EVALUATION OF GRAPEVINE ENDOPHYTIC BACTERIA FOR CONTROL OF PIERCE'S DISEASE

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ABSTRACT
We continued to screen our endophyte library for *in vitro* antagonism of *Xylella fastidiosa* (*Xf*) growth. Approximately 16 isolates exhibited antagonism out of the 150 strains that were screened. To date, we have screened approximately 650 isolates and identified 66 that showed some level of *Xf*-antagonism. We are continuing to screen the rest of the library and will conduct grapevine movement assays on all antagonists in the coming year.

Greenhouse testing of six grapevine endophytes that began in 2003 showed that three isolates provided statistically significant reduction in Pierce's disease (PD) symptom severity. Five months after these vines were removed from the greenhouse and planted in the field, all but one of the non-protected, *Xf*-inoculated, positive control vines were dead or had PD symptoms. No symptoms were observed in any of the ten vines that were inoculated with a *Cellulomonas* endophyte nor eight of ten vines inoculated with a *Bacillus* spp. These results indicate that these two endophytes have the ability to suppress populations of *Xf* within grapevines, because these vines initially tested positive for *Xf* four months following inoculation nor after transplanting these vines in the field.

A large greenhouse biocontrol experiment involving four of the Pseudomonads, or combinations of the Pseudomonads with *Pseudomonas viridiflava* to act as a movement facilitator, were established in August 2004. Unfortunately it appears that there was a low efficiency of inoculation using blue-green sharpshooters (BGSS) as well as mechanical inoculation because PD symptoms were not evident on the positive control vines four months following inoculation. The vines will be rated again for symptoms and planted in the field, which may help to induce PD symptoms.

Additional endophytes were isolated from "escape" vines; i.e. vines without apparent symptoms in vineyards with large PD losses on two occasions in Fall 2004. Representative colonies were grown in liquid media and stored at -80°C. During 2005 these isolates, as well as others from the original endophyte library will be screened for *Xf*-antagonism *in vitro*.

INTRODUCTION
Xf, the bacterium that causes PD, colonizes only xylem vessels, an ecologically distinct niche which supports the growth of comparatively few microbes. However, previous research conducted in Nova Scotia and in our lab has shown that a number of bacterial species can be isolated from grapevine xylem sap. While some of these bacteria are most likely wound inhabitants that cannot systemically colonize xylem elements, other species have been shown to move over 30cm, a distance that likely would involve the active degradation of xylem pit membranes that separate individual xylem elements. Previous research showed that some of the systemic colonizers were antagonistic to *Xf* in *in vitro* growth inhibition assays (see previous progress reports). Greenhouse grown grapevines that were inoculated with some of these strains did not prevent initial multiplication of mechanically inoculated *Xf*, however some of these strains prevented the subsequent development of PD (see below). If additional testing substantiates the protective properties of these bacterial endophytes against PD, these strains may provide a novel and environmental benign approach for minimizing losses to PD.

OBJECTIVES
1. Finish evaluating our existing library of grape endophytic bacteria to identify antagonists of *Xf in vitro*.
2. Evaluate the biocontrol abilities of endophytes against *Xf* including:
   i) prevention of infection
   ii) suppression of PD symptom development
   iii) long term health and survival of infected vines in field experiments.
3. Isolate additional endophytes from asymptomatic vines in infected vineyards (escape vines) and characterize these isolates for antagonistic traits.

RESULTS AND CONCLUSIONS
Continue in vitro screening the library of grape endophytic bacteria to identify antagonists of *Xylella fastidiosa* growth.
Details regarding the methods we used for the *in vitro* antagonism assay have been presented in previous progress reports. In brief, 100ul of a 10^8 CFU culture of the Fetzer or Temecula strain of *Xf* are spread over a plate of solid PD3 medium. A small amount of each endophyte isolate is removed from the -80°C glycerol stock and streaked out on the medium in which it was originally isolated to obtain single colonies. The *Xf* plates are incubated for approximately four days and then a small amount

of the endophyte is applied to the center of the *Xf* plate. The plate is then incubated for seven days until *Xf* colonies are clearly visible. If the endophyte has the ability to inhibit the growth of *Xf,* the size of the inhibition zone is measured and recorded.

