ENVIRONMENTAL FATE OF A GENETICALLY MARKED ENDOPHYTE IN GRAPEVINES

Project Leader:
Thomas A. Miller
Department of Entomology
University of California
Riverside, CA 92521

Collaborators:
Carol Lauzon
Department of Biological Sciences
California State University
Hayward, CA 94542

David Lampe
Department of Biological Sciences
Duquesne University
Pittsburgh, PA 19219

Consultants:
Frank Richards
Yale University
New Haven, CT 06520

Bob Rose
Frederick, MD

Reporting Period: The results reported here are from work conducted October 2004 to October 2005.

ABSTRACT
Alcaligenes xylosoxidans denitrificans (*Axd*), an insect and plant symbiotic bacterium, was genetically altered to carry a red fluorescent protein gene, *Ds*Red. The marked *Axd* (*RAxd*) was detected in stems of several grapevines two weeks post-inoculation at commercial vineyards in Temecula, Napa and UC Riverside. The amount detected at four weeks post-inoculation declined, and *RAxd* was absent six weeks post-inoculation. *RAxd* was not detected in grape berries, or in soil samples collected around *RAxd* positive grapevines nor in the roots of test plants. *RAxd* was found readily in the buccal cavity of the vector insect and in citrus xylem.

INTRODUCTION
Replacement therapy or symbiotic control (Beard et al., 2002) employs symbiotic bacteria to deliver anti-disease gene products to target pathogens to make vector insects unable to harbor the pathogen or to prevent pathogens from being transmitted. We are testing *Alcaligenes xylosoxidans denitrificans* (*Axd*), a xylem-limited endophytic symbiont and a commensal of glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*, for use in symbiotic control of Pierce's disease (PD) (Bextine et al. 2004). The marked recombinant strain was produced by inserting the *Ds*Red marker gene into *Axd* (to make *RAxd*) into mariner element plasmids (Ruben et al. 1999), which was the insertion vehicle. This field project was designed to determine the fate of *RAxd* when injected into grapevines in a future control strategy.

Vines in commercial vineyards were used to locate the test in as realistic a setting as possible and because we are aware that laboratory behavior of these plants and microbes does not reflect field behavior. We chose widely separated locations in California and more than one variety of grapevines to test. A top priority was to determine if the transgenic endophyte lodged in the grape berries or otherwise contaminated the product of the vineyards. These results follow similar protocols followed the year before. Permits from the Environmental Protection Agency (EPA) were required to conduct the field tests (Miller, 2004).

OBJECTIVES
1. Track the movement of *Alcaligenes xylosoxidans denitrificans* (*Axd*) in plants and the environment.
2. Characterize transmission of *Axd* by GWSS, *Homalodisca coagulata*.
3. Treat plants with excessive amounts of *Axd* to assess the effect on the plant and longevity of *Axd*.

RESULTS
In July 2004, field sites were arranged at commercial vineyards in Napa and Temecula Valleys and at UC Riverside. *RAxd* was applied to grapevines using the inoculation techniques used in previous years on this project. Grapevines were covered with insect-free screening (Figure 1A), to exclude arthropods from test plants. Samples were taken throughout the growing season and processed. Plants were burned at the end of trials (Figure 1B) as required by EPA permits.

Grapevines were needle inoculated with *RAxd* (Bextine and Miller 2004, Bextine et al. 2005) according to the schedule shown in Table 1. Over a 4.5 month period, from June 11 to October 15, 2004, grapevines, grape berries or roots were sampled every other week (Table 1).

Figure 1A. Experimental field cage.

Figure 1B. Burning grapevines at the end of the experiment as required by EPA (Miller, 2004).

Table 1. Schedule of events.

	Weeks After First Inoculation									
	0	2	4	6	8	10	12	14	16	18
Injected	YES	YES	YES	YES	YES	YES	YES			
Plant Samples Tested		YES	YES	YES	YES	YES	YES	YES		
Fruit Tested								YES	YES	YES
Root Tested										YES

Grapevine samples

Although the grapevines were consistently inoculated at two week intervals, these grapevines were not consistently positive throughout the growing season (Tables 2 and 3). In fact, nearly all grapevines tested positive two, four, and six weeks after the initial inoculations were made. No grapevines tested positive on the last two sample dates, despite the same inoculation treatments. These data are not consistent with error in the sampling methodology but may indicate incompatibility with the host during later stages of fruit development. No control grapevines tested positive.

Table 2. Number of RAXD positive shoots per cordon (n=30).

