Use of RFLP and T-RFLP for Characterizing Microbial Populations and Communities

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Importance of characterizing microbial populations and communities

- Fundamental exploration and comparison of diversity among different systems
- Association of specific populations with biotic and abiotic variables and functions
- Characterization of disturbances
- Testing ecological and evolutionary concepts
Why use RFLP and T-RFLP?

- RFLP: restriction fragment length polymorphism
- T-RFLP: terminal RFLP

- Complexity of microbial communities necessitates analyses of multiple samples with multiple populations

- Restriction analyses provide rapid screening of population or community structure through sequence diversity

- Screening can be adjusted to different levels of phylogenetic specificity

- Low cost and deterministic
Disadvantages:

- Low resolution of sequence diversity
- Highly complex communities result in TRFs from different taxa contributing to each distinct size class
Analytical trade-offs in profiling complex populations

- Domain-specific primers provide greater overview
- Genera- and species-specific primer sets provide greater discrimination
T-RFLP analyses

- Use one labeled and one unlabeled primer
- Amplify target sequences from mixed population
- Digest products with a restriction enzyme
- Separate DNA on polyacrylamide gels
- Size labeled terminal restriction fragments
- Compare peak areas of specific TRFs
- Determine source of TRFs/sequences
Restriction Analyses of Mixed Populations: Three Examples

1. Differences in bacterial populations in the phyllosphere of corn and soybean

2. *Bacillus* population profiling

3. Profiling of phlD producing *Pseudomonas* on Ohio fields and relationship to plant disease suppression
1. Phyllosphere case study

- Sample comparisons
  - Corn (C) vs. soybean (S)
  - New (N) vs. older (O) leaves

- Processing
  - DNA isolated from 0.5 g fresh weight leaf material
  - 16S sequences amplified with two different primer sets
  - Processed for FT-ARDRA
Generating sample profiles

Separation and identification of TRFs through automated sequencing systems. Each TRF is fluorescently tagged, and fragment size is compared to a standard.
Individual T-RFLP profiles are organized and aligned for further comparison and delimitation of regions of interest for analysis. For each profile, the x-axis indicates fragment size (base pairs) and the y-axis the intensity of the fluorophore which is proportional to TRF abundance.
Defining size limitations...

Regions of interest for analysis are selected based on fragment size. A specific size range can be selected, based on available information of the study group.
...and ranges for analyses
Defining OTUs

Individual peaks are identified and defined as operational taxonomic units (OTUs). The abundance of each OTU is recorded for each sample for further statistical analyses.
Quantify observations

- Generate data matrix for each sample
  - Named OTUs
  - Abundance of each

- Perform statistical analyses
  - Comparisons:
    - Number and abundance of OTUs (diversity of OTUs)
    - Correlate signals to other phenomena
  - Classifications:
    - Whole profile assessments
Comparison of TRF profiles obtained from old and new corn and soybean leaves. Profiles show differences in abundance of *Pseudomonas* in the phyllosphere. Samples were digested with *MspI*. 
Sample results: *Pseudomonas* profiles

Table 1. Significant differences in FT-ARDRA profiles of Pseudomonad populations inhabiting leaves. Leaf samples were taken 3 weeks post emergence from soybean (S) and corn (C). The newest (N) and oldest (O) leaves were sampled at the same time from replicate plants (n = 6) of each crop grown in a single 1 ha field. 16S sequences were amplified from leaf DNA using Psfor and Psrev. Amplification products were digested with MspI and separated under denaturing conditions. The size and abundance of HEX-labeled terminal restriction fragments were determined with Genescan 3.0. Terminal restriction fragments (TRFs) appearing in at least four independent replicates of at least one treatment were used for comparisons. The peak areas of defined TRFs were compared using Dunn’s procedure. Major signals are noted in **bold**. Treatments with significantly different (P < 0.05) amounts of amplified TRFs of a given size are distinguished by different letters.

<table>
<thead>
<tr>
<th>TRF (bp)</th>
<th>126</th>
<th>154</th>
<th>190</th>
<th>211</th>
<th>215</th>
</tr>
</thead>
<tbody>
<tr>
<td>S N</td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td>S O</td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>d</td>
</tr>
<tr>
<td>C N</td>
<td>b</td>
<td>a¹</td>
<td>a¹</td>
<td>a¹</td>
<td>a</td>
</tr>
<tr>
<td>C O</td>
<td>b</td>
<td>a</td>
<td>ab</td>
<td>a</td>
<td>b</td>
</tr>
</tbody>
</table>

¹The specified TRF was not detected in these samples.
2. *Bacillus* spp.

- Genome, biochemistry, and development of model strains well characterized
- Diverse species and subspecies varying in their biocontrol properties
- Isolates of several species identified with capacity to suppress plant pathogenic nematodes
- Most-widely marketed biocontrol bacteria
- Little known about ecology of native populations in agricultural systems
Bacillus-specific primers

Sequence alignment of Bacillus and closely related species. Red horizontal arrows indicate region corresponding to Bacillus-specific primers. Yellow box, as well as red and blue vertical arrows indicate variations in sequence.
Profile different *Bacillus* species from soil and root samples of two corn fields. 16S sequences were amplified using *Bacillus*-specific primers, and digested with *MspI*. Sizes of the TRFs, in bp, are shown on the x-axis. The relative abundance of TRFs, determined by fluorescence intensity is indicated on the y-axis.
Evaluation of TRF profiles

Magnified region of profiles of *Bacillus* species present in soil and root samples taken from adjacent fields. Black arrows indicate TRFs predicted for known species.
Bacillus population profiles summary

- Approximately 10 distinct ribotypes visible with a single digest

- Large majority of fluorescent signal corresponds to the targeted genera

- Significant variation in population structure is observed across microenvironment but not soil type nor crop
3. DAPG-producing *Pseudomonas* spp.

