Chapter 60

Marker-Assisted Selection of Novel Bacteria Contributing to Soil-Borne Plant Disease Suppression

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60.1 INTRODUCTION

It is well established that some bacteria can suppress plant diseases through the expression of multiple mechanisms, including the direct inhibition of plant pathogens and the indirect induction of plant host resistance pathways (Kim et al., 2011). Because of this, microbes that form close associations with plants are the primary source of active ingredients in biopesticides for the control of plant diseases. But for over a decade now, molecular techniques have provided a glimpse of the diversity of soil- and plant-associated microbes and the factors controlling their diversity and plant disease suppressing activities.

However, by themselves, culture-independent methods offer nothing more than a correlative window into the functions of bacterial populations whose true activities can only be studied in culture (Zengler, 2009). Over the past several years, culture-independent techniques have been successfully combined with culture-based methods to identify and recover novel species and subspecies with biocontrol properties. Here, we describe three marker-assisted approaches that have helped to transform the search for new and effective biocontrol bacteria.

The first approach relies on prior knowledge of the genetic mechanisms of biological control. Bacterial populations of interest can be targeted using gene-specific

primers, and different strains harboring variation in these genes are identified and selectively isolated. The second approach relies on removing the genetic redundancy inherent in most bacterial collections screened for biocontrol activities. The genetic diversity of a neutral marker, such as the 16S gene, can be amplified and screened using restriction analysis. By doing so, only the most distinct species and genera can be selected for bioassays. The third approach relies on comparative molecular analyses of bacterial community structure from healthy and diseased plant samples. Quantitative associations between the relative abundance of markers and the relative health of the plant samples can allow for identification of candidate biocontrol markers. Those markers can then be used to develop selective isolation protocols that are used to recover a much-reduced set of likely active bacteria.

60.2   APPROACH 1: MARKER-ASSISTED SELECTION USING FUNCTIONAL GENE MARKERS

Mechanistic studies have led to the identification of different genes and pathways involved in the biological control of plant pathogens by bacteria. Sequence analyses of those genes can be used to identify gene-specific primers with a relatively high degree of specificity for the target in a polymerase chain reaction (PCR)-based assay. Because sequence variation in functional genes has been previously associated with other genotypic and phenotypic variation at the subspecies level in bacteria, the use of functional gene probes can be used to rapidly identify bacteria of interest. Such an approach can be implemented using colony hybridization, fluorescence-activated cell sorting, or PCR. Because of the ease and efficiency of the latter, we describe marker-assisted selection of biocontrol bacteria using a PCR-based assay targeting a gene known to function in the biological control of plant pathogens.

This PCR-based approach to select novel biocontrol bacteria was first used for characterizing 2,4-diacetylphloroglucinol (2,4-DAPG)-producing *Pseudomonas* populations based on the amplification of *phlD* gene sequences (see Chapter 56). It has been used to quantify the abundance of and to directly characterize the genotype of the most abundant *phlD*$^+$ populations inhabiting the rhizosphere of various crops (McSpadden Gardener and Weller, 2001; McSpadden Gardener et al., 2005). The method has been applied to the screening and selection of *Bacillus* as well (Joshi and McSpadden Gardener, 2005) and it can be readily adapted to any other biocontrol bacteria for which a functional biocontrol gene target and semiselective culturing conditions can be applied. The approach was a significant improvement over

the colony hybridization protocol which was later shown to be both less sensitive and more costly (Landa et al., 2002). The use of multiwell plates and a multipipettor for preparing dilutions and PCR reactions can allow up to 20 times as many samples being processed per unit effort when compared with the colony hybridization procedure. By taking advantage of the correlation between the *phlD* restriction fragment length polymorphism (RFLP) pattern and genomic fingerprint (Mavrodi et al., 2001), one can directly characterize the most abundant *phlD*$^+$ populations without isolating individual colonies. RFLP data indicated that a combination of just two or three digests would directly and rapidly identify the genotype as previously defined by BOX-PCR (McSpadden Gardener et al., 2000). Because the PCR-based assay detects *phlD*$^+$ pseudomonads in mixtures, we were able to substantially reduce the effort of collecting bacteria. By targeting small subsets of samples with unique RFLP patterns of the amplified *phlD* gene, it was shown that the marker-assisted PCR-based assay could rapidly lead to the discovery and isolation of novel variants of DAPG-producing bacteria (McSpadden Gardener et al., 2005).

