FISHing for Bacteria-Eukaryote Associations
(and Other Epic Failures in Microbial Diversity)

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Introduction

Bacteria-eukaryote associations are ubiquitous and important, but many remain uncharacterized. My interest in such associations was inspired by arbuscular mycorrhizal fungi (AMF), which form symbioses with more than 80% of terrestrial plants (1). AMF are thought to influence soil stability, global nutrient cycling, and confer many benefits to plants, including pathogen resistance, enhanced nutrient uptake, and drought tolerance. There is some evidence that soil bacteria influence mycorrhizal activity, but the details of these interactions are poorly understood (2).

The aim of this project was to investigate the following questions:

1. Are bacteria-mycorrhizal associations common?
2. If so, how are bacteria spatially distributed around fungal hyphae?
3. Are some bacterial groups found more frequently than others around the hyphae?

These questions are more exploratory than hypothesis-driven, but I did expect that bacteria-mycorrhizal associations would be common near mycorrhizal plants, and that some bacterial groups would be more abundant around mycorrhizal hyphae than others. In order to pursue these questions, I conducted fluorescence *in situ* hybridization (FISH) on environmental samples and grew enrichment cultures. I hoped that FISH would allow me to simultaneously investigate microbial community composition and spatial dynamics. The enrichment cultures were intended to help identify specific hyphae-associated bacteria, and could also be used to further investigate the mechanisms responsible for bacteria-fungi interactions.

As is reported below, this project also explored the association of bacteria and archaea with protists in terrestrial and aquatic samples. Recent evidence suggests that these associations are common (3). However, like bactiera-mycorrhizal associations, bacteria-protist associations are still relatively unstudied. In this project, I used FISH and DAPI staining to search for bacteria and archaea near protists.

Materials and Methods

1. Sample Collection and Preparation

Plant root samples were collected from *Ammophila* (beachgrass) at Stoney Beach, *Lathyrus* (beach pea) near Stoney Beach, and mixed grass communities from the Swope Center lawn (Woods Hole, MA). Roots were washed gently with water to remove soil and sand. For enrichment cultures, fresh root samples were used to inoculate growth medium immediately. Root samples for FISH and DAPI were fixed with 4% paraformaldehyde at 4°C overnight. After fixation, roots were rinsed three times in PBS and stored in PBS:ethanol (1:1) at 4°C. A portion of the root samples were embedded in O.C.T. Tissue Tek and frozen at -80°C. The embedded

samples were sectioned into 20-50 µm sections with a cryomicrotome and mounted onto poly-L-lysine coated slides. Both lengthwise and cross-sectional samples were prepared.

Water, sediment, and decaying plant biomass samples were collected from Little Sippewissett Marsh (Falmouth, MA). One sample was stored as an intact microbial mat. The remaining samples were stored in glass bottles. After incubation for six weeks at room temperature, sub-samples were pelleted, resuspended in 1x PBS, and fixed with paraformaldehyde (4% final concentration) for 60 minutes at room temperature. Samples were then pelleted (at 1,500 g for 5 minutes) and rinsed three times with 1x PBS. Rinsed samples were mixed with .4% agarose at a ratio of 1:1. A drop of the sample-agarose mixture was smeared onto a glass slide and air dried at room temperature. Once dry, the agarose drops were further dehydrated using successive 5 minute ethanol baths in 50%, 75%, and 100% ethanol, and allowed to air dry at room temperature.

2. Enrichments for Microbes Associated with Roots

2.1. Enrichments on High Nutrient and Low Nutrient Agar Plates

Fresh root samples were plated onto 5YE plates (5 g yeast extract per L media) and oxalic acid plates (per L media: 10 ml 100x freshwater base, 25 mM oxalic acid, 10 mM $NaNO_3$, 1 mM $NaSO_4$, 1 mM KPO_4, 1 ml trace elements, 10 ml MES buffer at pH 5.5, final media pH adjusted to 5.8). Plates were incubated at 30°C. After 12 hours, roots were removed and plates were returned to incubation at 30°C. Growing colonies were streaked onto new plates for isolation. In preparation for FISH and DAPI staining, some plates were dissected into small slices (approximately 1 mm x 1 cm x 1 cm) and fixed with 4% paraformaldehyde overnight at 4°C and rinsed as described above.

