapes. Blue-green sharpshooters failed to efficiently inoculate wild-type *Xf* and green fluorescent protein-expressing (GFP) *Xf* into both grapes and alternate hosts; only one of 44 grapes inoculated with BGSS became infected. In order to generate GFP-*Xf* infected plants for microscopy, we are mechanically inoculating alternate hosts and grapes. Ongoing work focuses on refining microscopic techniques to visualize small numbers of *Xf* in plant stems, and generating large numbers of *Xf* infected grapevines to serve as new sources for sharpshooter bacterial acquisition.

INTRODUCTION
Alternate hosts of *Xf* were selected for their different patterns of *Xf* colonization after vector inoculation, lack of stem lignification, varying morphology, and absence of green autofluorescence under blue light. In previous experiments, *Xf*-carrying sharpshooters infected morning glory and sunflower more than 80% of the time. *Xf* spread systemically throughout both plants and reached populations over 10^5 colony-forming units (CFU)/gram. Quinoa and mugwort were less-frequently infected (32% and 16%, respectively) by *Xf* and supported lower bacterial populations (10^3 CFU/g for quinoa, 10^6 CFU/g for mugwort). *Xf* moved systemically to a limited extent in quinoa, but not in mugwort (8, 16). Grape cultivars with varying tolerance to PD selected for evaluation are tolerant 'Sylvaner', moderately susceptible 'Cabernet Sauvignon' and highly susceptible 'Pinot Noir' cultivars of *Vitis vinifera* (12, 13). Both blue-green sharpshooters (BGSS) and glassy-winged sharpshooters (GWSS) will be used to infect plants and assess the efficiency of insect acquisition of *Xf* (1, 7, 11).

We are using wild type and transformed isolates of Temecula *Xf* in our experiment. The transformed isolate continually expresses green fluorescent protein (GFP) when illuminated with blue light. GFP-*Xf* was transmitted by blue-green sharpshooters, retained typical virulence in grape, and allowed examination of plant tissues without the extensive fixation required with electron microscopy. With confocal microscopy, GFP-expressing *Xf* can be observed in small and large colonies in vessels, and passing through bordered pits between vessels in symptomatic 'Cabernet Sauvignon' petioles (10).

Anatomical comparisons between alternate hosts and grape cultivars included measurements of vessel length and number, and vascular bundle number and distribution based on the techniques of Tyson *et al.* (15), and Ewers and Fischer, modified to infuse the pigment via 100kPa pressure applied to the proximal end of the cutting (5). We evaluated primary vegetative growth rather than secondary xylem due to the difficulties in sectioning, culturing from, and feeding BGSS on partially lignified stems. GFP-*Xf* inoculation and colonization of all plants will be measured similarly in all plants: groups of four GFP-*Xf* carrying sharpshooters inoculated a 3-cm stem section, and the plants were evaluated for the presence of GFP-*Xf* approximately 8 weeks after inoculation. Colonized vessels will be counted, and populations estimated by culture on PWG media (2, 8).

We will measure *Xf* acquisition by sharpshooters from the alternate hosts and grape cultivars after completing the anatomical comparisons. Insects will be caged on *Xf* inoculated sites for 4 days to acquire the bacteria, and then be placed on another grape seedling for 2 days to determine their acquisition efficiency. Immediately following sharpshooter feeding, the stem site will be examined with confocal microscopy and tested with culture. Three stem cross-sections and three 1-cm long longitudinal sections per site will be sectioned and suspended in 50% glycerol on a depression slide. When illuminated with blue and ultraviolet light, both GFP-*Xf* and the individual vessels are visible, and it is possible to determine the proportion of vessels colonized, the extent of bacterial colonization inside them, and the distribution of colonized bundles. Bacterial populations will be determined by culture from remaining plant material of the same site, and symptom development and severity will be assessed. Since acquisition efficiency has been related to bacterial populations (9), we must separate the effects of bacterial distribution and proportion of colonized vessels from the effect of bacterial population. The number of plants we can evaluate via microscopy is a limiting factor. A maximum of 90 observations per experiment will allow examination of 5 inoculation sites for each of three species or cultivars, which should enable detection of a 20% difference in *Xf* colonization ($\alpha = 0.05$ and $\beta = 0.10$) (14).

OBJECTIVES

1. Describe the bacterial colonization of asymptomatic weed species and grape varieties of varying tolerance to Pierce's disease using an *Xf* strain that continuously expresses green fluorescent protein.
2. Determine the relationship between the pattern of colonization of a plant by *Xylella fastidiosa (Xf)* and the ability of that plant to be a source for bacterial acquisition by sharpshooter vectors.