During this period we screened approximately 150 more endophytes, bringing the total number of screened isolates to 650, which is approximately 2/3 of the total number of isolates that Dr. Darjean collected. Approximately 16 of the 150 isolates showed some degree of antagonism towards *Xf* growth *in vitro*. This brings the total number of endophytes that exhibited some degree of *in vitro* antagonism towards *Xf* to 66, or approximately 1/10 of the isolates that were screened. If this trend continues we would expect to identify approximately 100 endophytes that exhibit some degree of antagonism towards *Xf* from the entire library.

Table 1. Grapevine endophytes screened during 2004 that showed some degree of antagonism towards *Xf*.

Endophyte	RFLP Group	Zone of clearing
75	37	25 mm
110	15	25 mm
122	15	6 mm
127	15	10 mm
128	15	10 mm
138	15	15 mm
145	16	5 mm
174	Group 28	slower/complt
176	28	18 mm
178	19	6 mm
184	Group 80	3 mm
197	Group 7	20 mm
200	Group 7	5 mm
220	7	slower growth
221	7	5mm
310	33	10mm

Assess the ability of antagonists to colonize and move systemically in grape xylem.
Prior to her departure in October, 2004, Dr. Whisler screened 16 of the *Pseudomonas* endophytes for their ability to move in Chardonnay grapevines growing in the greenhouse. The vines were trained as a single cane and the cane was grown to approximately 1m. The endophytes were suspended in phosphate buffer to a density of approximately 10^7 CFU/ml and approximately 20ul of the suspension was pinprick inoculated into the stem using the same methods that we use to mechanically inoculate vines with *Xf*. Two grapevines were inoculated with each strain. After six weeks of growth in the greenhouse, 1cm stem sections were removed at 10cm and 30cm above the point of inoculation. The second petiole above the point of inoculation was also removed to assess whether the endophyte had the ability to cross the xylem pit membrane and enter into the petiole. The stem sections and petiole were surface sterilized in 10% bleach and 80% ethanol for 1 min each and then rinsed three times in sterile di-water. The stem sections were placed in sterile grinding bags with 2 mls of sterile phosphate buffer and the tissue was ground using a ball bearing grinder. One hundred ul of the homogenate was plated on the medium on which the endophyte was originally isolated. Colonies with morphologies that were similar to the inoculated endophyte were counted and one or two representative colonies were chosen at random for strain identification. As described in previous progress reports, the 16S rDNA from these colonies was PCR-amplified and sequenced to verify the identity of the putative endophyte. Table 2 summarizes the results of the movement assays for these 16 isolates.

Table 2. Movement and other characteristics of 16 Pseudomonad grapevine endophytes.

Pseudomonas subgroup	# of isolates	Vine Health[1]	Antagonism[2]	Movement[3]
1	5	Healthy	Complete[4]	10 cm
2	5	Healthy	Complete[4]	10 cm
3	3	Escape/Healthy	20-25 mm	Petiole/ 30 cm
4	1	Healthy	Complete[4]	10 cm
5	2	Healthy	5 mm	N/D[5]

[1]Condition of vine of origin at time of endophyte isolation.
[2]Antagonism of *Xf* growth is the zone of inhibition or the distance from the endophyte to the visible growth of *Xf*.
[3]Re-colonization and movement in grape xylem was assessed at 10cm and 30cm from the point of inoculation, and from the second petiole above the point of inoculation.
[4]Complete: no growth of *Xf* visible.
[5]N/D: not yet determined.

