CHARACTERIZE AND ASSESS THE BIOCONTROL POTENTIAL OF BACTERIAL ENDOPHYTES OF GRAPEVINES IN CALIFORNIA

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ABSTRACT

Over 1000 putative bacterial endophytes were isolated from healthy, PD-affected and "escape" (apparently healthy grapevines growing in a vineyard with high incidence of Pierces disease) grapevines growing in Napa and Davis, California from 2000 to 2002. There were no differences in total populations of endophytes isolated in Napa versus Davis, however higher populations of endophytes were recovered from PD-affected versus healthy vines. The endophytes were identified by RFLP and sequence analysis of a portion of the 16S rDNA. Sixty six RFLP groups were determined and sequence analysis showed a diversity of bacterial genera were recovered from grapevines; the most predominant genus was *Bacillus*. Nineteen of the isolates completely inhibited the growth of *Xylella fastidiosa* (*Xf)* in an *in vitro* plate assay. Approximately 80 of 138 isolates that were tested were classified as grapevine colonists because they could be recovered in high populations more that 6cm from the point where they were inoculated into grapevines. Thirteen of the endophytes were classified as both *Xf*-antagonists and grapevine colonizers. Five of these isolates were pin-prick inoculated into grapevines growing in the greenhouse. These vines were then challenged by *Xf*-infectious sharpshooters in the insectary at University of California, Berkeley. None of the endophyte-inoculated vines prevented infection by *Xf* or decreased the severity of PD that developed in these vines.

INTRODUCTION

Many higher plants contain internal populations of bacteria that apparently cause no damage to the plant. Some of these endophytic bacteria can colonize the xylem of plants; the same unique niche that *Xylella fastidiosa (Xf*)occupies. There has only been one published study on bacterial endophytes of grapevines and this study was undertaken in Nova Scotia, a climate significantly different from California. We undertook this study to determine the diversity and relative seasonal abundance of bacterial grapevines endophytes in California. We also wanted to determine whether any of these bacteria exhibited any antagonism towards *Xf* growing on petri dishes in the lab and whether isolates that exhibited *Xf* antagonism *in vitro* might colonize and protect grapevines from *Xf* infection. This effort comprised the PhD. research of Dawna Darjean-Jones who is now in the processing of writing her thesis. We will present a very brief overview of her results here.

OBJECTIVES

1. Isolation and identification of endophytes colonizing grapevines in California:
   i. Determine quantitative and qualitative differences in endophytic populations of grapevines between areas that support natural infections of PD (Napa) and areas where no natural infection of PD has been observed (Davis).
   ii. Determine whether endophytic populations vary quantitatively and qualitatively due to seasonal changes.
   iii. Compare endophytic populations of healthy grapevines with grapevines infected by *X. fastidiosa*
   iv. Compare endophytic populations of "escaped" grapevines (healthy grapevines growing among many infected vines that appear to have escaped disease) with that of healthy grapevines.
2. Determine if any grapevine isolates are antagonistic to or can prevent infection by *X. fastidiosa*.

RESULTS AND CONCLUSIONS

Isolation and identification of endophytes colonizing grapevines in California

Healthy established grapevines from two vineyards in the Davis area and two vineyards in the Napa area, as well as diseased and PD-escape vines from the Napa area were sampled periodically in 1999-2000. These same vines (when possible; diseased vines were sometimes removed without notification) were sampled continuously every other month beginning in September 2001 through December 2002. Xylem sap was expressed from shoots using a pressure chamber. Aliquots of xylem fluid were plated onto 3 microbiological media. Colonies were quantified, streaked to purity, then frozen at -80 ^{O}C until they identified. Using universal primers for the 16S rRNA gene, a PCR product was generated for each isolate.The PCR product was double-digested with restriction enzymes to generate a restriction fragment length polymorphism (RFLP) pattern for each strain. To reduce the redundancy in the identification process, the RFLP patterns of the strains were

subjected to computer analysis using the GelCompar program to avoid sequencing numerous isolates with the same RFLP pattern. 16S PCR products of representatives of each unique RFLP group were sequenced to identify the unknown bacteria.