Similar to all methods for studying the microbial ecology of natural environments, the PCR-based assay described has its limitations (Pepper, 1997). First, the described assay relies on the growth of *phlD*$^+$ bacteria on 1/3x KMB^{+++} media that is selective for pseudomonads. While detection from rhizosphere washes was possible, the theoretical limit of detection was approximately log 5.6 cells per rhizosphere, too high to be considered generally useful for studying population dynamics (McSpadden Gardener et al., 2001). Nonetheless, direct detection at log 5 *phlD*$^+$ cells per gram of rhizosphere may still be useful because such a large number of bacterial cells is actually required to achieve measurable levels of disease suppression, as was first noted in the take-all system (Raaijmakers and Weller, 1998). Another limitation of the assay is that it cannot directly address the relative activity of the *phl* biosynthetic locus or the cells themselves in the populations characterized. To determine what fraction of the uncultured *phlD*$^+$ cells might be metabolically active, it might be useful to combine the assay described above with a bromodeoxyuridine-labeling protocol (Borneman, 1999). And, while it was reported that the amount of 2,4-DAPG produced in the rhizosphere was proportional to the abundance of *phlD*$^+$ strains in wheat rhizospheres grown under controlled conditions (Raaijmakers et al., 1999), it seems likely that the amount of antibiotic produced in any given rhizosphere will depend on a variety of environmental factors present in that environment (e.g., moisture, temperature, pH, and/or the presence of antagonistic or synergistic microorganisms). Production of 2,4-DAPG *in vitro* has been reported to

vary among strains (Sharifi-Tehrani et al., 1998), so the level of disease protection may differ depending on the genotype of the most abundant $phlD^+$ strain present in the rhizosphere. Additionally, the correlation between population size and disease suppressiveness might not hold in some cases, that is when the biosynthetic operon containing the $phlD$ sequences detected is incomplete or nonfunctional. However, we assert that such a loss of function will likely be the exception, not the rule.

The use of functional gene markers has also revealed much about the ecology of biocontrol bacteria. On wheat, these bacteria increase in abundance in response to root infection, a response that alters rhizosphere community structure in ways that may ultimately impact crop health (McSpadden Gardener and Weller, 2001). On corn and soybeans grown in Ohio, native populations were frequently observed to exceed levels required for *in situ* pathogen suppression (McSpadden Gardener et al., 2005). Representative isolates of the most abundant genotypes were recovered and shown to be antagonistic to multiple soil-borne pathogens of corn and soybeans. Analyses of multiple field experiments indicated that the incidence and relative abundance of root-colonizing $phlD^+$ *Pseudomonas* spp. were influenced by crop rotation, tillage, organic amendments, and chemical seed treatments in subtle but reproducible ways (Rotenberg et al., 2007). Soil pH was negatively correlated to rhizosphere abundance of $phlD$ pseudomonads in no-till and nonamended soils, with the exception of the continuous corn treatments, indicating that biotic and abiotic controlling factors played significant roles in determining population activity. Interestingly, under low pH conditions inoculation seemed to have a peculiarly beneficial effect related to nutrient uptake (Raudales et al., 2009). Corn seeds inoculated with the strain Wood1R, a strain representative of the dominant $phlD^+$ pseudomonads found in Ohio, reduced the number of stress-induced lesions on foliage when grown on soils of low pH. In addition, tissue concentrations of P and/or Mn were significantly diminished in the plants that had been inoculated with Wood1R. Additionally, chemical seed treatments intended to control fungal pathogens and insect pests on corn also led to more abundant populations of $phlD$ in different tilled soils. Within a cropping sequence treatment, correlations between the relative abundance of $phlD$ and crop stand or yield were generally positive in corn plots and the strength of those correlations were greater in plots experiencing more root disease pressure (Rotenberg et al., 2007). Such observations were made possible by the power of PCR assays targeting the $phlD$ gene. Similar assays could provide a richer evaluation of the diversity of biocontrol bacteria and the subset of genes that significantly impact on plant health if only they were more widely applied.

60.3 APPROACH 2: MARKER-ASSISTED SELECTION USING NEUTRAL GENE MARKERS

In order to more fully screen, and subsequently exploit, the diversity of plant-associated bacteria, we can also start with a large and diverse collection of cultured bacteria and screen for activity using a tiered bioassay system. Such an approach has been the foundation of most efforts to recover and identify bacterial biocontrol agents for use as microbial inoculants (Desai et al., 2002; Fravel, 2005). However, it has only recently been that marker-assisted selection of subsets of large bacterial strain collections has been used to reduce the amount of bioassay effort required to identify useful lead candidates for microbial biopesticide development. Specifically, we screened a set of three different collections of bacteria for genotypic diversity using variation detected in the 16S genes. A multifactor selection approach was used to enhance the diversity of bacterial collections recovered from the phyllosphere and rhizosphere of plants and then screened to remove genotypic redundancy to identify subsets of bacteria of the greatest possible diversity.