RESULTS

There were no differences in the total vessel number, the proportion of short vessels, or the longest vessels between resistant and susceptible grape varieties between greenhouse-grown canes of similar length, age, and diameter. The longest vessel measured by paint infusion was 110 cm (Pinot Noir), although most vessels were less than 12 cm long in all cultivars (Figure 1). Cane length had a small but significant influence on longest vessel ($r^2 = 0.20$; $P = 0.02$, $n = 27$), but did not relate to the number of very short vessels. There was no relationship between stem length and vessel length in the other plants.

While more replication is needed, the longest vessel measured in any alternate host was 15 cm long (mugwort). In sunflower, 71% of vessels were less than 3 cm long. Other species had a wider range of vessel lengths, with about half their vessels less than 3 cm long (Figure 2). Mugwort had roughly twice as many vessels (592, $n = 3$) at the stem base than morning glory (217), quinoa (251) or sunflower (286) stems of comparable diameter and age. Sunflower, mugwort and quinoa all had vascular tissues in evenly distributed bundles wide interfasicular regions of parenchyma (4). Annual morning glory had large vessels distributed evenly along the cambium.

Table 1: Comparisons between canes of similar length, age, and diameter belonging to 3 grape cultivars.

Cultivar	Total # vessels at base of cane (SE)	% Vessels < 3 cm (SE)	Longest vessel (SE)	# Rays (SE)
Cabernet Sauvignon	515 (43)	21 (3)	53 cm (5)	34 (1)
Pinot Noir	474 (27)	20 (3)	64 (9)	34 (2)
Sylvaner	514 (38)	18 (5)	69 (9)	40 (2)
one-way ANOVA	(n = 27, $P = 0.67$)	(n = 27 , $P = 0.84$)	(n = 27, $P = 0.35$)	(n = 27, $P = .01$)

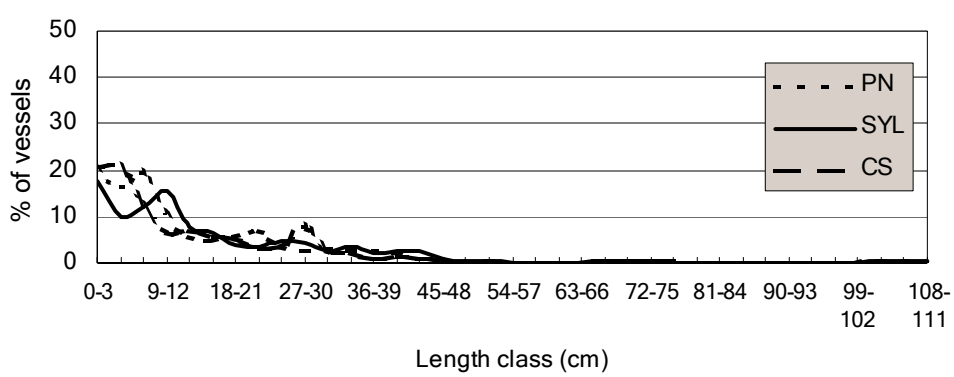

Figure 1: Vessel length distribution in greenhouse-grown Pinot Noir (PN), Sylvaner (SYL) and Cabernet Sauvignon (CS).

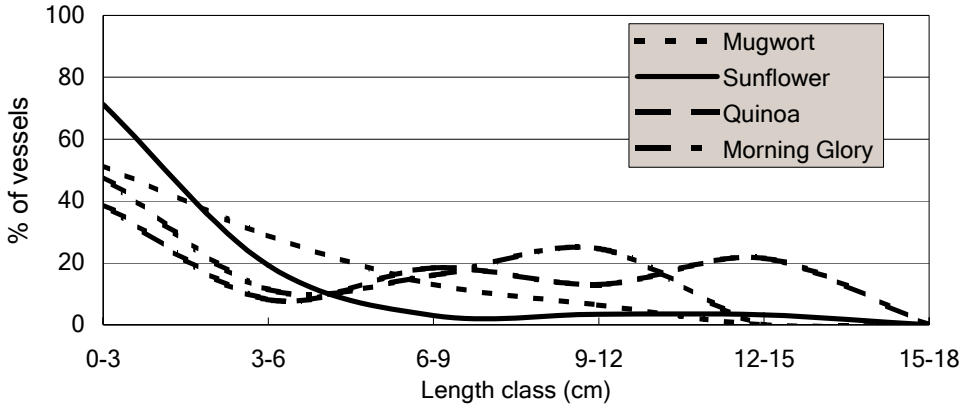

Figure 2: Vessel length distribution in greenhouse-grown annual morning glory, mugwort, quinoa and sunflower.