A total of 1018 endophytes were collected from healthy and PD-affected grapevines during the 3-year study. The total numbers of endophytes, both qualitatively and quantitatively, were similar in Davis and Napa healthy vines. Population of endophytes in PD-affected vines were slightly higher than healthy vines growing in Napa or Davis and more endophytes were isolated in the spring and fall than during the summer. Analysis using the GelCompar program has identified 66 RFLP groups of strains, thus reducing the number that had to be sequenced For examples, several *Bacillus* spp. have been collected from vines classified as healthy, diseased or escapes. Endophytes identified thus far include species from the following genera: *Bacillus, Pseudomonas, Agrobacterium, Erwinia, Streptomyces, Cellulomonas, Pantoea, and Paenibacillus.*

Identification of grapevine endophytes that are natural antagonists to *Xf* and capable of systemic movement in grapevine
A total of 138 endophyte isolates from 50 of the 66 RFLP groups were pinprick inoculated into greenhouse grown grapevines to assess potential movement within grapevine. Each endophyte was inoculated into two shoots from the same vine**.**

After 4 weeks sterilized razor blades were used to divide the surface sterilized stem into seven sections. Each sample was ground in 2 mL of buffer and 100 µl of ground plant suspension was plated onto isolation medium. Resulting colonies were visually compared to the original isolate, sub-cultured, PCR-amplified and the RFLP pattern was compared to the database pattern for the endophyte. Endophytes moving 6 or more cm from the point of inoculation were considered to be potential systemic colonizers of grapevines.
Thirty six of 138 isolates were not recovered from the point of inoculation or any area of the plant sampled. 80 of of the isolates multiplied and were recovered in numbers of at least $7X10^4$ / gram of tissue, i.e. more than 1000/100 ul of sap. About 58 of endophytes were recovered 6cm or more from the point of inoculation and were provisionally classified as systemic colonizers of grapevines.

Bacterial suspensions of *Xf* were spread plated onto solid PD3 medium to form a "lawn." Cultures were incubated for 3 days at 28^oC. Three, 5 µl droplets of overnight endophyte culture were placed onto the previously inoculated *Xf* plates and allowed to dry. Cultures were returned to the incubator for an additional 7 days. Growth of *Xf* and the endophyte were scored. Endophytes inhibiting the growth of *Xf* within a 1-3 mm zone were considered weakly antagonistic. Those inhibiting growth more than 3 mm were considered antagonistic.

Of the 125 grapevine endophytes that were tested for *in vitro* antagonism to the growth of *Xf*, 24 exhibited positive inhibitory activity and 19 isolates completely inhibited the growth of *Xf*. Of the 24 that were inhibitory, 13 isolates were classified as systemic colonizers of grapevines as described previously. Among the antagonists/colonizers were members of 6 *Bacillus* RFLP groups, a *Cellulomonas* sp., a *Rahnella* sp. and a bacterium belonging to the genus *Streptomyces*.

Five of the 13 antagonist/colonizer endophytes were pin-prick inoculated into each of 10 grapevines growing in pots in the greenhouse and allowed to colonize the plants for 1 month. With the generous assistance of the Purcell lab at UC Berkeley, the 50 endophyte-inoculated plants, plus a set of 10 non-inoculated control vines, were exposed to *Xf*-infectious blue green sharpshooters (BGSS) in the insectary. The vines were exposed to 5 BGSS for 48 hours and the insects were then removed. The vines were monitored for symptoms of PD for 5 months following exposure to BGSS. The vines were then rated on a scale of 0 (healthy) to 4 (dead vine) and all vines were tested for *Xf* by IC-PCR.

At least 60% of all the endophyte-inoculated vines and the non-inoculated controls developed symptoms of PD and tested positive for *Xf* using IC-PCR. There was no apparent reduction in the severity of disease in the endophyte-inoculated vines. Thus it appears that none of these 5 isolates has the ability to prevent *Xf* infection of grapevines, however this assay will be repeated and next time the endophytes will be given 2 months to colonize the vines before being challenged with *Xf*. The 8 other antagonist/colonizing endophytes will also be evaluated. We hope to identify xylem-colonizing bacteria that will decrease the ability of *Xf* to multiply to high populations and cause Pierce's disease.

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