- DAPG is toxic to diverse plant pathogens and may induce plant defenses
- Several well-studied biocontrol strains are known to produce DAPG
- Production of DAPG *in situ* correlates with root disease suppression on wheat
- Diverse genotypes of DAPG producers known to exist
- Populations high in some disease suppressive soils

![2,4-diacetylphloroglucinol (DAPG)]
Profiling DAPG-producers
McSpadden Gardener (2001) Phytopathology 91:44-54

Sample plants
Dislodge, dilute, and culture *Pseudomonas* spp.
Amplify *phlD*
Calculate population size
Digest amplified product from terminal dilutions
Identify genotypes
Isolate representative strains for more research and development
Populations of DAPG-producing *Pseudomonas* were studied in corn and soybean fields across Ohio.
Isolation and Characterization

- Representative strains of each genotype recovered from multiple sites
- All displayed capacity to produce DAPG and colonize corn and soybean roots
- \textit{In vitro} inhibition of pathogens varied by genotype and with media composition
## Distribution of DAPG producers

### Soybean cv. Kottman

<table>
<thead>
<tr>
<th>Year</th>
<th>Site</th>
<th>Incidence</th>
<th>Genotype Freq.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&gt;log3.4</td>
<td>&gt;log4.5</td>
</tr>
<tr>
<td>2001</td>
<td>Henry</td>
<td>1.0</td>
<td>0.75</td>
</tr>
<tr>
<td>2002</td>
<td>Henry</td>
<td>1.0</td>
<td>0.37</td>
</tr>
<tr>
<td>2003</td>
<td>Henry</td>
<td>1.0</td>
<td>0.50</td>
</tr>
<tr>
<td>2001</td>
<td>Huron</td>
<td>0.37</td>
<td>0.12</td>
</tr>
<tr>
<td>2002</td>
<td>Huron</td>
<td>0.75</td>
<td>0.50</td>
</tr>
<tr>
<td>2003</td>
<td>Huron</td>
<td>1.0</td>
<td>0.75</td>
</tr>
<tr>
<td>2001</td>
<td>Wood</td>
<td>1.0</td>
<td>0.50</td>
</tr>
<tr>
<td>2002</td>
<td>Wood</td>
<td>0.63</td>
<td>0.25</td>
</tr>
<tr>
<td>2003</td>
<td>Wood</td>
<td>1.0</td>
<td>0.75</td>
</tr>
<tr>
<td>2001</td>
<td>Clinton</td>
<td>0.75</td>
<td>0.25</td>
</tr>
<tr>
<td>2002</td>
<td>Clinton</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>2003</td>
<td>Clinton</td>
<td>1.0</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Most plants colonized, but <50% colonized by large populations. Dominant genotypes were revealed by RFLP of marker gene, *phlD*. 

*Note: Numbers in the table represent the proportion of plants colonized by different genotypes.*
Field Trials of Wayne1R on soybean cv. Kottman

<table>
<thead>
<tr>
<th>Site</th>
<th>Change in Stand (%)</th>
<th>Change in Yield (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>‘01</td>
<td>‘02</td>
</tr>
<tr>
<td>1</td>
<td>+10</td>
<td>+4</td>
</tr>
<tr>
<td>2</td>
<td>+22</td>
<td>+6</td>
</tr>
<tr>
<td>3</td>
<td>-6</td>
<td>+11</td>
</tr>
</tbody>
</table>

- Observed increases in stand (5%, P<0.05)
- Increased yields overall (71kg/ha, P<0.05)
- Greatest yield increases where native populations were lowest (125 kg/ha, P<0.10)
DAPG-producing *Pseudomonas*

Summary

- Native biocontrol populations present in most soils
- Rhizosphere colonization greater on corn than soybeans
- Genotypes vary on crops and with sites
- Plant yield most dramatically improved by inoculation where rhizosphere colonization by native populations is relatively low
Conclusions

- Multiple approaches to analyzing microbial diversity are available with most dependent on PCR in some way.

- RFLP and T-RFLP allow for rapid and flexible determinations of population structure, but limitations of resolution need to be considered when drawing conclusions.

- Correlations between the relative abundance of specific microbial populations and environmental variables indicate, but do not prove functional linkages.

- Targeting microbial populations with known functional importance in ecosystem, such as pathogens or biocontrol agents, reduces complexity of profiles and allows for stronger assertions of association/causality.
References


Brian McSpadden Gardener received his Ph.D. in Botany from Michigan State University in 1998. He began work in the field of biological control of plant pathogens as an ARS postdoctoral fellow under the guidance of Dr. David Weller. He joined the faculty at the Ohio State University in 2001 where he currently serves as an Assistant Professor. His research focuses on defining the diversity, population dynamics, and ecological functioning of microbial populations that colonize crop plants. Particular emphasis is given to studying *Pseudomonas* and *Bacillus* spp. with potential for development as biopesticides.
María-Soledad Benítez is a graduate student in the Department of Plant Pathology at The Ohio State University. She is studying the relationships between suppressiveness and *phlD* producing *Pseudomonas* spp. in organic farming systems. She obtained her undergraduate degree of Biological Sciences at the Universidad Católica in Quito-Ecuador. Her interests are in microbial ecology, specifically in relation to changes in microbial population structure as it relates to the functioning of agricultural and natural ecosystems.
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