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

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III: Summary:

Xylella fastidiosa is an "endophytic" bacterium—one that colonizes the internal tissues of the host plant. A key determinant of success for an endophyte (and symptom formation in the plant) is the ability to spread within the plant from the initial site of infection because the bacteria use up local resources as their population grows. As a large group of bacteria accumulates, they start behaving differently. There is strong evidence that *Xylella*, like its related plant pathogenic bacteria such as *Xanthomonas campestris,* secretes a gum that protects it, and produces degradative enzymes such as cellulases that break down plant tissues, but only after a large number of cells have developed at a site. These virulence traits, which allow them to expand into new vessels in a plant, would be ineffective for solitary cells. Hence the pathogen must repress the production of such traits at low cell numbers when it would be futile, and express such traits only when it has reached higher cell density, basically "ganging-up" on the plant. The bacteria keep tabs on their population size by communicating via a small diffusible signal molecule. Many different types of signals are employed by different species of bacteria, however more than one species often employ the same signal. In addition, some bacteria are capable of degrading the signal of another species, or blocking it with a "look-alike" analog. Our research involves finding other bacteria that can block or confuse the signals *Xylella* uses to spread in the plant. The first step in this research is to identify the signal used by *Xylella*. We

have found that *Xylella* employs the same signal molecule, DSF, as the related plant-pathogen *Xanthomonas*. We have engineered a collection of DSF "signal-sensing" strains of *Xylella* to be used as molecular tools that will allow us to monitor signaling in *Xylella* itself. These bacteria glow when they sense DSF and will indicate which genes in *Xylella* are being expressed in such a cell-density-dependent fashion. We are fortunate in finding that *Xylella* shares the same cell signaling system as *Xanthomonas*, since we can use a fluorescent "signal-sensing" strain of *Xanthomonas* to help us find other bacteria that grow with *Xylella* in the plant that can disrupt the signal. We have engineered a mutant strain of *Xylella* that should be unable to make the signal, but are still characterizing it. When finished, we will use this strain to verify the importance of signaling for survival and spread of the bacteria in the plant. We expect that the mutant will have a smaller population size and will be less efficient at spreading throughout the plant. We have collected potential DSF degrader and DSF analog producer strains of bacteria from infected grapevines and are preparing to test them with our signal-sensing strains to see if they can disrupt *Xylella* signaling. Such strains should be effective in reducing disease when introduced into plants as endophytes or if the genetic ability to produce these compounds or degradative enzymes is introduced into grape.

IV. Objectives and Experiments Conducted:

1) Characterize cell-cell signaling factors in *Xylella fastidiosa*

Cell-cell signaling and its role in virulence is well studied in *Xanthomonas campestris* pv. *campestris,* a bacterium with which *Xylella* shares a high level of DNA sequence similarity. The genes encoding the cell signaling apparatus of *Xanthomonas* are particularly similar between the two species, indicating that *Xylella* may use the same signaling compound (DSF). Experiments were undertaken to determine whether *Xylella* produces a DSF-like signal that is recognized by *Xanthomonas.* Two types of experiments were conducted. To obtain DSF samples, ethyl acetate extracts of PWG agar were prepared from plates on which *Xylella* or *Xanthomonas* had grown. As a negative control, extracts of fresh PWG plates also were made. The extracts were concentrated and soaked into sterile paper disks. These disks were placed on NYGB plates containing X-GLUC, the substrate of the GUS enzyme. The plates were then overlaid with a suspension of a DSF "signal-sensing" strain of *Xanthomonas* that expresses GUS in response to the *Xanthomonas* DSF, but cannot express GUS in the absence of exogenous DSF. This strain carries a *GUS* gene fused to the *engXCA* promoter (from a gene encoding a cellulase), which normally is activated only by DSF. Expression of GUS was detected as blue halos in the medium caused by accumulation of the cleaved GUS substrate. Blue halos were observed around paper disks soaked with extracts from both *Xanthomonas* and *Xylella* plates, but not around those control disks soaked with extracts of PWG agar from unused plates. This result indicates that *Xylella* produces a chemical that activates the *engXCA* promoter and suggests that *Xylella* produces DSF or a very similar compound. This is a very exciting result in that it indicates two things: 1) *Xylella* exhibits cell-density dependent signaling which almost certainly involves virulence factors such as cellulases and galacturonases involved in movement between vessels in grape stems. 2) Since the signaling factor produced by *Xylella* seems to be the same or very similar to that produced by *Xanthomonas*, we can assume that much of what is known about the types of genes regulated by DSF and their pattern of regulation in *Xanthomonas* may apply to

