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

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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 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.

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* 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 *Xylella fastidiosa* 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 27 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 *Xylella fastidiosa* 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. Dr. 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 *X. fastidiosa* (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 *X. fastidiosa*. 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

Several DNA extraction and PCR amplification protocols were tested over the past year. Most procedures yielded too many clones that were of plant origin. Even extracted plant sap contained considerable plant DNA, of mitochondrial and chloroplast origin, that amplified with different versions of prokaryotic-specific ribosomal DNA primers. The use of filtration with various pore sizes to remove plant material from extracted sap also did not eliminate plant DNA from the samples. Finally, we recently succeeded in selectively extracting and amplifying bacterial DNA from grapevine sap using differential centrifugation to remove DNA of plant origin (naked or in organelles). Plant sap was extracted from grapevines with a pressure pump and centrifuged at 8,000 rpm for 1 hr. The pellet was suspended in 1 ml phosphate buffered saline and loaded onto a tube containing percoll. After centrifugation for 30 min at 22,000 rpm, fractions were collected and subjected to DNA isolation. Isolated DNA was amplified with rDNA primers and cloned (Table 1). Fractions containing bacteria yielded only one plant-derived DNA clone out of 58 in the first experiment, and similar results were obtained when the experiment was repeated. A full-scale extraction and amplification from symptomatic and asymptomatic grapevines from the field is in progress.

Table 1. Bacterial species identified from rDNA sequences amplified from grapevine sap in preliminary tests.

Acidovorax sp.
Agrobacterium sp.
Bacillus macroides
Burkholderia sp.
Caulobacter sp.
Escherichia coli
Escherichia fergusonii
Pseudomonas putida
Pseudomonas syringae
Rhizobium tropici
Shigella flexneri
Teichococcus ludipueritiae
Xylella fastidiosa
Unidentified Acinetobacter
Unidentified Proteobacterium
Unidentified Sphingomonas

CONCLUSIONS

Most of the endophytic species that we detected through cloning of bacterial rDNA sequences were not detected in previous culture-based approaches to identify endophytes in grapevine (Bell et al., 1995; Kirkpatrick, 2003). Since the 16 species that we detected were identified among just 58 clones in our recent preliminary studies, we expect that our full surveys of endophytic bacteria in grapevine this year will yield a far greater diversity than previously known. 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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