2.2. DNA Extraction and 16S rRNA Sequencing of Isolates

DNA was extracted from colony isolates by boiling in nuclease-free water for 5 minutes. PCR was used to amplify the 16S rRNA sequences with the 8F and 1492R primers. PCR products were submitted for sequencing. Sequences were BLASTed against the NCBI database to find closest matches.

2.3. Preparation of Agarose Slides

Two concentrations of yeast extract media (.5 g yeast extract and 5 g yeast extract per L) were prepared for the construction of mini-enrichment plates on agarose slides. Small circles were cut out of foam sheets and attached to glass slides to create wells for the media. Warm media was poured into the wells. Once the media had solidified, the foam was removed, root samples were placed on top of agarose, and the slides were incubated at 30°C. After 12 hours, the roots were removed and the slides were returned to incubation at 30°C. In preparation for FISH and DAPI staining, the agarose slides were dried down using successive 5 minute ethanol baths in 50%, 75%, and 100% ethanol, and allowed to air dry at room temperature.

3. Catalyzed Reporter Deposition Fluorescence in situ Hybridization (CARD-FISH)

3.1. Permeabilization and Inactivation of Endogenous Peroxidases

Fixed roots were permeabilized inside Eppendorf tubes with lysozyme solution (10,000 units of activity per mL in .05 M EDTA, .1 M Tris-HCl, pH 8) for 60 minutes at 37°C. After

permeabilization, roots were washed in excess MilliQ water. Endogenous peroxidases were inactivated by incubating the roots in .1 M HCl for 5 minutes, followed by 3% H_2O_2 for 30 minutes. Roots were then rinsed with excess MilliQ water, rinsed with 95% ethanol, and left to air dry.

3.2. Hybridization and Washing

Individual probes were mixed with aliquots of hybridization buffer (900 mM NaCl, 20 mM Tris-HCl, 35% Formamide, .01% SDS) at a ratio of 1:300. The BET42a, EUB338-I-III, and NON338 probes were used. During most CARD FISH attempts, a probe-free control was also used to assess nonspecific binding of the tyramide. Root samples were incubated with the hybridization-probe solution inside Eppendorf tubes for 3 hours at 46°C. After hybridization, roots were washed in pre-warmed washing buffer (.5 ml .5 M EDTA, 1 ml 1 M Tris-HCl, 700 µl 5 M NaCl, MilliQ water to 50 ml, 25 µl 20% SDS) for 10 minutes at 48°C, followed by incubation in PBS for 15 minutes at room temperature, and blotting on filter paper.

3.3. CARD

Fresh H_2O_2 (.15% in PBS) was prepared and mixed with amplification buffer (4 ml 10x PBS, .4 ml 10% Blocking Reagent, 16 ml 5 M NaCl, milliQ water to 40 ml, 4 g dextran sulfate) at a ratio of 100:1. Fluorescently labeled tyramide was added at a ratio of 1:1000. Roots were incubated with this solution inside Eppendorf tubes for 30 minutes at 46°C. After incubation, excess liquid was blotted of with filter paper and roots were incubated in 1x PBS for 10 minutes at room temperature in the dark. Roots were then washed three times with excess MilliQ water, once with 96% ethanol, and left to air dry in the dark.

4. Standard FISH

Hybridization buffer was mixed with probes at a ratio of 18:2. For standard FISH on root samples, EUB338-I-III and NON338 probes were used. For standard FISH on protist samples, EUB338-I-III and ARCH915 probes were used. Samples were incubated in this solution for 3 hours at 46°C. After incubation, samples were incubated in washing buffer for 15 minutes at 48°C, washed in MilliQ water, and air dried at room temperature in the dark.

5. DAPI Staining

Fresh DAPI solution was prepared by diluting 1 µg of 1000x DAPI in 1 ml of 1x PBS. Roots, agar slices, agarose slides, and protist were incubated in 20 µl of DAPI working solution on top of glass slides for 5 minutes at room temperature. After incubation, samples were rinsed three times in DI water, once in 96% ethanol, and left to air try on filter paper at room temperature in the dark.

6. Sample Mounting

Unmounted samples were mounted onto clean glass slides. All mounted samples were covered with a drop of Citifluor/Vectashield mounting medium (mixed at a ratio of 4:1). Cover slips were sealed to the slide with nail polish. Slides were stored at -20°C until viewing.

7. Microscopic Evaluation

Samples were viewed with a Zeiss epifluorescent microscope equipped with filter sets for DAPI, Cy3, and GFP.