Xylella. More importantly, *Xanthomonas campestris* strains can be used as surrogates in many steps used to study DFS signaling and interference. This is a great advantage since MUCH more rapid progress can be made with *Xanthomonas* strains since they grow so much faster and easier than *Xylella* strains.

In a second experiment designed to test for the presence of DSF in *Xylella* media, ethyl acetate extracts of PWG agar from *Xylella, Xanthomonas*, and fresh plates were concentrated and resuspended in methanol, then were added to LB medium inoculated with the *Xanthomonas rpfF* mutant. This mutant grows in clumps rather than planktonically in LB unless exogenous DSF is added to the medium. We found that cultures were planktonic if *Xanthomonas* extract was added and were mostly planktonic if *Xylella* extract was added. Cultures were completely clumped if PWG extract or methanol alone were added. Because the number of cells on a *Xylella* plate is much lower than that of a *Xanthomonas* plate, we suspect that if a more concentrated extract of *Xylella* medium were added then cultures would be completely planktonic. This result supports our finding that *Xylella* produces a DSF-like compound.

In order to detect DSF directly in *Xylella,* we are in the process of engineering several potential signal-sensing strains of *Xylella*. Three *Xylella* genes are likely activated by DSF based on the *Xanthomonas* model: a *gumB* homologue (involved in extracellular polysaccharide production) and two *engXCA* homologues (encoding cellulases involved in movement through the xylem). The promoters of these genes were cloned from the *Xylella* Temecula strain and each fused to the gene encoding green fluorescent protein (GFP), and to three variant forms of GFP that have different half-lives in the cell. These variant forms of GFP and the different promoters employed will allow us to choose a signal-sensing strain that is optimally balanced, expressing a strong signal but only in the presence of DSF. These constructs were introduced into plasmids we engineered to integrate into the *Xylella* Temecula genome. Analysis of transformants is underway.

We have made good progress in developing a genetic system for *Xylella*. In a companion project in our lab that addresses the role of adhesion in the process of Pierce's disease, Dr. Helene Feil has shown that gene knockouts are possible in *Xylella*. Following the example of Monteiro, et al (2001), Dr. Feil developed plasmid vectors that enable partial replication ability in *Xylella*; such vectors are ideal for gene knockouts and gene replacement strategies. Specifically, into a plasmid such as pUC18, which will not replicate in *Xylella*, the chromosomal origin of replication from the Temecula PD strain was cloned. Since the sequence of the Temecula strain was available through the Brazilian sequencing efforts, the *ori*C region was readily obtained by PCR amplification. This "shuttle vector" apparently is poorly maintained in *Xylella* after introduction by electroporation. Antibiotic resistance markers such as *kan* thus can be rescued after integration of the entire vector into the chromosome if sequences homologous to *Xylella* are present on the vector. In the first demonstration of this process, homologous recombination between the vector and the chromosome occurred within the chromosomal *pil*A gene since this gene involved in pilus production was introduced into the plasmid. Thus the shuttle vector apparently replicates sufficiently well to allow ample opportunity for recombination to occur after introduction into *Xylella*, but sufficiently poorly that it is lost after several generations of growth of *Xylella*, which allows the rescue recombination events after eviction of the plasmid. As noted below, we are in the process of developing different variants of this shuttle vector and different strategies of making gene knockouts that should prove useful

to us as well as other *Xylella* workers. These molecular tools will be useful in making gene-specific knockouts as well as for introducing gene fusions to monitor patterns of gene expression.

