Investigation of molecular methods for the analysis of microbial community composition and diversity in complex environmental samples

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Introduction: Microbial ecologists are interested in analyzing species diversity and composition in natural communities for a variety of reasons. One important reason is to determine whether total species diversity is directly related to the function and stability of ecosystems, and another is to determine whether the occurrence of specific community members is related to a specific ecosystem function.

Unfortunately, microbial community analysis in complex environments is very difficult to achieve. Most of our knowledge of bacterial species and communities is generated from data derived from culture techniques, but less than 1 to 10% of total bacteria in natural samples are culturable (Hugenholtz and Pace, 1996). Extraction of total DNA and amplification by the polymerase chain reaction, in conjunction with analytical methods such as denaturing gradient gel electrophoresis (DGGE) (Ferris et al, 1996), checkerboard hybridization (a sophisticated form of dot-blotting), in situ hybridization (Kampfer et al, 1996; Aßmus et al, 1997), and restriction fragment length polymorphism (RFLP) (Liu et al, 1997) have been utilized to describe and contrast phylogenetically-based community structure in complex environmental samples. However, all these methods are limited to some extent by the culturable species database, and all of these methods generate biased reflections of community composition. These limitations must be considered when utilizing these methods to describe and compare microbial communities. For example, all PCR-based techniques contain biases generated by the PCR process (Suzuki and Giovanni, 1996). In addition, these methods must be optimized for the system being investigated. To learn more about the utility of these techniques, I employed several of these methods to analyze two types of environmental samples: Spartina alterniflora roots grown in sludge-amended and unamended sediments (for information about the sludge-amended plots, see Bohannon/Sobczak report), and several pink microbial aggregates, all collected from the Great Sippewissett salt marsh.

Materials and methods:
Plant tissue collection and preparation: Spartina alterniflora plants were sampled from sludge-amended and unamended plots at the Great Sippewissett salt marsh on two dates approximately one week apart. Small clumps of plants were removed (soil, roots, stems) with a shovel, placed into buckets, and returned to the laboratory for analysis. Roots were carefully removed from soil
with sterile tweezers and scissors and soil particles were removed by gentle agitation in 75% sterile seawater. Roots from the entire plant clump were bulked into a single sample.

**Microbial aggregate collection:** Aggregates were collected from microbial mats and tidal pools at the Sippewisset salt marsh in Falmouth, MA. Samples were collected in sterile tubes from 1) pink aggregates found in cyanobacterial mats near the bridge, 2) intense filamentous pink aggregations in a very small pool near mats at the beach, 3) large pink aggregations in the stream sand, 4) pink putatively faecal "worms" from a tidal pool near the beach, and 5) a pink microbial "berry" from a tidal pool.

**DNA extraction:** DNA was extracted with a protocol modified from the DNA lysis procedures described in the course handout. Root tissue was frozen in liquid nitrogen and ground with 180 ul of sterile 0.01 mM Tris buffer, pH 7.6, in a sterile mortar and pestle. Aggregate samples were suspended in buffer, vortexed, and spun down at 3,000 x g for 1 min. Pink masses were then collected from the top of the solids and resuspended in buffer. For bacterial isolates, single colonies were suspended in buffer. A spatula full of glass beads, 100 ul of phenol and 100 ul of suspended sample were added to a bead beater tube and bead-beat for 3 mins. Samples were cleaned with 2 phenol/chloroform extractions and one chloroform extraction. DNA was precipitated on ice after the addition of 0.1 volume Na Acetate and 1.0 volume 2-propanol, centrifuged for 5 min. at 13,000 x g, and dried.

**Community analysis:** Extracted DNA was amplified for checkerboard, DGGE, RFLP (single enzyme digestion with HinP1), and sequencing analysis (see course handouts).

**PCR protocols:** 16S rDNA was amplified with the following reaction mix: 5 μl 10X buffer, 6 μl MgCl₂ (25 mM stock), 4 μl dNTP mix (25 um stock), 1 μl universal forward, 1 μl universal reverse, 32 μl dH₂O, and a bead of Promega TaqBead polymerase. PCR was run at 94°C for 5 min, 30 cycles of 94°C (45 sec)/53°C (45 sec)/72°C (90 sec), and 4°C thereafter. PCR products were then loaded onto 1% agarose gels with ethidium bromide in 1X Tris Borate EDTA buffer at 100 mV.