Blue-green sharpshooters failed to efficiently inoculate *Xf* into both grapes and alternate hosts in three separate attempts from 7/03 to 4/04; only one of 44 grapes became infected. Though the *Xf*-infected source plants had fully developed symptoms and were positive for *Xf* by culture, there may have been nutritional or physiological factors that prevented them from being good sources of bacterial acquisition. We are mechanically inoculating alternate hosts and grapes to generate GFP-*Xf* infected plants for microscopy practice. Because the distribution of *Xf* in an insect-inoculated stem is likely different from a mechanically inoculated stem, we still plan to use insect-inoculated plants when we compare sharpshooter acquisition and bacterial distribution in alternate host stems. Ongoing work focuses on refining microscopic techniques to visualize small numbers of *Xf* in alternate host stems, and generating large numbers of *Xf*-infected grapevines to serve as new sources for sharpshooter bacterial acquisition.

CONCLUSIONS

Three things are required for the development of Pierce's disease in grape: the pathogen *Xylella*, a sharpshooter insect vector, and a susceptible plant host. We are systematically examining the interactions between plants and the pathogen, and the role that host resistance plays in the ability of the vector to acquire *Xf* and spread Pierce's disease. The only significant difference between grape varieties was that tolerant 'Sylvaner' had approximately 20% more rays per stem compared with susceptible 'Cabernet Sauvignon' or 'Pinot Noir'. In grapes, rays are composed of dense parenchyma cells, without tracheids or vessels, and separate the water-conducting xylem into longitudinal zones (3). Perhaps this limits the lateral spread of *Xf* to the zone it is originally inoculated into. While additional work is needed, the vessels of other hosts were approximately 75% shorter than vessels of grapes, limiting the passive spread of *Xf* via xylem sap movement, and are found in bundles separated by parenchyma cells, which may also limit the lateral spread of *Xf.* Additionally, it is likely *Xf* movement between bordered pits is an active process (10); anatomical and biochemical differences in pit structure may explain differences between cultivar susceptibility to *Xf.*

In grapes, electron and confocal microscopy showed *Xf* densely packed in individual vessels, with adjacent vessels empty or containing a few cells (10, 15). Alternate hosts or tolerant grape cultivars with low overall populations may have just a few vessels with bacteria, so acquisition would be highly variable and dependant upon sharpshooters encountering the few colonized vessels while feeding. In symptomatic grape petioles, 13% of vessels were colonized to some extent with GFP-*Xf,* though only 2.1% of all vessels were completely blocked with bacteria (10). Though is it not known how many probes a sharpshooter makes in a given feeding session, glassy-winged sharpshooters can generate multiple salivary sheaths in one insertion, adjacent to vessels and xylem parenchyma cells (6). Sharpshooter acquisition of *Xf* increased along with bacterial populations in infected grapes (9), and a similar positive relationship is expected if the proportion if colonized vessels increases insect acquisition of *Xylella*.

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DOCUMENTATION AND CHARACTERIZATION OF *XYLELLA FASTIDIOSA* STRAINS IN LANDSCAPE HOSTS

Project Leaders:

Frank Wong
Dept. of Plant Pathology
University of California
Riverside, CA 92521
frank.wong@ucr.edu

Donald A. Cooksey
Dept. of Plant Pathology
University of California
Riverside, CA 92521
donald.cooksey@ucr.edu

Heather S. Costa
Dept. of Entomology
University of California
Riverside, CA 92521
heather.costa@ucr.edu

Cooperators:

James Downer, UCCE, Ventura County
Mike Henry, UCCE, Riverside County
John Kabashima, UCCE, Orange County
John Karlik, UCCE, Kern County
Michelle LeStrange, UCCE, Tulare County
David Shaw, UCCE , San Diego County

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ABSTRACT

To better understand the impact of *Xylella fastidiosa* on the urban environment and the potential for ornamental hosts to serve as reservoirs for agronomically important diseases caused by the bacteria, a survey project was initiated to document and characterize strains of the bacteria harbored in landscape plants. Targeted sampling of 122 landscape species either symptomatic for bacterial scorch or testing positive for *X. fastidiosa* by ELISA in 2003 was performed. Of the 830 samples, 321 tested positive by ELISA (representing 77 of the 122 species tested). *X. fastidiosa* was also detected in 23 species by PCR-amplification using *X. fastidiosa* specific primers. Twenty-seven isolates from 13 host species were obtained from samples testing positive by ELISA. Isolates from plants not previously reported as hosts in southern California urban environments included mulberry, heavenly bamboo, magnolia, day lily, western redbud, jacaranda and peach. Genetic characterization of these isolates by 16S-23S rDNA sequencing distributed these isolates amongst previously characterized strain groups: almond leaf scorch (crape myrtle, ornamental plum, liquidambar, gingko, olive), Pierce's disease (magnolia, peach, western redbud), mulberry leaf scorch (mulberry, heavenly bamboo), and oleander leaf scorch (magnolia, jacaranda, day lily). The role of some *X. fastidiosa* strains in their ability to cause disease is presently being tested by fulfilling Koch's postulates in glasshouse experiments. The data collected from this study strongly suggest that *X. fastidiosa* is causing a number of scorch diseases in the urban landscape, and that strains of agronomic importance may be harbored in this environment.