The genotypic diversity of each collection was assessed by amplified ribosomal DNA restriction analysis (ARDRA; see Chapter 9) and the occurrence of different banding patterns was noted. Single-enzyme ARDRA using *Msp*I led to the identification of approximately 100 distinct genotypes for every 500–1000 isolates examined. Statistical analyses indicated that all three bacterial collections were skewed in their distribution of genotypes. The five most common genotypes represented approximately 40% of all isolates examined. In contrast, only about 15% of isolates represented singlets or doublet isolations in the collections. Because we were interested in identifying novel bacteria, the use of this simple genotyping step helped to focus our bioprospecting efforts, with selection and subsequent bioassay efforts focused on the different genotypes.

To improve the efficiency of recovering novel biocontrol strains, we chose to screen only 419 bacterial isolates, or only about 13% of our entire collection, *in planta* based primarily on genotypic differentiation. These included 129 phyllosphere isolates representing 80 different genotypes from the collection and 82 rhizosphere isolates representing another 67 genotypes. From a primary screen targeting 6 different plant diseases, 48 isolates displaying the greatest biopesticidal potential were retested and also assayed for their ability to induce system resistance to disease caused by a phytobacterial pathogen. Among the top 20 isolates, nearly half of the strains performed as well or better than a commercial biopesticide on one or more bioassays, with overall performance scores for the unformulated cultures being between 50% and 75% of the formulated

product. Interestingly, the genotypes of approximately half of the top performing isolates occurred at a frequency of less than 1% in our collections. Remarkably, these 14 isolates represented 10 different bacterial genera, some of which have been only rarely noted for their biocontrol activities, for example, *Curtobacterium*, *Exiguobacterium*, and *Chryseobacterium* (Rodriguez-Diaz et al., 2008). To date, there have been no commercial biofungicide products developed containing several of the identified genera (Copping, 2005). These data provide a clear indication that a marker-assisted, multifactorial sampling and selection strategy can be used to improve the efficiency of bioprospecting efforts.

60.4   APPROACH 3: MARKER-ASSISTED SELECTION USING MARKERS DERIVED FROM COMMUNITY ANALYSES

Microbial community profiling of samples differing in a specific ecological function, such as, soil-borne plant disease suppression, can be used to mark, recover, and ultimately identify the bacteria responsible for that function. The microbial basis for plant-pathogen suppression has been well established (Baker, 1987), and the components of suppressiveness have been described for multiple pathosystems (Hoitink and Boehm, 1999), especially for those involving a specific pathogen and microbial antagonist (Weller et al., 2002; Borneman and Becker, 2007). The use of community analyses for the identification of biocontrol agents involved in specific disease suppression has been previously reviewed (Borneman and Becker, 2007). And while such approaches can be applied to all sorts of functional gradients (Borneman et al., 2007), uncovering the majority of microorganisms associated with general soil-borne disease suppression remains challenging. Anyone wishing to find success with this approach must recognize the sampling and statistical challenges associated with the diversity and complexity of microbial communities on individual plants and handfuls of soil.

While many different profiling approaches exist, they vary in their relative cost of use. Because microbial communities of soils and rhizospheres are remarkably diverse, sample-to-sample variation can be remarkably high. Because of this, large numbers of independent samples must be assayed separately, and statistical analyses have to be applied to relatively large data sets to draw meaningful associations between population structure and activities. Metagenomics has offered much promise (Leveau, 2007) but, until very recently, per sample costs of such an approach have precluded useful applications of high-throughput sequencing to the quantitative

identification of markers associated with plant disease suppression. The PhyloChip and GeoChip have been used to identify variations in community structure; however, no subsequent use of the identified markers to isolate and validate the functionality of the associated bacterial populations has been described in the literature. In contrast, two low-cost and relatively low-tech approaches to community profiling have been successfully applied to the identification and marker-assisted selection of biocontrol microorganisms. The first successful application involved the application of oligonucleotide fingerprinting of ribosomal genes (OFRG) to the identification (Yin et al., 2003) and ultimate recovery of fungi associated with sugar beet cyst nematode suppression (Olatinwo et al., 2006). The second successful application involved the use of terminal restriction fragment length polymorphism (TRFLP; see Chapter 9) analyses to identify (Benítez et al., 2007) and ultimately recover (Benitez and McSpadden Gardener, 2009) two new species of biocontrol bacteria. Details of the latter approach are described here as a road map to others wishing to pursue this avenue of marker-assisted selection of biocontrol bacteria.