Results and Discussion

1. CARD FISH on Roots and Fungi Samples

The first round of CARD FISH on root and fungi samples appeared to successfully label bacteria on hyphae and root hairs surrounding the root (Figure 1). However, because this was conducted as a preliminary methods trial, only the EUB338-I-III probe was used. Also, DAPI stained the roots and hyphae very brightly and could not be used to verify that the signals from the CARD FISH stain were bacteria.

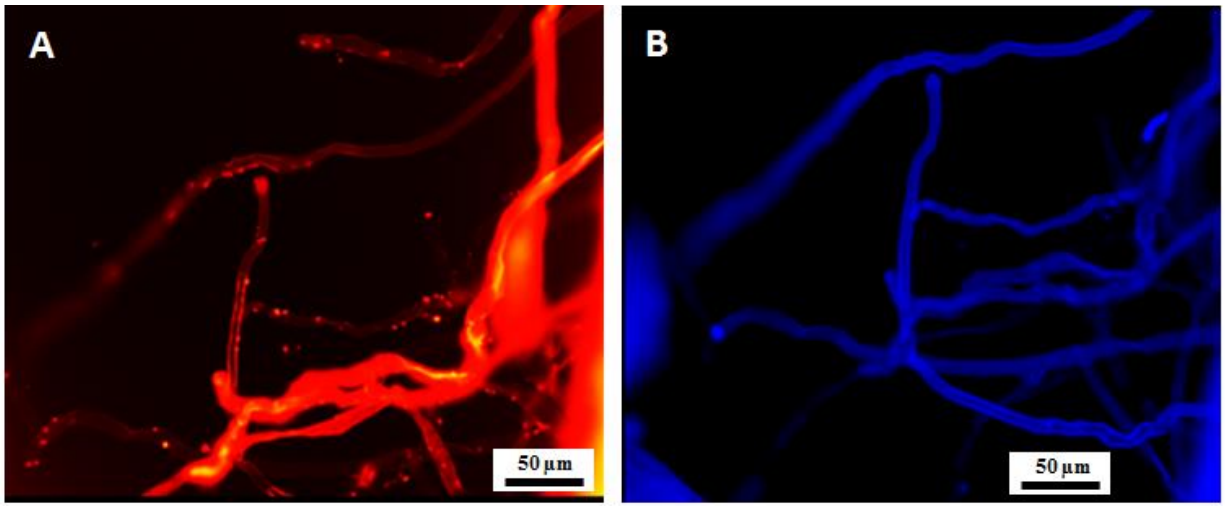

Figure 1. A) Beachgrass roots stained with CARD FISH (EUB338-I-III). B) Beachgrass roots stained with DAPI.

Unfortunately, in each of the many repeated attempts at CARD FISH, no specific signal could be detected with any of the probes used. This may have been due to high root autofluorescence, nonspecific tyramide binding, failure to inactivate endogenous peroxidases of the roots, insufficient incubation times, or other reasons. Cryomicrotome sectioning and subsequence staining did not reduce autofluorescence (Figure 2).

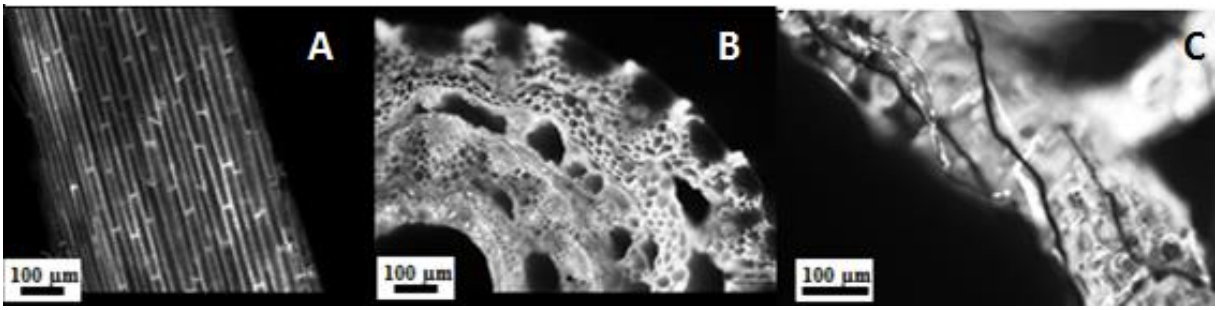

Figure 2. Beachgrass and unidentified lawn grass root samples stained with CARD FISH EUB338-I-III probe: A) beachgrass longitudinal section, B) beachgrass cross section, and C) unidentified lawn grass root and fungal hyphae.

