CULTURE-INDEPENDENT ANALYSIS OF ENDOPHYTIC MICROBIAL COMMUNITIES IN GRAPEVINE IN RELATION TO PIERCE'S DISEASE

Project Leaders:
Donald A. Cooksey and James Borneman
Department of Plant Pathology
University of California
Riverside, CA 92521

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ABSTRACT
Culture-independent, nucleic acid-based methods of assessing microbial diversity in natural environments have revealed far greater microbial diversity than previously known through traditional plating methods. If true for grapevines, then this has important consequences for Pierce's disease (PD) management strategies that involve the establishment of introduced bacteria systemically in the grapevine xylem. Such establishment will likely be influenced by the presence of yet uncharacterized microorganisms, and knowledge of endophytic communities and their dynamics will therefore be important to the successful implementation of these strategies. In addition, analysis of microbial community composition in different hosts and conditions could lead to the identification of new biological control agents. We are employing a novel method, called oligonucleotide fingerprinting of rRNA genes (OFRG), that was recently developed by the Co-PI for analyzing microbial community composition in environmental samples. In a replicated comparison of symptomatic and asymptomatic grapevines, 558 OFRG fingerprint clusters, or taxonomic groups, were revealed in an analysis of 8,094 total clones, and several clusters were significantly correlated with healthy vs. diseased plants.

INTRODUCTION
In recent years, culture-independent, nucleic acid-based methods of assessing microbial diversity in natural environments have revealed far greater microbial diversity than previously known through traditional plating methods (Amann et al., 1995). This is true for water, soil, the plant rhizosphere, and the plant leaf surface (Yang et al. 2001). A recent culture-independent analysis of bacterial populations inside of citrus plants in relation to *Xylella fastidiosa (Xf)* also suggested that bacterial endophytic populations are much more diverse than previously realized (Araújo et al., 2002). If true for grapevines, then this has important consequences for Pierce's disease management strategies. Several strategies are being investigated to biologically control *Xf* in grapevines, including the use of antibiotic-producing endophytes (Kirkpatrick et al., 2001), endophytes that disrupt cell-to-cell signaling by the pathogen (Lindow, 2002), endophytes that degrade xanthan gum (Cooksey, 2002a), and the use of nonpathogenic strains of *Xylella* for competitive exclusion of pathogenic strains (Cooksey, 2002b). These strategies have in common the need to establish an introduced strain systemically in the grapevine xylem. Such establishment will likely be influenced by the presence of yet uncharacterized microorganisms, and knowledge of endophytic communities and their dynamics will therefore be important to the successful implementation of these strategies. In addition, analysis of microbial community composition in different hosts and conditions could lead to the identification of new biological control agents.

We are employing a novel method that was recently developed by the Co-PI for analyzing microbial community composition in environmental samples. This method can be used to characterize both bacterial and fungal communities (Valinsky et al., 2002a; 2002b). Previous culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE), generate only superficial descriptions of microbial community composition (Araújo et al., 2002). A far more complete view of total microbial community composition can be achieved by amplifying, cloning, and sequencing of conserved rRNA genes from the hundreds or thousands of microorganisms present in an environmental sample, but this is prohibitively expensive for any significant number of experiments. The new methodology, called oligonucleotide fingerprinting of rRNA genes (OFRG), represents a significant advance in providing a cost-effective means to extensively analyze microbial communities. The method involves the construction of clone libraries of rDNA molecules that are PCR amplified from environmental DNA, arraying of the rDNA clones onto nylon membranes or specially-coated glass slides, and subjecting the arrays to a series of hybridization experiments using 37 different end-labeled DNA oligonucleotide discriminating probes (Borneman et al., 2001). The process generates a hybridization fingerprint and identification for each clone that is essentially like sequencing the individual clones.