	Weeks After First Inoculation						
Bacterial Concentration Inoculated	2	4	6	8	10	12	14
10^9	19	22	19	8	5	0	0
10^6	20	23	23	5	3	0	0
χ^2	0.073	0.089	1.270	0.884	0.577	30	30
p-value	0.787	0.765	0.259	0.347	0.447	1	1

Table 3. Number of RAXD positive grapevines (n=15).

	Weeks After First Inoculation						
Bacterial Concentration Inoculated	2	4	6	8	10	12	14
10^9	12	15	12	8	5	0	0
10^6	13	13	14	4	2	0	0
χ^2	0.240	2.143	1.154	2.220	1.677	30	30
p-value	0.624	0.143	0.283	0.136	0.195	1	1

Fruit samples
No fruit samples were confirmed positive on any date (Table 4). Fruit extracts were tested three times by two QRT PCR methodologies (twice with TaqMan and once with SYBR® Green). During the initial screening of fruit about 14% of samples (52 of 360) from week 14 were labeled "questionable." Upon re-testing these samples, no *RAxd* was detected. No fruit samples from the other two collection dates tested positive.

Table 4. Fruit samples tested for the presence of RAXD.

	Weeks After First Inoculation		
	14	16	18
Individual Grapes	0/300	0/300	0/300
Bunch Stem	0/60	0/60	0/60

As with all PCR-based detection systems, QRT PCR comes with a certain degree of ambiguity so positive samples have to be confirmed. In the case of the week 14 fruit samples, fluorescence increased at the end of the reaction, slightly below the positive threshold using the *Taq*Man chemistry. Because these samples were close to the threshold, they were tested twice more (again by *Taq*Man and using the SYBR® Green chemistry). In these subsequent reactions, no samples tested positive.

Root samples
No root samples tested positive.

CONCLUSIONS
*Ds*Red *Alcaligenes xylosoxidans* var. *denitrificans* (*RAxd*) survives in grapevines in commercial vineyards as a recombinant endophyte for less than one month following injections; titers decline below detectable levels after a few weeks. Re-treatment restores the titer. *RAxd* does not spread extensively throughout the grapevine and was not found in the roots, in the petioles or in grapevine berries. From a regulatory and residue standpoint, this is an ideal result. Moreover, the grapevines withstood injection of large amounts of this endophyte with no ill-effects. These tests were not designed to demonstrate control of PD, merely the possibility of delivery of a "biopesticide." Regulatory permission to test the ability of *RAxd* to deliver an anti-PD strategy would require increased pressure from the grape and wine industry in California. The possibility of delivering an anti-PD strategy with the symbiotic control approach using trap crops associated with vineyards and the possibility of native leafhoppers acquiring and moving the recombinant endophyte to other plant hosts were to be the subject of further testing in 2005-2006; however, the funding needed was not obtained, so those tests were cancelled.

REFERENCES

Beard, C. B., C. Cordon-Rosales, and R. V. Durvasula. 2002. Bacterial symbionts of the Triatominae and their potential use in control of Chagas disease transmission. Ann. Rev. Entomol. 47:123-141.

Bextine, B., and T. A. Miller. 2004. Comparison of whole-tissue and xylem fluid collection techniques to detect *Xylella fastidiosa* in grapevine and oleander. Plant Disease 88: 600-604.

Bextine, B., C. Lauzon, S. Potter, D. Lampe, and T. A. Miller. 2004. Delivery of a genetically marked *Alcaligenes* sp. to the glassy-winged sharpshooter for use in a paratransgenic control strategy. Curr. Microbiol. 48:327-331.

Bextine, B., M. Blua, D. Harshman, and T. A. Miller. 2005b. A SYBR green-based real-time polymerase chain reaction protocol and novel DNA extraction technique to detect *Xylella fastidiosa* in *Homalodisca coagulata*. J. Econ. Entomol. 98: 667-672.

Bextine, B., D. Lampe, C. Lauzon, B. Jackson, and T. A. Miller. 2005a. Establishment of a genetically marked insect-derived symbiont in multiple host plants. Curr. Microbiol. 50: 1-7.

Miller, T. A. 2004. Rachel Carson and the adaptation of biotechnology to crop protection. Amer. Entomol. 50: 194-198.

Rubin, E. J., B. J. Akerley, V. N. Novik, D. J. Lampe, R. N. Husson, and J. J. Mekalanos. 1999. *In vivo* transposition of mariner-based elements in enteric bacteria and mycobacteria. Proc. Nat'l. Acad. Sci. 96:1645-1650.

FUNDING AGENCIES

Funding for this project was provided by the USDA Animal and Plant Health Inspection Service, and the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

Additional Note: All of the field tests were conducted under a permit from the EPA. A report of the tests was submitted to the EPA and the sponsors.