ENVIRONMENTAL FATE OF A GENETICALLY MARKED ENDOPHYTE IN GRAPEVINES

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ABSTRACT
Symbiotic control employs symbiotic bacteria to deliver anti-pathogen compounds to disrupt transmission of the pathogen to new hosts. *Alcaligenes xylosoxidans denitrificans* (*Axd*), an insect and plant symbiotic bacterium, occupies same niche as the plant pathogen *Xylella fastidiosa* (*Xf*) which causes Pierce's disease. We determined the fate of genetically altered *Axd* (*RAxd*) after introduction into grapevines to assess its feasibility as a symbiotic control organism to control *Xf*. *RAxd*, which expresses a fluorescent protein (DsRed), was applied to grapevines by needle inoculation, foliar spray application, or soil drench. The plants were covered with insect-resistant screening, to exclude arthropods from test plants. *RAxd* were detected in stems of several grapevines 2 weeks post-inoculation from each inoculation type. The amount detected at 4 weeks post-inoculation declined, and *RAxd* was absent 6 weeks post-inoculation. *RAxd* was not detected in grape berries or soil samples collected around *RAxd* positive grapevines. This work demonstrated that transgenic *Axd* became established in grapevines in the field but did not thrive there. A limited lifespan of transformed *Axd* in grapevines would keep its population increase in check in that host plant. Re-inoculation of grapevines at 6 wk intervals would be sufficient to keep anti-pathogen products present. *RAxd* thrives in GWSS and citrus. Therefore, there is a good chance that GWSS would pick up the *RAxd* as an antimicrobial symbiont from nearby sources to render GWSS vector-incompetent.

INTRODUCTION
Replacement therapy or symbiotic control employs symbiotic bacteria to deliver anti-disease compounds to target pathogens of plants to make vector insects unable to harbor the pathogen or to prevent a pathogen from being transmitted to healthy plants (1). *Alcaligenes xylosoxidans denitrificans* (*Axd*), was selected for further study and a fluorescent marker gene inserted. We followed the movement of genetically altered *Axd* (*RAxd*) in grapevines and in the vector insect, glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*.

Regulatory and industry acceptance of this approach requires knowing the fate of *Axd* in various locations and in plants at different times of the year. Our current detection methods employ PCR (polymerase chain reaction) and fluorescence microscopy (3, 4). QRT-PCR provides a quantitative measure of bacteria in the samples, which is missing from existing methods. This is important because it allows determining optimum doses and timing for application of the delivery organism and its expression of anti-*Xylella* products.

Fluorescent protein gene markers are now commonly used in genetics and are considered environmentally benign since they are based on natural products. The bacterial transformation cassette was inserted with so-called jumping genes (mobile or transposable elements) originally identified in *Drosophila mauritiana* and called mariners (7). The mariner elements have had their jump mechanism removed (so the inserted gene will not be remobilized) and all antibiotic genes used for selection have been removed (so no antibiotic factors can be moved inadvertently to other bacteria). The resulting transgenic strains are very stable and grow readily in culture. Little or no mutation or reversion has been observed.

Since the marker genes were placed next to an open reading site that is designed to contain the future anti-*Xf* compound, the bacteria we are using now are nearly complete. In other words it is close to the final product. Thus, we can study the biology of the genetically altered vehicle bacterium, *RAxd*, and its behavior in the vineyard ecosystem.

We prefer to do this in commercial vineyards because the laboratory experiments are never fully indicative of behavior in the field. We chose widely separated locations and in California and more than one variety of grapevine 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.

OBJECTIVES

1. Track the movement of *Alcaligenes xylosoxidans denitrificans* (*Axd*) within plants with or without insect involvement and track movement in the environment.
2. Characterize transmission of *Axd* by glassy-winged sharpshooter (GWSS, *Homalodisca coagulata*).
3. Develop an application method for transgenic *Axd* into the xylem of grape plants for delivery of an anti-*Xylella* strategy.

RESULTS

In July 2003, field sites were established at four locations in the state of California; Napa, Bakersfield, Temecula, and Riverside. At the Napa, Bakersfield, and Temecula sites, *RAxd* was applied to grapevines using 3 inoculation techniques; needle inoculation, foliar spray application, and soil drench. These plants were covered with insect-free screening, to exclude arthropods from test plants. Samples were taken throughout the growing season and processed. Grapevines at the Riverside field site were needle inoculated with *RAxd* and three concentrations of GWSS (0, 10, and 50) were placed on the plants to test the affect of GWSS feeding pressure on the translocation of *RAxd* in grapevines. We collected mature grapes and plant parts for analysis from grapevines at all four field sites.

Detection of RAxd in Grapevine Xylem: Napa Field Site

Grapevines were inoculated 41 days prior to harvest. Pre-harvest grapevines xylem samples were collected three times (2, 4, and 6 weeks post-inoculation). Only single samples from 2 of 15 test plants tested positive for the presence of *RAxd* at 2 weeks post-inoculation. These positives were from plants treated by needle inoculation and soil drench. Two weeks later, only a single sample from the soil drench-treated plant tested positive. There were no positive samples collected 6 weeks after inoculation. No control plants tested positive for the presence of *RAxd* on any date.