The state of knowledge of the relationship between *Xf* and the resident endophytic flora of grapevines is at a very early stage. Work to date has been limited to the culturing of endophytes from grapevines, but even this has led to the realization that grapevine xylem sap contains a complex community of microorganisms. Bell et al. (1995) cultured over 800 bacterial strains from grapevine xylem fluid in Nova Scotia. Bruce Kirkpatrick has also isolated several hundred bacterial strains from grapevine xylem fluid in two counties of California (Kirkpatrick et al., 2001). In citrus, the culture-independent DGGE method of microbial community analysis was compared with culturing of endophytes in relation to the citrus variegated chlorosis strain of *Xf* (Araújo et al., 2002). It was found that DGGE detected the major bacteria that were cultured from citrus xylem, but it also detected other bacterial species that had not been cultured. In addition, this method showed differences in

microbial communities in different plant varieties, and most importantly, between citrus that was infected vs. non-infected with *Xf*. This provides support to our hypothesis that there are likely to be important interactions between *Xylella* and indigenous microflora in grapevines. With the greater resolving power of the oligonucleotide fingerprinting technique proposed in our study, we expect to make considerable advances in our knowledge of grapevine microbial communities and their interactions with *Xylella* or with other endophytes being considered for establishment as biological control agents.

OBJECTIVES

1. Characterize the diversity and community structure of endophytic microorganisms in healthy and infected grapevines.
2. Compare endophytic microbial populations in different susceptible and tolerant grapevine cultivars, in different hosts that support high or low populations of *Xylella*, and in plants grown under different conditions.
3. Characterize the potential interactions of endophytic populations with *Xylella* and introduced biological control agents through experimental manipulations.

RESULTS

Last year, several DNA extraction and PCR amplification protocols were tested, and a method involving differential centrifugation to remove DNA of plant origin was developed. This year, a full-scale extraction and amplification from symptomatic and asymptomatic grapevines from the field was conducted. Plant sap was extracted with a pressure pump and analyzed from six replicates of grapevines with Pierce's disease symptoms (three plants per replicate) and six replicates of asymptomatic plants. Isolated DNA was amplified with rDNA primers and cloned, and 768 clones were picked from each sample. Amplified rDNA from the clones were arrayed onto nylon membranes and subjected to a series of hybridization experiments with 37 different end-labled DNA oligonucleotide discriminating probes. The quality of some of the hybridizations was poor, so we were not able to use data from all 37 probes. These will be repeated to obtain more definitive identification of the clones. However, from the data we were able to analyze from 8,094 of the clones, 558 different OFRG fingerprint clusters, or taxonomic groups, were identified. Further, eight of the groups were more significantly more prevalent in asymptomatic vs. symptomatic grapevines. Tentative identification of these seven groups placed six of these groups in the Proteobacteria and one in the Firmicutes. The only bacteria that were more prevalent in symptomatic vs. asymptomatic plants belonged to the Xanthomonadaceae, and were probably *Xylella*. A phylogenetic tree showing the different clusters and more definitive identification of clones will be constructed after data from all 37 probes are analyzed. The following table shows the numbers of clones belonging to groups that were more prevalent in asymptomatic vs. symptomatic grapevines.

Table 1. Numbers of clones belonging to groups significantly ($P<0.1$) more prevalent in healthy vs. diseased grapevines.

Group number	General classification	Diseased	Healthy
Gp192	Proteobacteria	23	60
Gp277	Proteobacteria	9	25
Gp196	Gamma-Proteobacteria	2	14
Gp14	Firmicutes	1017	1769
Gp153	Proteobacteria	14	20
Gp316	Beta-Proteobacteria	4	28
Gp107	Proteobacteria	4	13
Gp284	Proteobacteria	4	12

CONCLUSIONS

Last year, our preliminary analysis of just 58 clones revealed 16 different species, including several that had not been detected in previous culture-based approaches to identify endophytes in grapevine (Bell et al., 1995; Kirkpatrick, 2003). This year, our larger-scale analysis of symptomatic and asymptomatic grapevines confirmed that there is considerable diversity of endophytes in grapevines, with 558 bacterial taxa out of 8,094 clones. We also showed that some bacterial groups were more prevalent in healthy vs. diseased plants. An additional experiment with samples taken from healthy and diseased grapevines at different times during the season is in progress. Researchers working on biological control of the pathogen, as well as disease resistance in grapevine cultivars, will benefit from the information gained in this work. The work should enhance discovery of potential biological control agents for Pierce's disease and the implementation of biological control efforts underway.

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