After consistent failure to detect bacteria using CARD FISH, I decided to broaden my search from bacteria associated with mycorrhizal fungi only (which necessitated staining the

roots that serve as hosts to mycorrhizae), to bacteria associated with any fungi from soil and root samples. By incubating root samples on agar plates or agarose slides for 12 hours, I was able to enrich for fungi and bacteria that had grown in the presence of roots, but could be fixed and processed without roots, thereby reducing associated challenges (see Materials and Methods for a description of agarose slide preparation). Unfortunately, CARD FISH on these samples was not successful either, possibly due to agar autofluorescence, or the probes' difficulty to travel to the target through the agar and agarose media.

2. Standard FISH on Fungi and Bacteria Grown on Agarose Slides

Because CARD FISH was unsuccessful, I decided to try the standard FISH technique, which uses monolabeled oligonucleotide probes. I hoped that due to their smaller size, these probes would be able to reach their target through the agarose media that I had prepared. Upon first examination, standard FISH did not appear to show any specific staining of bacteria. However, when further examined under a different microscope, it appeared that some bacteria had been stained successfully with the EUB338-I-III probe (Figure 3).

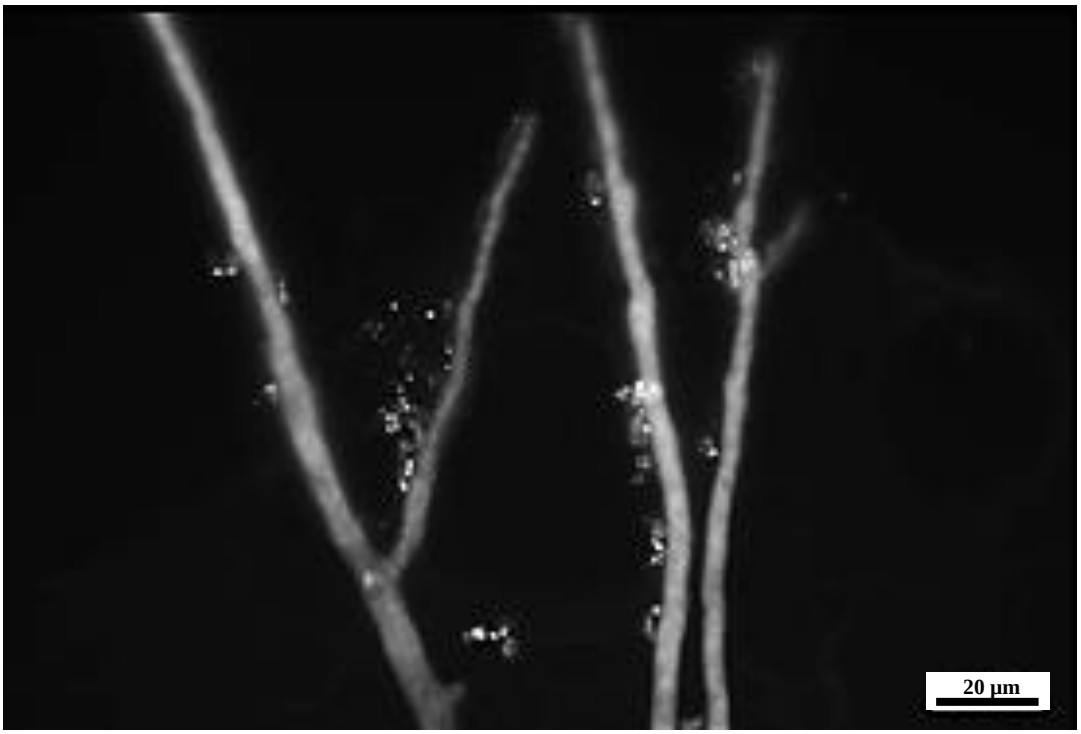

Figure 3. Hyphae surrounded by bacteria stained with the EUB338-I-III mono probe.

3. DAPI Staining on Root and Fungi Samples

DAPI staining of hyphae and associated bacteria was not successful on root samples. Root autofluorescence was too high to detect any specific staining of fungi and bacteria. However, DAPI staining on agar slices and agarose slides revealed many hyphae that were surrounded by bacteria (Figure 4).