INTRODUCTION

Xylella fastidiosa (*Xf*) is a xylem-limited, insect-vectored, plant pathogen that can cause severe damage to a wide range of host plants. Diseases caused by this pathogen include Pierce's disease of grapevine (PD), oleander leaf scorch (OLS) and almond leaf scorch (ALS). In 2003, a survey of landscape plants in five urban locations in southern California was initiated to document the incidence of the *Xf* infection in landscape ornamental hosts and to characterize strains existing in these hosts that may prove a threat to landscape ornamentals or crops of agronomic importance. Two hundred twenty one samples (29%) representing 48 species tested positive by ELISA. Ten isolates of *Xf* were obtained from eight plant species (*Fatsia japonica, Ginkgo biloba*, *Lagerstroemia indica*, *Liquidambar styraciflua*, *Morus alba*, *Nandina domestica*, *Olea europea*, and *Prunus cerasfiera*) not previously described as hosts of *X. fastidiosa* in southern California.

Based upon the results of the first year, targeted sampling of host species testing positive by ELISA was performed primarily in the Riverside and Redlands areas in order to obtain additional isolates for characterization. To prove the role of *Xf* in causing disease in previously identified hosts, test plants were inoculated in glasshouse experiments to fulfill Koch's postulates for these isolates, and to determine if they were able to cause disease in grapevine and oleander.

OBJECTIVES

1. Use laboratory methods to identify landscape host species that are infected with *X. fastidiosa.*
2. Secure isolates from these hosts to document infection and provide material for genetic characterization of the *X. fastidiosa* strain(s) involved.
3. Genetically characterize the strains of pathogen in landscape plant species.
4. Confirm pathogenic infection through inoculation studies with specific isolates.
5. Test ability of new strains to infect agricultural crops including grape, olive, and almond.

RESULTS

Objective 1

In 2004, 830 samples from 122 landscape plant species were collected. Sampling focused on plant species that were symptomatic or had tested positive by ELISA in 2003 surveys. Three hundred twenty one samples (39%), tested positive by ELISA. At least one sample from 77 of the 122 species tested was positive by ELISA (63%). Attempts to isolate the

pathogen from these positive samples yielded only a small number of isolates (see next section). PCR testing (Minsavage 1994) was performed on a subset of the samples collected using a modification of the published methodology. Briefly, petioles and midveins from leaves were chopped in sterile water, tissues were allowed to sit in the water for several minutes to allow for the release of *Xf* from the tissues and then DNA extracted from the water. Results were greatly improved using this method, and *Xf* was detected in 23 species tested (Table 1). PCR testing of additional species testing positive by ELISA is continuing on species from which isolates could not be obtained.

Table 1. ELISA, isolation and PCR results for 23 of 122 species tested for *Xf*.

Plant Name	Common Name	#Tested	#ELISA(+)[a]	Culture(+)[b]	PCR(+)[c]
Albizia julibrissin	Silk Tree	6	5		yes
Cercis occidentalis	Western Redbud	4	3	yes	yes
Ginkgo biloba	Maidenhair Tree	15	6	yes	yes
Hemerocallis	Day Lily	9	5	yes	yes
Jacaranda mimosifolia	Jacaranda	49	24	yes	yes
Juglans	Walnut	2	2	no	yes
Lagerstroemia indica	Crape Myrtle	17	5	yes	yes
Lavandula dentata	Lavender	4	4	no	yes
Ligustrum lucidum	Glossy Privet	7	5	no	yes
Liquidambar styraciflua	Liquidambar	19	7	yes	yes
Magnolia grandiflora	Southern Magnolia	31	18	yes	yes
Morus alba	White Mulberry	3	2	yes	yes
Nandina domestica	Heavenly Bamboo	20	3	yes	yes
Nerium oleander	Oleander	3	3	yes	yes
Olea europaea	Olive	6	5	yes	yes
Phoenix reclinata	Senegal Date Palm	2	2	no	yes
Prunus cerasifera	Ornamental Plum	12	7	yes	yes
Prunus dulcis	Almond	3	3	yes	yes
Prunus persica	Peach	5	2	yes	yes
Rosmarinus officinalis	Rosemary	13	8	no	yes
Vitis labrusca 'Concord'	Concord Grape	2	2	yes	yes
Vitis vinifera 'Red Flame'	Red Flame Grape	2	2	yes	yes
Vitis vinifera 'Thompson Seedless'	Thompson Seedless Grape	5	5	yes	yes

[a] denotes number of samples testing positive using a commercial *Xf*-specific ELISA kit
[b] denotes if an *Xf* isolate was successfully obtained from at least one sample
[c] denotes if PCR-amplification using RST31/33 primers from plant tissue was successful for at least one sample