NitH DNA was amplified using the above reaction mix but with nitH primers instead of universal 16S primers. The primer sequences were GCIWYTAYGGAARGGIGG (19F) and AAICRCRCAIACRRT (407R). R represents A or G, I represents inosine, W represents A or T, and Y represents C or T (Ueda et al, 1995). The reaction conditions were 40 cycles of 95°C (30 secs), 50°C (1 min), and 72°C (30 sec) (Ueda et al, 1995).
**Microbial isolations:** Root tissues were surface-sterilized by dipping in 95% ethanol and flaming (Dong et al, 1994). Sterilized roots were then homogenized in a sterile mortar and pestle with sterile 75% sea water and plated on two media: A) solid agar composed of 0.05% yeast extract, 1% carbon source (dextrose, carboxymethylcellulose (CMC), starch and casitone), 75% sea water, and 1.5% agar. B) semi-solid media consisting of 0.2 g K2HPO4, 0.6 g KH2PO4, 0.002 g Na2MoO4·2H2O, 0.01 g FeCl3·6H2O, 1.8 g agar, 1% carbon source (citrate, dextrose, starch and CMC), and 75% seawater for the isolation of nitrogen-fixing bacteria (Cavalcanente and Dobereiner, 1988; Palus et al, 1996).

**Root tissue preparation for scanning confocal laser microscopy:** Roots were cut into 4-6 mm sections and sent to the Marine Research Center, fixed with formalin and ethanol, mounted in paraffin, sectioned into 6 μm and 15 μm sections, mounted on positively-charged slides, and probed with rhodamine-labelled universal probe PrA (Nierzwicki-Bauer protocol, 1997). Slides were examined by fluorescence and scanning confocal laser microscopy.

**Results and discussion:**

**Single-enzyme RFLP analysis of 16S rDNA extracted from roots.**

1. The root-associated microbial communities of sludge-amended and unamended plots were diverse. RFLP patterns of one unamended and two sludge-amended samples are presented in Figure 1. The first lane in the top section represents a 100bp ladder, lanes 2-9 represent 16S rRNA clones from roots harvested from unamended sediments, and lanes 10-17 represent 16S rDNA clones from roots collected from sludge-amended sediments. In the bottom section, lane 1 represents a 100 bp ladder, and lanes 2 and 5-8 represent clones from a second root sample collected from a sludge-amended sediment. Of 20 clones from the three samples, eleven or twelve appeared as distinct RFLP patterns. There were 7 distinct patterns from 8 clones in the roots harvested from the unamended sediment, and 4 distinct patterns from 8 clones in the roots harvested from the sludge-amended plots. One RFLP pattern appeared five times in eight clones in the sludge treatment. However, in the second sludge treatment sample, there were four distinct patterns in five clones (fewer clones were generated because tops popped off PCR tubes during amplification!). It would be necessary to collect many more samples per treatment and restrict many more clones per sample to generate a realistic picture of the diversity and evenness of the microbial community in the two sediment types. Furthermore, even after exhaustive cloning, restriction analysis and sequencing, the proportion of the community described by this analysis would remain unknown. However, cloning/RFLP analysis might be the most appropriate of the methods described in this paper to estimate community diversity in complex environments.
2. Root-associated organisms containing nifH genes were present in pink aggregates and root samples. NifH amplification products of roots harvested from sludge-amended (gel samples 8 and 17) and unamended sediments (gel samples 6 and 7), as well as pink aggregates 1 and 2 (gel samples 1 and 2, respectively) (Fig. 2a), sludge-amended and unamended sediments are presented in Fig. 2a. The nifH genes were also diverse. RFLP analysis of clones generated from the amplified nifH genes is presented in Fig. 2b. Lane 1 is a 100bp ladder, lanes 2-4 are clones from roots collected from unamended sediments, and 6-10 represent clones from root collected from sludge-amended sediments. While cloning of the nifH genes into E. coli was only marginally successful (only 33-50% of the clonal colonies resulted in amplification products), there were at least 4 distinct patterns from 8 amplified clones. The 390 bp nifH gene of three clones was not cleaved by the HinP enzyme; these clones are likely the same organism but they must be restricted with a second enzyme and/or sequenced. As explained above, it would be necessary to collect many more samples per treatment and restrict many more clones per sample to generate a realistic picture of the diversity and evenness of the PCR-amplifiable nifH-containing microbial community in the two sediment types.

NifH genes were also amplified from two pink microbial aggregates (pink aggregates from a mat and filamentous pink masses from near the mats). This is not surprising, as nitrogen fixation is the largest source of nitrogen supporting primary productivity in mat environments (Paerl et al., 1995). Cyanobacterial probes were not lit up by these same samples in the RCH grid (see below). This is also not surprising, however, as a wide variety of non-cyanobacterial mat-inhabiting bacteria are capable of nitrogen fixation, including photosynthetic, chemolithotrophic, and heterotrophic bacteria (Chlorobium, Thiobacillus, Azospirillum, Klebsiella, Clostridium)(Paerl et al., 1995). In addition, the accuracy of the probes in the RCH analysis has not been thoroughly tested. While I cloned these samples, I did not have time to complete RFLP analysis of the clones. It would be interesting to compare the RFLP patterns of the two pink aggregate samples and the root samples.