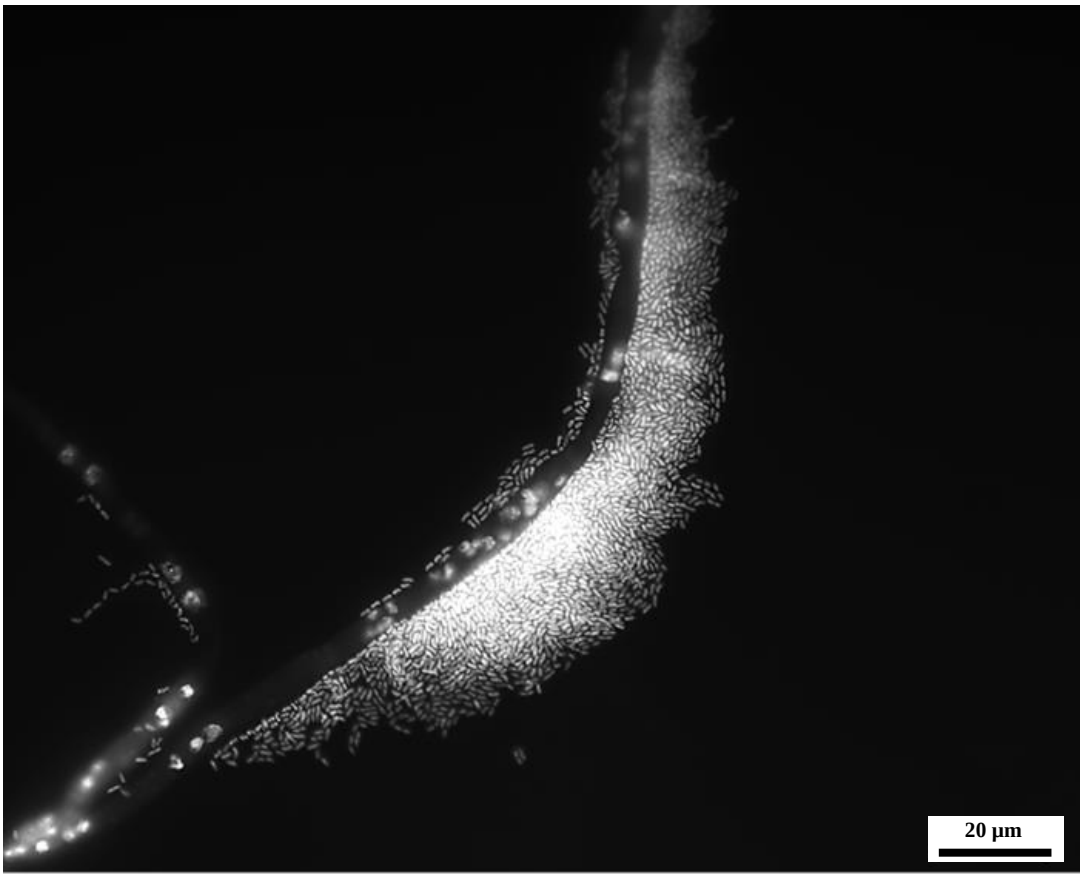

Figure 4. Bacteria and fungal hyphae stained with DAPI.

4. Enrichments for Bacteria Associated with Fungi on High and Low Nutrient Agar Plates

During the three weeks of incubation, growth on the 5YE plates was more rapid and morphologically diverse than growth on the oxalic acid plates (Figure 5). Colonies isolated and sequenced from the 5YE plates included putative members of *Bacillus* and *Actinomycetes*, in addition to many unidentified bacteria. Only one putative bacterial isolate was sequenced from the oxalic acid plates. However, according to BLAST results, there is relatively low confidence in its inclusion in the domain Bacteria (90%). It is not clear if this is due to sequencing error, a failure to achieve a true colony isolate, or other factors.