Objective 2

Twenty-seven isolates (from 13 host species) were obtained from samples testing positive by ELISA (Table 2). Isolation of the pathogen from samples, even those testing strongly positive from ELISA, was not always possible. Briefly, samples were washed in soapy water, soaked for 1 min in 70% ethanol, 1 min in 20% bleach, then triple rinsed in sdH_2O. Samples were then sliced into 1-2 mm pieces and soaked in PBS. Fifty microliters of the PBS buffer was then plated onto PW media with or without the addition of 25 ppm of cycloheximide. The failure to obtain isolates from all samples testing positive by ELISA suggests that specific methodologies need to be determined for specific tissue types from different hosts as a general isolation protocol may be inadvertently killing the pathogen, the pathogen may be highly irregularly distributed in host tissues, or the commercially available ELISA kit may be generating a high number of false positives due to non-specific interactions with host tissue.

Objective 3

Collected isolates were confirmed as being *Xf* by extraction of the DNA from the cultures using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA) and subsequent PCR amplification with the RST31/33 primer pair. Isolates were further characterized by amplification and sequencing of the 16S-23S ribosomal DNA intergenic spacer region as described by Hendson et al. 2001. All the 16S-23S rDNA sequences were aligned using the clustalX program (Thompson *et al*., 1994) and their relationship was analyzed with the PHYLIP program (Felsenstein, 1995) with the sequence of the *Xanthomonas vesicatoria* (AY288080) as an outlying group (Figure 1).

Two strains isolated from mulberry (Morus024 and Morus012) showed 99.41% identity with the previously reported mulberry-VA strain from the eastern U.S. (Huang and Sherald, 2004), while Nandina065, Morus059 and Morus063 showed a 100% of identity with the same strain. For the two peach isolates, Peach018 showed 100% identity with previously reported Pierce's disease strains (AO5) while Peach018 showed a little less identity (99.41%), but both grouped with PD strains. The Cercis050 strain also grouped with PD strains (99.61% identity). Strains isolated from Magnolia showed just 98.44 % identity between them. Since Magnolia038 was more closely related to Oleander leaf scorch (OLS) (99.02% identity) while

Magnolia002 showed more identity (99.41%) to PD strains. For isolates from Hemerocallis and Jacaranda, they showed 100% identity between them and showed to be more closely related to oleander strains (99.22%)
Gingko, olive, liquidambar and some ornamental plum strains showed to be closely related to the Dixon almond leaf scorch strain (100% identity). Some ornamental plum strains showed divergence amongst them (97.86% identity) and from ginkgo, olive and liquidambar, but all of them grouped together with the Dixon strain. Lastly, the strain isolated from a yet to be identified host (nicknamed "*negrito"*) showed slight differences from the ornamental plum, liquidambar and olive isolates. None of the isolates grouped with plum leaf scald, phony peach, oak leaf scorch group or with citrus variegated chlorosis and coffee leaf scorch strains.

Table 2. *Xf* isolates collected in 2004 surveys.

Host Scientific name	Common Name	Isolate designation
Cercis occidentalis	Western Redbud	Cercis050
Hemerocallis	Day Lily	Hemerocallis034
Jacaranda mimosifolia	Jacaranda	Jacaranda028
Liquidambar styraciflua	Liquidambar	Liquidambar020
Magnolia grandiflora	Magnolia	Magnolia038
Magnolia grandiflora	Magnolia	Magnolia 002
Morus alba	White Mulberry	Morus012
Morus alba	White Mulberry	Morus024
Nerium oleander	Oleander	Oleander031
Nerium oleander	Oleander	Oleander028
Prunus cerasifera	Ornamental Plum	Pcerasifera057
Prunus cerasifera	Ornamental Plum	Pcerasifera086
Prunus cerasifera	Ornamental Plum	Pcerasifera047
Prunus cerasifera	Ornamental Plum	Pcerasifera052
Prunus cerasifera	Ornamental Plum	Pcerasifera053
Prunus dulcis	Almond	Almond036
Prunus persica	Peach	Peach018
Prunus persica	Peach	Peach.019
Unknown species	'negrito'	Negrito005
Vitis labrusca 'Concord'	Grape	Grape153
Vitis labrusca 'Concord'	Grape	Grape154
Vitis vinifera 'Red Flame'	Grape	Grape155
Vitis vinifera 'Red Flame'	Grape	Grape156
Vitis vinifera 'Thompson Seedless'	Grape	Grape149
Vitis vinifera 'Thompson Seedless'	Grape	Grape150
Vitis vinifera 'Thompson Seedless'	Grape	Grape151
Vitis vinifera 'Thompson Seedless'	Grape	Grape152