All of the Pseudomonads moved at least 10cm above the point of inoculation, however work done in the Labavitch laboratory has shown that a small proportion of xylem elements are greater than 10cm in length thus these positive isolations may reflect inoculation into some of the longer elements. Isolates from subgroup 3, which were phylogenically most similar to *Pseudomonas viridiflava*, moved the greatest distance, at least 30cm from the point of inoculation. These isolates were also the only ones recovered from the petiole, suggesting they had the ability to degrade pit membranes that presumably occur between xylem vessels in the stem and the petiole. These isolates were plated on a sodium polypectate medium that can detect the production of polygalacturonase (PG), an enzyme that degrades pectin-like polymers. Clearing zones around the colonies proved that these isolates, like true *Pseudomonas viridiflava* strains produce PG. Ms. Caroline Roper, a student working jointly in the Labavitch and Kirkpatrick labs, has shown that the production of PG is absolutely necessary for the movement of *Xf* in grapevines (2004 PD/GWSS Conference). It would appear that *Pseudomonas viridiflava* would be an excellent candidate as a potential *Xf*-antagonist or it could act as a "movement facilitator" which, when co-inoculated with a stronger *Xf* antagonist, could facilitate the movement of the stronger antagonist by degrading xylem pit membranes.

Continued evaluations of biocontrol experiment initiated by Dr. Darjean-Jones in 2003
A previous PD progress report presented many details about a biocontrol project that was initiated by Dr. Darjean-Jones in 2003. The following provides an update of the field evaluation of these plants that was done in October 2004 and 2005.

Six bacterial grapevine endophytes that exhibited antagonism to *Xf in vitro* and which moved 15cm from the point of inoculation into grapevines growing in the greenhouse were evaluated as potential biocontrol agents for PD. Each strain was inoculated into 10 Cabernet Sauvignon vines in April, 2003 and allowed to colonize the vines for six weeks in the greenhouse. With the assistance of Purcell's group at UC Berkeley, the vines were then exposed to *Xf*-infectious BGSS. The vines were returned to UC Davis and kept in a greenhouse. Four months later, in September 2003, they were tested for *Xf* by IC-PCR and their symptoms were rated on a severity scale of 0 (healthy) to 4(dead). These results are shown below:

Table 3. Greenhouse evaluation of endophyte vines four months following *Xf* inoculation (10 reps each endophyte).

Endophyte Inoculated	*Xf* PCR (+)	Average Disease Severity (0-4)[z]
Bacillus megaterium	9/10	2.0
Streptomyces spp	7/10	2.3
Bacillus spp –147	9/10	1.5*
Bacillus spp –161	9/10	1.4*
Cellulomonas	9/10	1.5*
Agrobacterium $F_2 5$	10/10	2.2
Control, no endophyte	9/10	2.1

z. disease severity calculated with PCR (+) only. 0=healthy; 4=dead.
* statistically significant difference at p=0.05.

PCR analysis showed that: 1) there was a high rate of successful transmission using the BGSS and 2) none of the endophytes provided protection against initial infection by *Xf*. However, three of the endophytes provided a statistically significant reduction in the severity of PD symptoms after four months of growth in the greenhouse. These vines were kept in the greenhouse over the winter, during which time some of the vines died from PD. In spring 2004, all of the remaining vines were planted in the field at UC Davis. The vines were fertilized and watered with a drip system. In October 2004 and 2005 the vines were rated for PD symptoms. Table 4 presents those results.

Table 4. Disease evaluation of endophyte-inoculated vines planted in the field approximately 1 ½ and 2 ½ years following inoculation with *Xf*.

Endophyte Inoculated	Healthy/Vine Vigor[z]		PD Symptomatic		Dead
	October 04	October 05	October 04	October 05	
Bacillus megaterium	0/NA	1/3	1	0	9
Streptomyces spp.	5/2.6	6/2.5	1	0	4
Bacillus spp –147	6/2.5	6/2.8			4
Bacillus spp –161	8/2.8	8/2.4	1	0	1
Cellulomonas	10/2.8	10/2.8			0
Agrobacterium F_25	5/2.5	5/3			5
Control, no endophyte	1/1.8		2	2	7

z. Vigor rated on a scale of 3= comparable to other non-endophyte inoculated vines; 1= poor growth