Because the *Xanthomonas engXCA* promoter responds to a signal from *Xylella*, it will be possible to detect the *Xylella* signal using *Xanthomonas* signal-sensing strains. Therefore, we are in the process of constructing promoter-*gfp* constructs using the *Xanthomonas engXCA* and *gumB* promoters for transformation into *Xanthomonas*. These signal-sensing strains, which have the advantage of much faster growth rate and less specific media needs than *Xylella* strains, will be very useful for objectives 3 and 4.

2) Determine role of signaling factors on virulence and transmissibility of *Xylella*

The most efficient way to address the role of signaling factors is to compare wild type *Xylella* with *Xylella* that is unable to produce signaling factors. To engineer *Xylella* that is unable to synthesize DSF, we are knocking out the *rpfB* gene, which is required for DSF production in *Xanthomonas,* by two approaches. In the first, the *rpfB* gene was cloned from the *Xylella* Temecula strain and mutated such that it lacks a promoter, its start codon is mutated, its 3'end is truncated, and an in-frame stop codon is inserted upstream of the truncation. This truncated form of *rpfB* was cloned into a plasmid that carries a gene for GFP driven by a constitutive promoter. We expect that chromosomal integration of this plasmid should occur by a single recombination event, resulting in two non-functional *rpfB* loci in antibiotic resistant, GFP positive transformants. One *rpfB* locus will lack a promoter and start codon while the other *rpfB* locus will produce a severely truncated form of the protein. We expect this will abolish gene function. *Xylella* Temecula strain transformants have been made and both their genetic structure as well as virulence features are being analyzed.

A potential drawback to the first approach is the possibility for intrachromosomal recombination to cause loss of the plasmid sequence concomitant with restoration of *rpfB* gene function. In the second approach, a stable mutation formed by a double recombination event is sought. A region of the genome that includes the complete *rpfB* coding sequence was cloned from *Xylella* Temecula strain. A large proportion of the coding sequence was replaced with a cassette that provides antibiotic resistance and constitutive GFP activity. This marker-replaced version of *rpfB* will be cloned into a plasmid carrying a second antibiotic resistance gene designed for transformation into *Xylella*. We expect plasmid integration by a single recombination event to yield transformants that are positive for GFP, both antibiotic resistance markers, and rpfB activity, but plasmid integration by a double recombination event will yield transformants that are sensitive to the antibiotic carried on the plasmid and that are rpfB negative.

3) Identify degraders of signaling factors of *Xylella*

(Objectives 3 and 4 are being completed together)

4) Identify inhibitory analogs of signaling factors of *Xylella*

Endophytic bacteria that grow in close association with *Xylella* were collected from grape in vineyards that had active Pierce's disease and were bordering riparian areas. Canes were cut from vines that were asymptomatic (stage 0), had an isolated infection (stage 1), had spreading

symptoms (stage 2), or were overcome by disease (stage 3). Canes also were taken from stage 0 vines that were surrounded by many stage 3 vines. In addition, canes were collected from both asymptomatic and symptomatic wild (American) grapevines growing in the bordering riparian areas. Canes were surface sterilized and trimmed, tissue was homogenized and plated on 5%TSA. Colonies have been stockpiled in the freezer and are ready to be tested with the signal sensing strain being constructed in Objective 1.

Because *Xylella* apparently employs the same signal as *Xanthomonas*, it is likely that degraders of *Xylella* signaling factors may be found growing in close contact with *Xanthomonas* cells in infected plant tissues. *Xanthomonas campestris* causes a wilt and necrosis of crucifers such as cabbage, often called black rot. This disease is common in California. An advantage of testing for co-inhabitants of *Xanthomonas*-infected plants is that there will be many more such bacteria much more closely coincident with the pathogen in such a disease, and hence we would expect a higher likelihood of finding strains that interfere with this pathogen. Such strains should also interfere with *Xylella* since it shares a signaling system with *Xanthomonas*. Therefore we plan to collect further strains from *Xanthomonas*-infected cabbage plants, and have initiated collaboration with Steve Koike, Cooperative Extension Plant Pathologist in Monterey County, who will be providing us with infected plant material. Samples will be processed and strains will be tested as for the grapevine canes.