Objectives 4 and 5

Eight characterized strains of *Xf* collected from the landscape in 2003, plus an oleander and a grape strain, were inoculated into their host plants of origin in glasshouse assays. Strains used were Almond276, Ginkgo, Lagestroemia02 (crape myrtle), LiquidambarUI12 (liquidambar), Morus069 (mulberry), Nandina065, Olive AC12 , Pcerasifera076 (ornamental plum), Riverside3 (oleander), GrapeA05. These same eight strains were also used to inoculate grapevine and oleander. Briefly, isolates were grown on PW media for two weeks from which a suspension of 1 x 10^9 CFU in sterile phosphate buffer was obtained. Plants were needle inoculated on three to four sites per plant using the needle-stab technique described by Hill and Purcell (1995). Approximately 25 plants were used for the inoculation studies. All plants were tested by ELISA prior to inoculation to ensure that they were *Xf* free. Starting approximately three months after inoculation, plants were ELISA tested and attempts were made to isolate the pathogen from positive plants. *Xf* cultures have been obtained from some hosts testing positive by ELISA and have been confirmed as *Xf* by PCR, namely those from mulberry inoculated with the Morus069 isolate. Isolation and characterization studies from these test inoculations are currently underway for the rest of the test plants and *Xf* isolates.

CONCLUSIONS

The results of the study do indicate that there are a number of landscape hosts that are harboring different strains of *Xf* in southern California. Of the new isolates characterized, it appears that new hosts have been identified for a number of strain groups: Pierce's disease (magnolia, peach, western redbud), oleander leaf scorch (magnolia, jacaranda, day lily), mulberry leaf scorch (heavenly bamboo), and almond leaf scorch (ornamental plum, crape myrtle, liquidambar, gingko, olive). Inoculation tests appear to have confirmed the role of *Xf* in causing mulberry leaf scorch in California, while other tests await completion. It does appear that new methodologies will have to be developed to successfully obtain or test for *Xf* in a number of ornamental plant species. The role of *Xf* infections in landscape hosts does appear to have a significant impact on

several species; however, additional studies must be completed to further elucidate the role of this pathogen in causing widespread disease in the urban setting as well on crops of agronomic importance in California.