The *Cellulomonas* and *Bacillus*-161 strains provided good suppression of PD symptoms in the field. Petioles from these vines also tested negatively for *Xf* by PCR while symptomatic leaves from some of the other vines tested positively for *Xf*. This would suggest that these strains greatly suppressed the growth of *Xf* from the time when they tested positive four months following inoculation to the time they were tested one and two years later. Xylem sap from a few of the *Cellulomonas*- and *Bacillus*-inoculated vines was plated on endophyte media and some colonies that morphologically resembled these strains were seen. However, the identity of these colonies was not proven by analysis of their 16S rDNA. Additional xylem sap has been extracted from these vines and identity of isolated bacteria is now being done by a new graduate student, Margot Wilhelm. We will re-inoculate these endophyte strains into young and five year old Cabernet Savignon and Chardonnay, which is more susceptible to PD, in 2006. Budwood will be collected from the *Cellulomonas* and *Bacillus*-161 vines during the winter and rooted in spring 2006. Xylem sap will be examined for the presence of the endophytes in some of the rooted vines while others will be mechanically inoculated with *Xf* to determine if propagated vines possess any resistance to PD.

2004 biocontrol experiment initiated by Dr. Whistler

A large biocontrol experiment was initiated by Dr. Whistler beginning in July 2004. This experiment focused on the Pseudomonad group 7 that exhibited good *in vitro* inhibition. There were a total of 24 treatments with 10 Chardonnay vines per treatment. Four strains, 197 (*Pseudomonas viridiflava*, a strong grapevine colonizer), 205, 329 and 403 (strains that strongly inhibited *Xf in vitro*) were individually inoculated in 10 vines/trmt using the pinprick inoculation procedure routinely used to inoculate *Xf*. In addition, a movement facilitator treatment using strain 197 in combination with 205 or 329 or 403 was also inoculated into 10 vines/trmt. To assess the potential impact of the endophyte on grapevine growth, the strains were also individually inoculated into 10 vines/trmt. Ten vines of each endophyte individual or combination treatment were mechanically inoculated with *Xf* and ten vines of each treatment were insect inoculated using putatively infectious BGSS in cooperation with Sandy Purcell's lab. Ten vines not inoculated with anything served as controls to monitor greenhouse environmental conditions. Ten vines each were inoculated with buffer alone, or buffer then *Xf* inoculated mechanically or with BGSS. In total this experiment had 240 potted vines.

Unfortunately, subsequent infectivity tests by the Purcell lab found that the batch of BGSS that was used to inoculate the vines had poor transmission rates to test plants kept at Berkeley. Because of the long latent period, typically 12 to 14 weeks, for PD symptoms to show, we did not know these results until it was too late to acquire more BGSS for another inoculation attempt. In addition, the mechanically inoculated, positive control vines still appeared healthy in December 2004 and April 2005, three and seven months after *Xf* inoculation. In May 2005, the vines were transplanted into the field in the hope that PD symptoms would develop in the fall. Unfortunately none of the non-protected, positive control vines developed symptoms, which indicates that, for reasons unknown, the *Xf* inoculation was unsuccessful. In addition, because the vines were inadvertently sprayed with *Bacillus thurengensis* (BT) to control a caterpillar infestation and BT has very resilient endospores, attempts to at least measure how effectively the endophytes colonized the control vines were ruined because

surface sterilization failed to kill the BT spores and isolation plates were completely contaminated with BT. This was obviously a great disappointment for all involved. Because the Pseudomonads looked so promising in the initial screening process we will repeat this screen in 2006.

Isolate additional endophytes from asymptomatic vines in vineyards with a high incidence of PD

Two isolations from 10 "escape vines" were made in late August and early October 2004 in order to verify that the vines were truly asymptomatic. Xylem sap was expressed from these vines using the pressure chamber as previous described. Aliquots of the sap were plated on the same media that we have used throughout this study. Representative colonies were individually grown in liquid medium, the culture was adjusted to 15% glycerol and frozen and -80°C. In 2006, we will screen these isolates for anti-*Xf in vitro* activity in the manner previously described.

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