Initially, a series of soils that differed in their capacity to suppress endogenous and introduced oomycetes pathogens that caused damping-off of tomatoes and soybeans were identified (Baysal et al., 2008). The suppressiveness had been induced by mixed hay cropping and was shown to be durable, occurring over varying greenhouse and field conditions. The bacterial population structure in bulk soil and the rhizosphere of both crops was then characterized using TRFLP analyses of amplified 16S rDNA sequences (Benítez et al., 2007). Principal component analysis (PCA) revealed changes in the relative abundance of bacterial terminal restriction fragments (TRF) in response to transition strategy and/or compost amendment in eight different experimental contexts. In each context, a different subset of TRF substantially contributed to the variation along the first two principal components. However, terminal restriction fragment M148 contributed significantly to the observed variation in six out of the eight experiments. Nonparametric analyses of variance revealed that the relative abundance of TRF differed among treatments. While the responsive subsets identified varied somewhat by experimental context, M137, M139, and M141 were more abundant in samples from the mixed hay transition strategy in multiple experimental contexts. Subsequent correlation analyses revealed that TRF associated with disease suppressive treatments were frequently negatively correlated with damping-off disease incidence. As a group, these TRF were disproportionately associated with lower disease levels further indicating their role in disease suppression, although the proportional differences were not particularly extreme. Subsequent *in silico* analysis of the bacterial 16S rDNA

sequence database revealed that the TRF identified in the 2007 study (e.g., M137, M139, M141, and M148) might correspond to well-characterized genera of bacterial biological control agents. However, the size of the TRF did not provide conclusive evidence for the presence of the bacterial species noted. From that work, it was hypothesized that the bacteria giving rise to the TRF associated to damping-off suppression were generally able to contribute to the observed suppression and that general soil-borne disease suppression was at work in the studied system.

Subsequently, TRFLP data were used to rapidly identify and direct the isolation of novel and ecologically important microbial populations (Benitez and McSpadden Gardener, 2009). This was achieved by using sequence analyses of the cloned TRF to design a directed isolation strategy to recover the bacteria giving rise to those TRF. This included the use of LM media that had not been used previously for the isolation of biocontrol bacteria, but had been used to isolate the type strain of *Mitsuaria* from ocean samples. (Note: This observation also justifies modification of culturing strategies to increase the diversity of potential candidate strains as noted earlier in Section 60.3.) Ultimately, two novel groups of rhizosphere bacteria were recovered using the molecular markers previously associated with the ecologically important function of soil-borne plant disease suppression. The functionality of these strains was further evidenced by the pathogen-suppressing activities expressed by all of our isolates *in vitro* and *in situ*. All of the novel *Mitsuaria* and *Burkholderia* isolates obtained were observed to be active against multiple soil-borne pathogens on different crops. This contrasts with traditional random-screens that typically show fewer than 10% of isolates to be active against any single pathogen and even fewer with activity against multiple pathogens. For example, in our laboratory's screen of *Bacillus* isolates found fewer than 1% of isolates to be active against a similar spectrum of pathogens *in vitro* (Joshi and McSpadden Gardener, 2005). The efficient recovery of active strains was not surprising when one considers that the correlative evidence for the involvement of M139- and M141-marked bacteria in disease suppression was generated in multiple experimental contexts with varying levels of disease pressure (Benítez et al., 2007, Baysal et al., 2008). The general utility of these new bacterial species as biocontrol inoculants, in different soils and on different crops, is currently under investigation.

60.5 CONCLUSION

It is important to not only describe differences in microbial communities between soils with different levels of disease suppression, but also to identify the microorganisms most likely involved in this phenomenon (Janvier et al., 2007).

The novel bacteria identified using the marker-assisted approaches described above represented just a small percentage of the total soil- and root-inhabiting bacteria. For example, DAPG-producing *Pseudomonas* spp. typically represent between 0.1% and 1% of the bacteria cultured on semiselective media (McSpadden Gardener et al., 2001; McSpadden Gardener et al., 2005). And, based on our TRF data, the relative abundance rarely exceeded 1–10% of the total 16S signal (Benítez et al., 2007), a pattern reinforced by the low frequency of recovery of the TRF-marked *Mitsuaria* and *Burkholderia* bacteria identified in the follow up (Benitez and McSpadden Gardener, 2009). While it may be surprising that such a small percentage of the plant-associated bacterial community can have a significant functional impact on soil-borne diseases, past research has correlated similarly small percentages of native and introduced populations of DAPG-producing pseudomonads with significantly higher stands and yields of crops (Raaijmakers and Weller, 1998). Given the environmentally driven variation in soil- and plant-associated bacteria (Garbeva et al., 2004), the identity, distribution, and relative abundance of pathogen-suppressing populations in different environmental contexts will be difficult to assess. Thus, the marker-assisted approaches described above will likely be limited to identifying a limited, but ever growing, portion of the diverse pathogen-suppressing antagonists present in any given set of samples. Only through iterative application of such methods in multiple systems will the full range of pathogen-suppressing bacteria be selected for testing as microbial biocontrol agents.

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