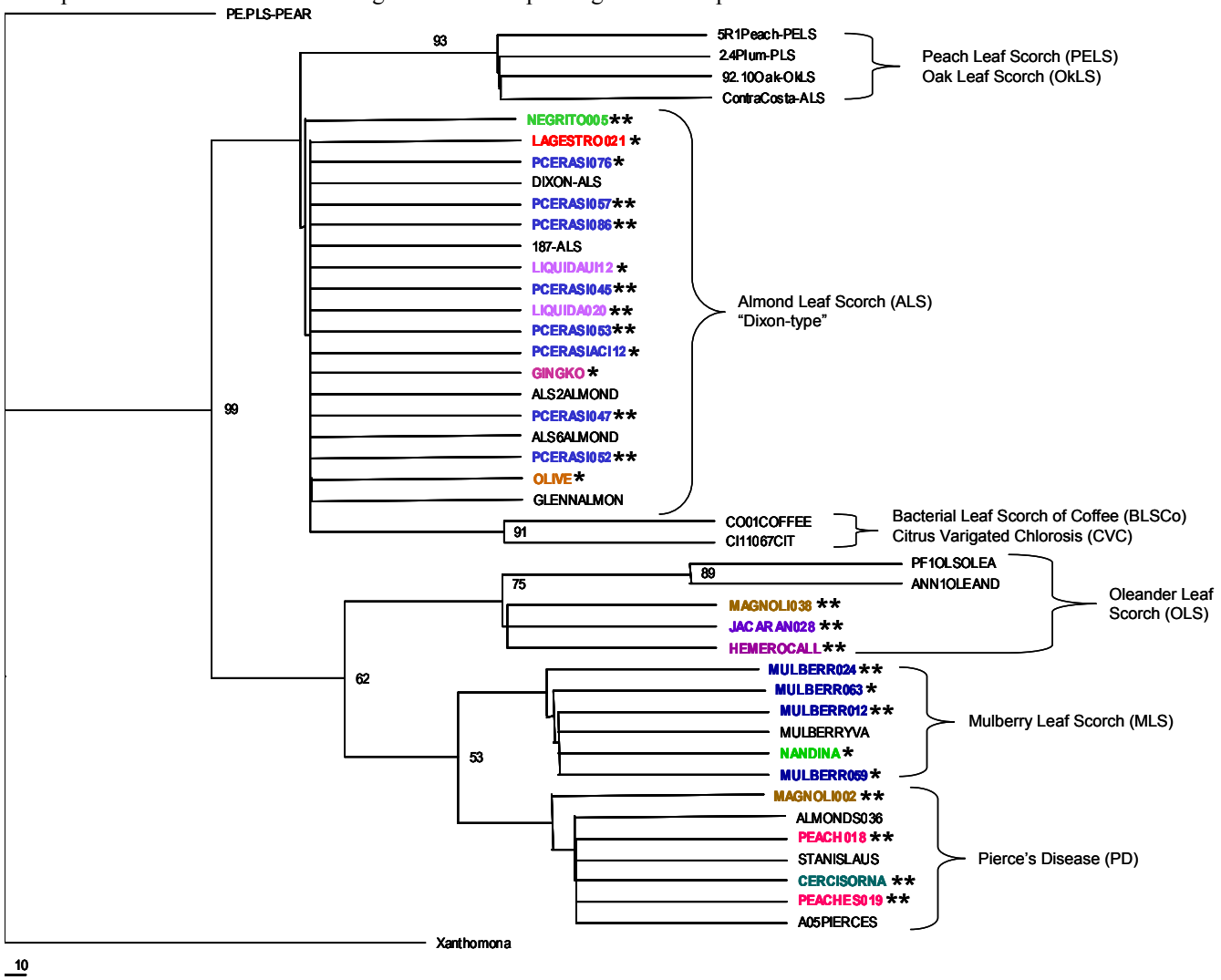

Figure 1. Preliminary phylogenetic tree constructed using the neighbor joining method, based on 16S rDNA sequence data for *Xylella fastidiosa* with the sequence of *Xanthomonas vesicatoria* (AF203392) as the outgroup. The numbers above the branches represent bootstrap values obtained for 100 replications. * Indicates isolates collected in 2003, ** indicates isolates collected in 2004.

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PLASMID ADDICTION AS A NOVEL APPROACH TO DEVELOPING A STABLE PLASMID VECTOR FOR *XYLELLA FASTIDIOSA*

Project Leaders:
Glenn M. Young
Dept. of Food Science and Technology
University of California
Davis, CA 95616

Michele Igo
Section of Microbiology
Division of Biological Sciences
University of California
Davis, CA 95616

Cooperator:
Bruce Kirkpatrick
Dept. of Plant Pathology
University of California
Davis, CA 95616

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INTRODUCTION

Current approaches to understanding the progression of Pierce's disease are limited by the lack of genetic techniques that can be used to study the biology of *Xylella fastidiosa* (*Xf*). In particular, extrachromosomal elements, such as plasmids, having long-term stability in *Xf* when grown in lab cultures or *en planta,* have not yet been satisfactorily developed. We will develop vectors that exhibit stable maintenance by *Xf* by adapting previously described genetic and microbiological techniques. Our particular research efforts will focus on taking advantage of a well-studied bacteriological phenomenon called plasmid addiction (2, 4, 10). The major mechanistic principle of plasmid addiction is that the plasmid carries a genetic trait that the host bacterium requires for viability. The trait does not affect the metabolic properties of the bacterium nor does it affect reproduction. However, loss of the plasmid-encoded trait is a lethal event, so by definition plasmid addiction ensures vector stability. In addition, we will systematically evaluate other genetic mechanisms for increasing plasmid stability including multimer resolution and active partitioning systems. Finally, we will examine the stability of each of the newly developed vectors for *Xf in vitro* and *en planta*. The results of this analysis will allow us to construct one or more stable plasmid vectors that can be used by all researchers using genetic approaches to develop methods that limit *Xf*-related diseases.

Xylella fastidiosa is a Gram-negative, endophytic bacterium, which is responsible for a number of economically important plant diseases (for recent reviews, see (5, 7, 8)). Diseases that are important to the California agricultural economy include Pierce's disease of grapevine, almond leaf scorch, alfalfa dwarf, and oleander leaf scorch. Some strains of *Xf*, such as the Pierce's disease strains, have very wide host ranges and are capable of colonizing the xylem of widely divergent plant species. In many plant species, infection by *Xf* does not provoke symptoms or noticeable distress. However, the colonization of certain plants, such as grapevines, leads to the development of disease symptoms and of plant decline. Although the specific details of the disease process are not fully understood, it is known that *Xf* forms a biofilm within xylem vessels that has a major impact on the movement of sap within the xylem tissue. Disease symptoms seem to be dependent on the rate and extent of colonization of the xylem tissue by *Xf*. Some of the symptoms observed in infected grapevines include leaf marginal necrosis, severe leaf scorch, and dieback.

Another important aspect of the disease cycle involves the insect vector. *Xf* is transmitted from plant to plant by xylem-feeding insects including the glassy-winged sharpshooter (5, 7, 8). The insect vectors acquire the bacterium by feeding on infected plants. Since the Pierce's disease strain can colonize numerous plant species, the source of inoculum can be infected grapevines or symptomless plants present in the riparian habitats surrounding the vineyard. In vectors showing the highest transmission efficiencies, *Xf* is present as a polar biofilm in the insect foregut and is transmitted to uninfected plants during subsequent feeding events. In susceptible plants, efficient transmission of *Xf* occurs at low bacterial cell numbers (<100 cultivable cells per insect head).