Bakersfield Field Site

Grapevines were inoculated 33 days prior to harvest. Pre-harvest grapevines xylem samples were collected two times (2 and 4 weeks post-inoculation). Multiple samples from 8 of 15 test plants tested positive for the presence of *RAxd* at 2 weeks post-inoculation. Of these *RAxd* positives plants, 3/5 from foliar spray, 2/5 from needle inoculation and 3/5 from soil drench. Two weeks later, only two plants from the foliar spray-treated grapevines tested positive. No control plants tested positive for the presence of *RAxd* on any date.

Temecula Field Site

Grapevines were inoculated 43 days prior to harvest. Pre-harvest grapevines xylem samples were collected 3 times (2, 4, and 6 weeks post-inoculation). No samples on any collection date tested positive for the presence of *RAxd*.

Riverside Field Site

Grapevines were inoculated 26 days prior to harvest. Pre-harvest grapevines xylem samples were collected two times (2 and 4 weeks post-inoculation). Only 10 samples collected 2 weeks after inoculation were positive for the presence of *RAxd*. Six of the positive samples were from grapevines with no GWSS included, while 4 of the positive samples were from grapevines with GWSS included. No significant differences in *RAxd* presence in grapevines could be attributed to the presence of GWSS (X^2=0.24 df=1, p value=0.624).

Detection of RAxd in Soil. RAxd

Detection of *RAxd* in soil. *RAxd* was not detected in soil samples collected from the base of any grapevines at any locations using the culture methods or RT-PCR.

Detection of RAxd in Grape Berries

In grape cluster samples collected on the date of harvest (Napa Aug. 27, Bakersfield Sept. 3, Temecula Sept. 2, and Riverside Aug. 18), *RAxd* was not detected by RT-PCR in whole grape samples from any location. Furthermore, *RAxd* was not detected in dissected grape berry samples of flesh, veins, seeds, peduncle, or stem from any location.

Detection at the Time of Field Plot Destruction

RAxd was not detected in grapevine, root, or soil samples at the time of removal.

Table 1. Detection of *RAxd* in grapevines from three field sites (2003).

	Application Method	*RAxd* positive samples[1]						
		Weeks post-inoculation					During grapevine removal[2]	
		0	2	4	6[3]	Berries[4]	Canes	Root[5]
Bakersfield	Foliar Spray	0	**3**	**2**	ND	0	0	0
	Needle Inoculation	0	**2**	0	ND	0	0	0
	Soil Drench	0	**3**	0	ND	0	0	0
	Control	0	0	0	ND	0	0	ND
Napa	Foliar Spray	0	0	0	0	0	0	0
	Needle Inoculation	0	0	0	0	0	0	0
	Soil Drench	0	**1**	**1**	0	0	0	0
	Control	0	0	0	0	0	0	ND
Temecula	Foliar Spray	0	0	0	0	0	0	0
	Needle Inoculation	0	0	0	0	0	0	0
	Soil Drench	0	0	0	0	0	0	0
	Control	0	0	0	0	0	0	ND

[1]Represents 6 samples from 5 grapevines per treatment per field site (n=30 per grapevine).
[2]Grapevines were removes >14 weeks after inoculations at all locations.
[3]ND = not determined.
[4]Berries were collected during final collection date.
[5]Root samples were taken only from *RAxd* treated vines.

2004 Field Project
Data are not complete and will not be reported here.

CONCLUSIONS
Grapevine inoculations were made after 50-80 days following grapevine flowering at all locations. After flowering, both the xylem and the phloem begin to fill the fruit with fluid (6). Between 60 and 70 days after flowering, the xylem stops filling the fruit, and phloem contributes all fluid for the development of the fruit. This flow continues to 120 days after flowering which is the average number of days to fruit harvest. At all field sites, *RAxd* inoculations were made 26 (Riverside) to 43 (Temecula) days prior to harvest. Considering grapevine physiology, inoculations were made after the xylem ceased to contribute fluid directly to the fruit in all cases. Therefore, it was not surprising that *RAxd* was not found in fruit at any location because it is a xylem-associated bacterium (3).

The most probable explanation for the inability of *RAxd* to survive in grapevines after 4 weeks was its lack of competitive fitness associated with the transgenic organism. Xylem contains diverse and sometimes extensive communities of microbes (2). In greenhouse studies, a strain of EGFP protein-expressing *Axd* was introduced into seedlings of several plant species (3). In that study, the genetically marked bacterium moved readily within the xylem vessels of the plants and was recovered 10 months later. However, presence of a well-established microbial community may have restricted the growth and colonization of transformed *Axd*, ultimately leading to its demise. Chromosomally transformed organisms are commonly less fit than native bacterial species due to the cost of the genetic insert (5). A comparison of the genetically modified *Axd* to the native *Axd* showed that the transformed strain was less fit in laboratory cultures (Lauzon, unpublished data). Although the experiments were not designed to test the relative fitness of transgenic *Axd,* the bacterium's inability to persist longer than 4 weeks provides additional support for the theory of reduced fitness.

Given the ubiquitous nature of *Axd* and its ability to colonize several plant hosts, including grapevines, in the greenhouse (3), we expected it to persist longer in field-grown grapevines. Even so, viability of 4 weeks may offer a large enough window for the delivery agent in a symbiont control strategy to dispense the necessary anti-pathogen factors to negatively affect *Xf*. Additionally, re-application of the symbiotic control agent may be necessary. Additionally, reduced fitness offers an internal controlled mechanism that will guard against transformed *Axd* population spread and persistence in the environment or consumer products.

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