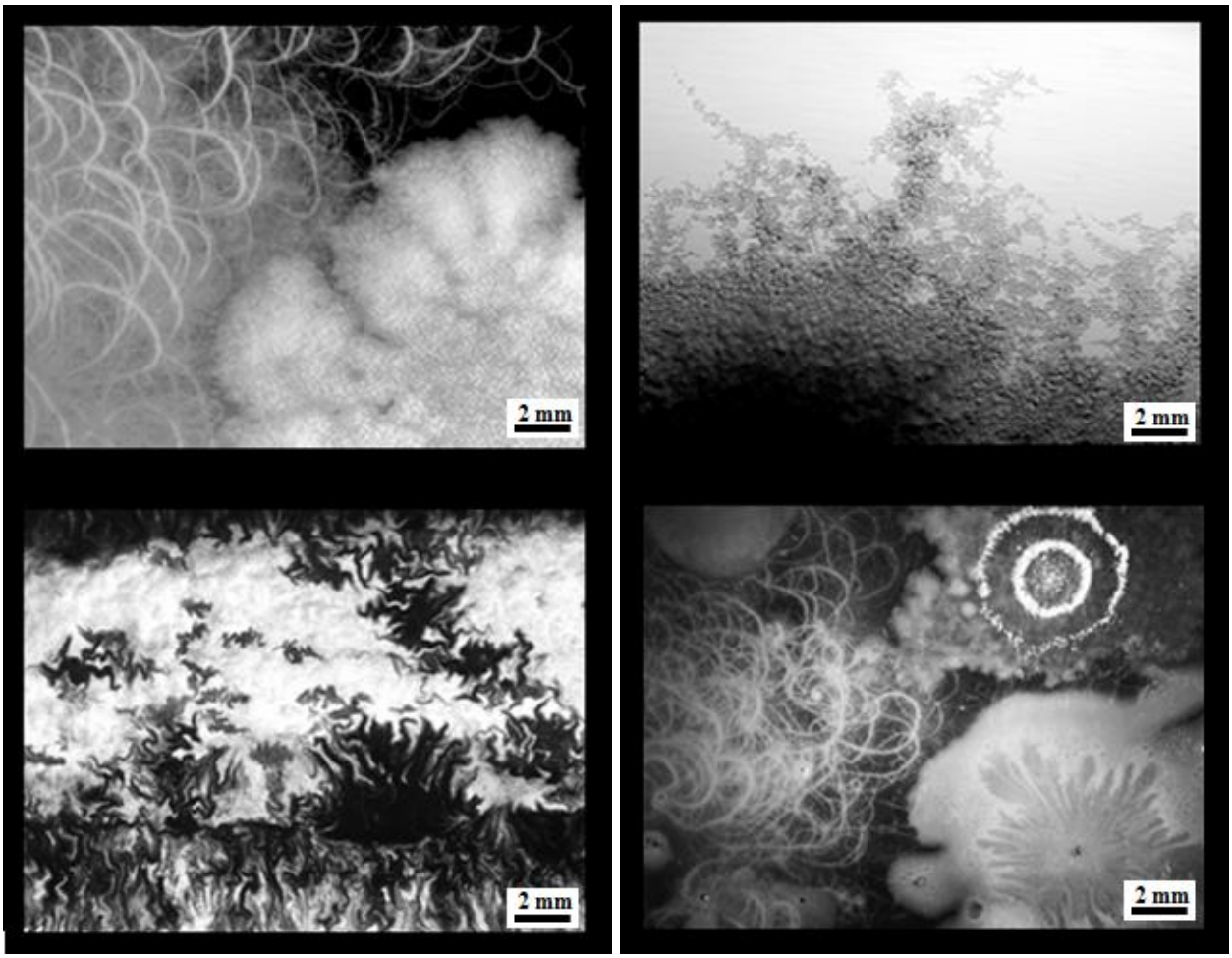

Figure 5. Microbial growth on 5YE plates inoculated with root samples.

5. FISH and DAPI Staining of Bacteria and Archaea Associated with Protists

Standard FISH and DAPI staining showed that bacteria are often associated with protists. Every protist counted in this study was surrounded by at least three bacterial cells. Some protists were surrounded by hundreds of bacteria (Figure 6). Mean bacterial counts per cell are reported in Table 1. Additionally, some archaea were found around protists.