Thus, an important feature of the *Xf* infectious cycle is the ability of this pathogen to colonize and interact with the xylem tissue of plants and the foregut of insect vectors. Successful colonization of these hosts is dependent on the ability of *Xf* to subvert host defense networks and to acquire essential nutrients. To better understand how *Xf* survives in and interacts with its hosts, many research laboratories have been working to identify genes important for virulence and nutrient acquisition. However, rapid progress in this area is affected by the lack of genetic and molecular tools necessary to investigate the contribution of *Xf* genes to the infection process. One extremely important tool that is needed to advance these studies is a plasmid that is maintained by *Xf* throughout the infectious cycle. The goal of our project is to develop this type of plasmid. Plasmid-addiction systems consist of a pair of genes that specify two components: a stable toxin and an unstable antidote (for recent reviews, see (2, 4, 10). When a bacterium looses the plasmid harboring one of these addiction systems, the cured cells loose the ability to produce the unstable antidote and, as a result, the lethal effect of the stable toxin kills the bacterium.

Thus, to remain alive each living bacterium in a sample must retain the plasmid to continue producing antidote. We will test the two different types of addiction modules that have been identified in bacteria. The first type of addiction system consists of a toxin that is encoded by a stable mRNA, but expression of the toxin is limited by the antidote, which is a small unstable antisense RNA molecule that blocks mRNA translation. The antisense mRNA antidote is produced as long as the plasmid is retained. Both the *hok/sok* system of plasmid R1 and the *pnd* locus of plasmid R483 utilize this mechanism of establishing addiction. Inclusion of the *hok/sok* system has been shown to successfully stabilize engineered plasmids in divergent species of bacteria including *Escherichia coli*, *Salmonella typhi*, *Pseudomonas putida*, and *Serratia marcescens* (3).

The second type of addition system consists of a stable protein toxin and an unstable antitoxin protein. Similar to the previous example, antitoxin is produced as long as the plasmid is retained. One of the best characterized of this type of addiction system is the *parDE* system from the broad-host range plasmid RK2 (also called RP4). Addition of a region of RK2, which includes the *parDE* system, to a poorly maintained plasmid has been shown to enhance stability of a wide range of bacteria such as *Alcaligenes eutrophus, Alcaligenes latus, Azotobacter chroococcum, Klebsiella pneumoniae, Pseudomonas aeruginosa, P. putida,* and *E. coli* (1, 9). Interestingly, placing more than one type of plasmid addiction module onto the same plasmid provides an additive effect on plasmid stability (6). Thus we will also evaluate whether placing the two different types of plasmid addition system leads to additional plasmid stability in *Xf*.

OBJECTIVES

1. Develop a stable plasmid vector for *Xf*.
   A. Evaluate the potential of various plasmid addiction systems for the ability to convert plasmids known to replicate in *Xf* into stable vectors.
   B. Evaluate how plasmid maintenance by *Xf* is affected by other genetic mechanisms known to affect plasmid stability, such as systems for multimer resolution and active partitioning systems.
2. Evaluate the stability of the newly developed plasmid vectors when propagated in *X. fastidiosa en planta*.

RESULTS

This report summarizes the goals of a new project focused on constructing a stable plasmid vector to aid genetically based studies of *Xylella fastidiosa*.

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GENETIC VARIABILITY OF *XYLELLA FASTIDIOSA* STRAINS ISOLATED FROM TEXAS GRAPES AND OTHER PLANT RESERVOIRS

Project Leaders:

Kristi Bishop
University of Houston-Downtown
Houston, TX 77002

Lisa Morano
University of Houston-Downtown
Houston, TX 77002

Prince Buzombo
University of Houston-Downtown
Houston, TX 77002

ABSTRACT

Pierce's disease is a serious threat to the burgeoning Texas wine industry. Evaluation of the ecology and epidemiology of the disease in Texas may also be of significant scientific value for other areas of the country. We have begun a molecular biological evaluation of the genetic variability of *Xylella fastidiosa* (*Xf*) strains in Texas using small, established primers for creation of diagnostic banding patterns (REP, ERIC, and BOX primers). Cloning and sequencing of amplicons using RST31-33 primers resulted in little genetic difference between strains if one considers the error rate of *Taq* polymerase. However, priming with the small diagnostic primers resulted in differential banding patterns among *Xf* isolates across Texas. Based on these patterns, some vineyards had genetically distinct isolates and others genetically identical isolates. Vineyards may also contain more than one isolate. Analysis of *Xf* from a non-*Vitis* species showed a high distinct banding pattern suggesting broad genetic variability within Texas. Indirect immunofluorescence on *Xf* isolates also supports significant genetic variability within Texas, as there is differential antigen localization among several strains.