**Denaturing gradient gel electrophoresis (DGGE)**

A DGGE gel of two “berries” and isolates from pink aggregates are shown in Fig. 3. The DGGE gel generated different band patterns for the two microbial “berries” (lanes 12 and 13). DGGE data also indicated that isolates from tidal pool sediments 14-16) may not be prominent members of the berry community. Interestingly, of four clones generated from the “berry” (lanes 8-11), one clone generated from the “berry” did not appear in the “berry” DGGE band pattern (lane 11). This might be an indication of PCR bias, or that community members of low abundance may not show up in DGGE analysis. Therefore, absence of DGGE bands in a particular position does not definitively indicate that an organism whose DNA denatures at that position is not present in the community. Furthermore, this indicates that even in a relatively simple microbial community
such as the berry, cloning/sequencing and DGGE analyses generate different community compositional data.

No bands were generated in the root tissue DGGE analysis, lanes 17 and 18 (Fig. 3). One problem associated with the DGGE method is the relatively poor amplification of environmental DNA by the DGGE primer, which carries a 40 base GC tail. Poor amplification compared to amplification by other primers (DIG- and cloning-universals) was reported also for “berry” and cockroach gut samples. Other students amplified DNA several times, bulked samples, and concentrated the DNA into a single more concentrated sample. In this way they were able to generate a DGGE band signal, although for the cockroach gut samples the resulting DGGE bands remained a smear, and in sediment samples they generated a single band which clearly does not approximate the composition of the sample. This problem remains unresolved. In addition, Elke Jaspers has never been able to generate more than 12 bands from an aquatic community which is known to contain at least 40 different type of organisms. According to many users, the method is difficult to operate and achieve accuracy and reproducibility. There are many questions which remain unresolved about DGGE analysis.

Reverse checkerboard hybridization (RCH)

RCH analyses of roots and root isolates, and microbial aggregates are shown in Figs. 4a and b, respectively. In Fig. 4a, lanes 10-13 represent root samples. Only a single root sample from a sludge-amended sediment was successfully hybridized (lane 13). Lanes 14-18 represent root isolates 1-5. Again, there was no hybridization with the universal probes for root isolate 2. The lack of hybridization is due to deterioration of PCR products during storage. I have not been able to explain this phenomenon. I also tried several times to reamplify the original DNA to replace the deteriorated samples but with varying success. Checkerboard analysis of root community DNA and root isolates revealed little about community structure, as the probes in the grid were not optimized for the root system.

RCH did, however, generate useful information about the bacterial groups and species present in microbial aggregates most likely because the “berry” is a relatively simple system and the method was nominally optimized for that system. In Fig. 4b, lanes 27 and 28 represent microbial aggregates 1 and 2, respectively, while eight different “berries” are represented in lanes 32-39. Another student in the class had extracted DNA and generated clones from the microbial “berry” aggregate. He then both designed probes and selected probes which should identify “berry” community members, and these probes were used for the analysis of both his “berries” and my aggregate samples. As my aggregate samples were collected from environments similar to those of the “berries”, the probes selected for “berry” community analysis also hybridized successfully with my environmental DNA. I was able to identify several bacterial groups and even species in my two aggregate samples, and compare community composition of my aggregates with the composition of the berries. The Vibrio
spp. probe originally cultured from the "berry" did not appear in the "berry" by RCH analysis, but hybridized successfully with DNA extracted from the microbial mat pink aggregate. Probes specific for sulfate-reducing bacteria hybridized successfully with "berry" DNA but not with my aggregate-extracted DNA.

RCH requires probe development and optimization to generate useful information. Another potential problem with RCH is the requirement that all probes be optimized for the same hybridization conditions, which may result in less-than-optimal hybridization conditions for some probes. Dot blotting might be required for some probes so hybridization could be optimized under different conditions. In addition, controls must be run for each probe utilized in the grid to ensure that each probe is specific to the group or population it is designed to identify. PCR bias is unavoidable with checkerboard analysis. Dot-blotting of total extracted DNA would avoid this bias and would also permit more quantitative assessment of probed groups. However, very large quantities of extracted DNA might be required to generate signal for bacterial species of low density. Nonetheless, some form of DNA probing system is likely to be the best candidate for comprehensive studies of community composition in changing, complex environments.