Note on related work: In the past year since we proposed this strategy of interfering with cell-cell signaling in *Xylella* as a new means of disease control, this principle has been demonstrated in a different bacterial disease by a research group in Singapore. *Erwinia carotovora* rots plants such as potato tubers by means of enzymes such as pectinases and proteinases that it secretes into the plant. Such macerating enzymes are produced only after relatively large populations of the pathogen have grown in the plant. The pathogen coordinates production of these virulence factors by production of a diffusible factor called an n-acyl homoserine lactone (HSL). (This signal factor is different from that produced by Xylella). The Singapore group isolated a Bacillus strain that produced a lactonase that inactivates HSLs (the enzyme cleaves the lactone ring on this signal molecule, thus inactivating it). The gene encoding this enzyme could be easily cloned from Bacillus, and when introduced into potato resulted in potatoes that were virtually immune to infection by *Erwinia* after inoculation. Apparently the enzyme, when in the plant, degrades the signal molecule as fast as it is made by the pathogen, thus preventing the pathogen from accumulating enough signal in the plant to induce virulence gene expression. We find these results to be VERY EXCITING since they verify the strategy that we are pursuing toward control of Pierce's disease. While the strains/genes found by this group will not work for *Xylella* control, the strategy that they took to find such strains is the same as we are pursuing, and their results indicate that the chances for success of our strategy in grape are very high.

5) Evaluate disease management using signaling factor degrading organisms, enzymes, and inhibitory analogs

This Objective is dependent upon completion of Objectives 3 and 4.

V. Summary of Major Research Accomplishments and Results

1) Characterize cell-cell signaling factors in *Xylella*

We have determined that the *Xanthomonas* signaling apparatus responds to a signal from *Xylella*, and that the *Xylella* signal is most certainly the same or a very similar compound as *Xanthomonas* DSF.

We have engineered a collection of signal-sensing strains of *Xylella* that will allow us to monitor DSF signaling in *Xylella* itself.

2) Determine role of signaling factors on virulence and transmissibility of *Xylella*

We have engineered potential *rpfB* knockout strains, which are being tested.

3 and 4) Identify inhibitory analogs of signaling factors of *Xylella* and identify degraders of signaling factors of *Xylella*

We have collected potential DSF analog producer and DSF degrader strains from infected grapevines and are preparing to test them.

We have made a GFP-based signal sensing system in *Xanthomonas campestris* that will allow us to adopt a rapid high-through-put screening system for indigenous bacterial strains that interfere with or degrade the signaling compound of *Xylella*.

5) Evaluate disease management using signaling factor degrading organisms, enzymes, and inhibitory analogs

VI. Outside Presentations of Research

December 2001. Pierce's Disease Research Symposium. "The role of cell-cell signaling in host colonization by *Xylella fastidiosa."*

VII. Research Success Statements

We have determined that *Xylella* produces a diffusible signal factor that is very likely involved in the expression of virulence traits required for disease symptoms. We have developed a simple screening system that will allow us to test thousands of indigenous bacteria for the ability to interfere with or degrade the signaling system in *Xylella*. Such strains can then be tested directly for their ability to control disease when introduced into grape. Alternatively, the genes encoding such interfering compounds or degradative enzymes can be introduced into grape so that *Xylella* virulence genes can be prevented from being expressed, hence preventing disease symptoms. We have developed a genetic system that enables gene-specific knockouts to be made, as well as in gene replacements to enable monitoring of gene expression using various reporter genes. While this system is still being refined, this is a major breakthrough that will enable us, as well as others working on the pathogen, to understand better the processes leading to disease.

VII. Funds Status

Work on this project was initiated in August 2001, after funds were transferred to UC-Berkeley and Dr. Newman started work on the project. The expenses for the project, while initially low, are increasing throughout the remainder of this first year of the project since an increasing number of genetic constructs are being analyzed, bacteria are being isolated and screened for antagonistic activity toward *Xylella*, and inoculations of grape by *Xylella* mutants are being performed in the greenhouse.