Table 1. Counts of Bacteria Associated with Protists

Sample Source	Protist Types	Mean Bacteria Count per Protist
Microbial Mat	3	29.69 (SE 7.3)
Water + Plant Biomass	10	32.67 (SE 12.9)
Sediment	3	23.25 (SE 2.6)

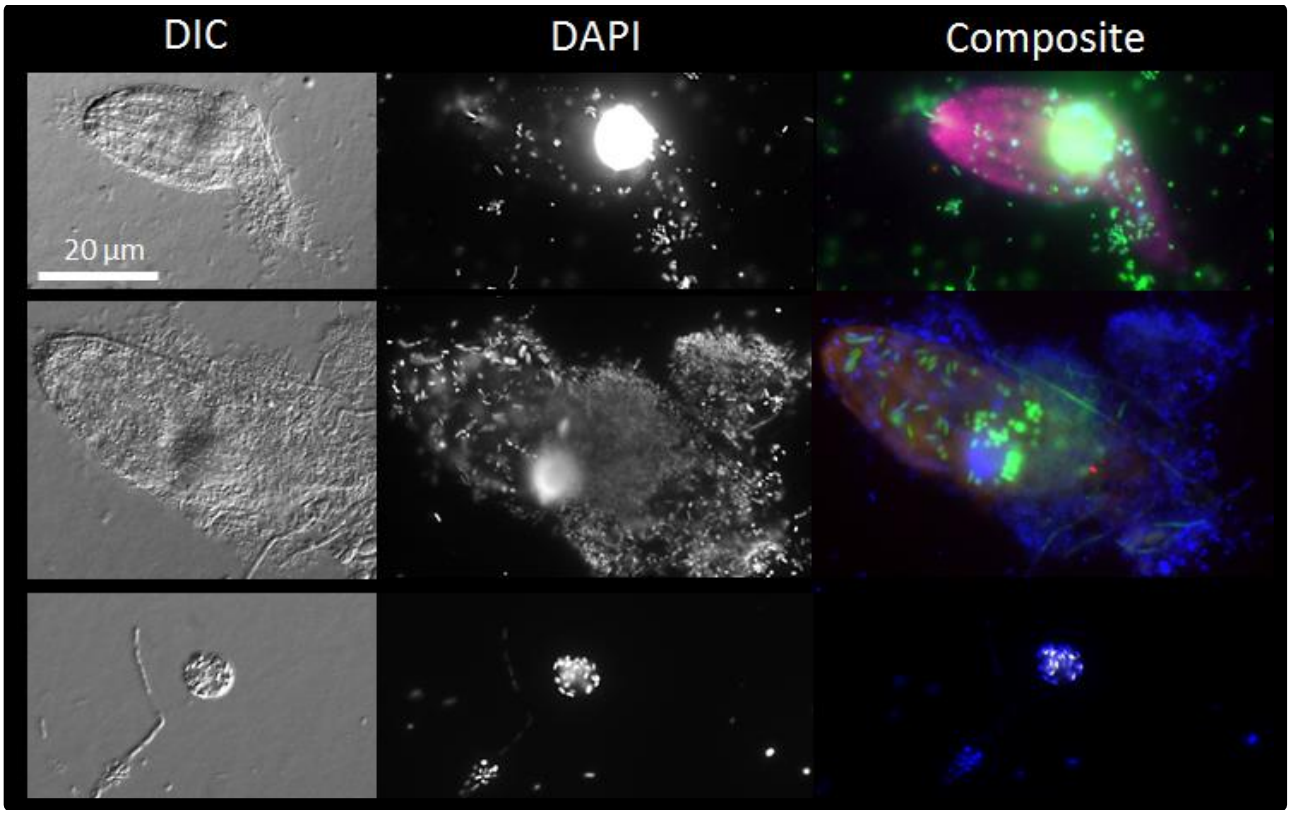

Figure 6. DIC, DAPI, and composite images of DAPI and standard FISH staining with the ARCH915 probe for Archaea and the EUB338-I-III probe for Bacteria indicate that bacteria and archaea are associated with protists from different environmental samples. The archaeal probe fluoresces red, the bacterial probe fluoresces green, and DAPI fluoresces in blue. The red fluorescence inside the protist cells may be due to autofluorescence. The 20 µm scale bar applies to each image.

Conclusion

Standard FISH and DAPI staining suggested that bacteria-eukaryote associations are common in soil fungi and protists. It the future, it would be interesting to use a wider range of FISH monoprobes to try to differentiate between different classes of bacteria. Next generation sequencing could also be used to identify bacterial species present and to help guide the choice of FISH probes and enrichment culture conditions. For protist samples, it would also be interesting to use confocal microscopy to determine whether bacterial cells were located inside or outside organisms. The use of high quality microscopes is imperative, as demonstrated by my ability to detect a FISH signal with one microscope, but not with another.

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