In-situ hybridization

No information was generated by in-situ hybridization of plant tissues. Paraffin was used to stabilize plant tissues for microtome sectioning. Unfortunately, paraffin and plant tissues autofluoresce in the same regions as the rhodamine probe, so no bacteria could be visualized by rhodamine probes. Autofluorescence is observed in root tissues regardless of the filter type used on the fluorescence microscope. Autofluorescence was also observed by students working with microbial aggregates and termite intestinal tissues embedded in paraffin. Probes may become sorbed into the paraffin, creating fluorescence throughout tissues embedded in paraffin. This problem might be overcome by the use of a different embedding material or direct fixation of the plant material on the slide (McNaughton et al, 1996; Aßmus et al, 1997), or by more sophisticated use of the MetaMorph image processing software (see MetaMorph handout, Cold Spring Harbor course), but there was no time to work this out during this course. I have found no papers that have successfully probed bacteria in plant tissues containing chlorophyll, which also emits considerable autofluorescence. In situ hybridization should, however, be a very powerful tool for localising specific organisms in complex environments and for determining the proportion of specific organisms or groups in total bacterial communities.

Bacterial isolations: Four distinct bacterial isolates (as determined by colony morphologies) were cultured from surface-sterilized roots harvested from sludge-amended and unamended treatments on solid media (Table 1). A single bacterial isolate was cultured from a micro-aerobic crack in the starch-amended semi-solid nitrogen-free medium inoculated with surface-sterilized roots harvested from unamended sediment (Table 1). As many nitrogen-fixing
organisms are anaerobic, more isolates may have been obtained if these plates had also been incubated anaerobically (this was not done in cited references). Gram staining and the catalase test were conducted on very old cultures, so those data should not be considered reliable. PCR products of extracted DNA were sent to Michigan State for sequencing, but the sequences returned were very poor. Two sequences generated phylogenetic identifications as Bacillus spp. If these identifications can be considered reliable, they could be either internal plant tissue inhabitants or external colonists which may have survived sterilization as spores (e.g. these Bacillus spp.).

Conclusions:

All these molecular methods for the analysis of microbial diversity and community composition are potentially very powerful, but are also easily misapplied and misinterpreted. Each method has both strengths and weaknesses, and these should be taken into consideration when choosing a method or methods to apply to a research question. None of these methods can presently adequately describe either community composition or diversity at the level required to relate either to ecosystem function. Furthermore, all methods would require many preliminary validation experiments before the method could be applied with confidence to a complex (or simple) system. These might include (depending on the method): the determination of the minimum population size required for population detection (see Boyle report), an estimation of the proportion of the total bacterial community detected by the method (for example by comparison with dot-blotting of total extractable DNA), and the determination of probe/primer specificity and hybridization condition optima. In addition, a rigorous examination of the impact of DNA extraction and PCR protocols on community analytical data (regardless of method) should be conducted. As demonstrated nicely in student reports by Banin, Wenuganen, and Hughes/Zurek, several of these methods (along with other methods not considered here) applied to the same research question can generate more robust information than any one method used alone.
Fig. 1  RFLPs of 16S rDNA clones from *Spartina alterniflora* root samples

Lanes 2-9  
Unamended

Lanes 10-17  
sludge-amended
Fig. 2a
NifH primer amplified DNA: pink aggregates and sludge- and unamended root samples
Fig. 2b  RFLPs of nifH
Spartina alterniflora root samples
Fig. 3.
DGGE gel of berries, berry clones, non-sulfur isolates and Spartina roots
Checkerboard hybridization

16S PCR products from samples

sediments, plant roots, Al's heterotrophs  artificial are boxed
Checkerboard hybridization

Berries, cyanos, sediments (see key)
<table>
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<tr>
<th>Inoculum source</th>
<th>Carbon source</th>
<th>Colony type</th>
<th>Morphology</th>
<th>Motl</th>
<th>Gram</th>
<th>Cat. test</th>
<th>16S rRNA identification</th>
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</thead>
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<tr>
<td>roots</td>
<td>casitone</td>
<td>orange, opaque</td>
<td>small coccoid rods</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Bacillus sp.</td>
</tr>
<tr>
<td>unamended roots</td>
<td>CMC</td>
<td>small, pale orange</td>
<td>coccoid rods</td>
<td>-</td>
<td>-</td>
<td>+?</td>
<td>NI</td>
</tr>
<tr>
<td>roots, sludge</td>
<td>starch</td>
<td>tiny, transparent</td>
<td>curved rods</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Bacillus sp.</td>
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<tr>
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<td>+?</td>
<td>NI</td>
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References


