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Fluorescence In Situ Hybridization and Spectral Imaging of Coral-Associated Bacterial Communities

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Microbial communities play important roles in the functioning of coral reef communities. However, extensive autofluorescence of coral tissues and endosymbionts limits the application of standard fluorescence in situ hybridization (FISH) techniques for the identification of the coral-associated bacterial communities. This study overcomes these limitations by combining FISH and spectral imaging.

Microbial communities of corals play substantial roles in normal and perturbed coral physiological states. Recently, studies using phylogenetic analysis have demonstrated high microbial diversity associated with coral tissues (8, 16, 29, 32, 33, 41) and coral mucus (29). Coral microbial communities appear highly complex; they are distinct from those in the water column, are coral species specific, are spatially and temporally stable, and have roles in the physiological function of the coral holobiont (16, 29, 32, 33). Putative roles include nitrogen fixation, carbon fixation, nutrient accumulation, antibiotic production, pathogen protection, and prevention of fouling and colonization (13, 33, 36, 41). Yet to better understand the complexity of the coral microbial community, we need to know the architecture of the coral-microbe association. Understanding how microorganisms interact with corals is important, given that they are increasingly implicated in coral disease and reef degradation. Fluorescence in situ hybridization (FISH) is widely applied in diverse and complex microbial systems (2) where traditional microbial studies are limited. It is estimated that as few as 0.1% of environmental microbes can be isolated in pure culture; in situ studies overcome this limitation (1, 18, 39).

Extensive autofluorescence has been the major limitation to the application of FISH in coral studies. Coral tissues are highly fluorescent due to the presence of pocilloporins (green fluorescent protein-like molecules) (14, 15, 34) and high densities of endosymbiotic dinoflagellates containing chlorophyll (22, 42). This extensive autofluorescence confounds coral microbial FISH studies, as image analysis is unable to resolve the sources of fluorescence (12). Bythell et al. (9) conducted the first study of FISH using enzyme amplification, previously reported to provide a 10- to 20-fold increase in signal intensity (26, 39). The study of Bythell et al. (9) was able to distinguish coral-associated bacterial communities; however, autofluorescence and nonspecific probe binding confounded image quality. Subsequently, Wegley et al. (40) used peptide nucleic acid probes for visualizing *Archaea* within coral tissue slurries. While the technique appeared effective for enumeration of probe binding, cellular morphology and image clarity were low. While the authors examined the binding potential of the probes with microbial cultures, controlling for sources of fluorescence emission such as coral tissue fragments and nonspecific probe binding were not considered. Amann et al. (2) suggested that improvements with peptide nucleic acid probes are confounded by nonspecific binding; therefore, the reliability of this method is questionable when it is applied to complex coral tissue slurries where confirmation of cell type and sources of fluorescence are not available. Rowher et al. (33) noted that there is yet to be a clear picture of the ecological roles of the diverse coral microbial associates. The present study describes a method that can address these questions by characterizing the architecture of the coral-bacteria association.

Recently, spectral imaging has been applied to plant research where tissue autofluorescence is a limiting factor (5, 6). Spectral profile images enhance the ability to interpret a fluorescent image by analyzing the spectral emission curve data of each pixel, thus providing an added dimension for image analysis (6, 43). Spectral imaging provides a means of distinguishing sources of fluorescence emission, allowing coral autofluorescence and FISH probe-fluorochrome emissions to be distinguished. Our study applied conventional FISH techniques and spectral imaging to accurately visualize the architecture of bacterial communities associated with reef-building corals.

Branching (*Acropora aspera*, *Acropora formosa*, and *Acropora nana*) and tabulate (*Acropora cytherea*, *Acropora clatharata*, and *Acropora hyacinthus*) acroporids, as well as nonacroporids (*Seriatopora hysterix* and *Stylophora pistillata*), were collected from Heron Island on the Southern Great Barrier Reef (23°44′17″S, 151°91′25″E), Australia. Corals were collected from March through September 2004 (at a depth of 1 to 8 m), transported to the Heron Island Research Station, and held in flowthrough seawater aquaria prior to fixation in 4% (wt/vol) paraformaldehyde in sterile phosphate-buffered saline (1) for 12 h. Samples were embedded in 1.5% (wt/vol) agarose (9) prior to decalcification with 20% (wt/vol) EDTA in phosphate-buffered saline (37). Tissues were processed sequentially through 70%, 80%, 95%, and 100% ethanol and three xylene and three paraffin washes, each for 40 min, prior to paraffin embedding.

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TABLE 1. Bacterial group probes used in FISH for identification of bacterial populations

Probe	Target group	Oligonucleotide sequence	% Formamide	Reference
ALF969	α-Proteobacteria	5-′TGGTAAGGTTCTGCGCGT-3′	20	23
BET42a	β-Proteobacteria	5′-GCCTTCCCACATAGTTT-3′	35	23
GAM42a	γ-Proteobacteria	5′-GCCTTCCCACATCGTTT-3′	35	23
CF319	*Cytophaga-Flavobacterium*	5′-TGGTCCGTGTCTCAGTAC-3′	35	24
HCG69a	*Actinobacteria*	5′-TATAGTTACCACCGCCGT-3′	25	30
LGC354	Firmicutes	5′-TGGAAGATTCCCTACTGC-3′	35	25

Serial tissue sections (4 μm) were collected onto Superfrost Plus slides (Menzel, Germany). Visualization of bacterial communities associated with coral tissues and lesions was conducted with the bacterial probe suite EUBmix (3, 11) using a conventional FISH protocol (3, 4, 18). Sequential adjacent tissue sections were probed with bacterial group probes (Table 1). All oligonucleotides were labeled with Cy3 (Thermo Electron Corp.). The hybridization was conducted in buffer (0.9 M NaCl, 0.01% sodium dodecyl sulfate, 0.01 M Tris-HCl, pH 7.2) for 1.5 h at 46°C, followed by a 10-min wash in prewarmed buffer (0.08 M NaCl, 0.01% sodium dodecyl sulfate, 0.01 M Tris-HCl, 0.05 M EDTA). Image analysis was conducted on a Meta 510 confocal scanning laser microscope (Zeiss, Germany) with the Zeiss Image Browser software. Several coral tissue sections treated with the FISH protocol without the application of probe were used for spectral profiling of autofluorescence without confounding sources of fluorochrome fluorescence. This was repeated for coral species to determine variability in fluorescence profiles. Coral tissue sections washed with 4% paraformaldehyde-fixed cultures of *Escherichia coli* were used as positive controls for the combination FISH and spectral imaging procedure.

The application of spectral imaging successfully separated coral and endosymbiont autofluorescence from the probe fluorochrome, thereby overcoming previous autofluorescence problems that limited in situ analysis. The success of spectral imaging of the autofluorescence was evident by distinguishing the fluorescence conferred by EUBmix-Cy3-labeled oligonucleotides hybridized to individual *E. coli* cells. Individual *E. coli* cells coating coral tissues sections could be easily distinguished from the background fluorescence (Fig. 1).

The FISH technique and spectral imaging were successfully applied to visualize and identify bacterial communities associated with predation lesions of corals. Individual bacterial cells and communities of bacteria associated with the lesion created by predation of *A. formosa* by the snail *Drupella* sp. were identified. The mixed bacterial community formed a mat that penetrated the coral gastroderm and tissue layers and coated the epithelium (Fig. 2). Two morphologically distinct bacterial communities (filamentous and coccoid) were associated with

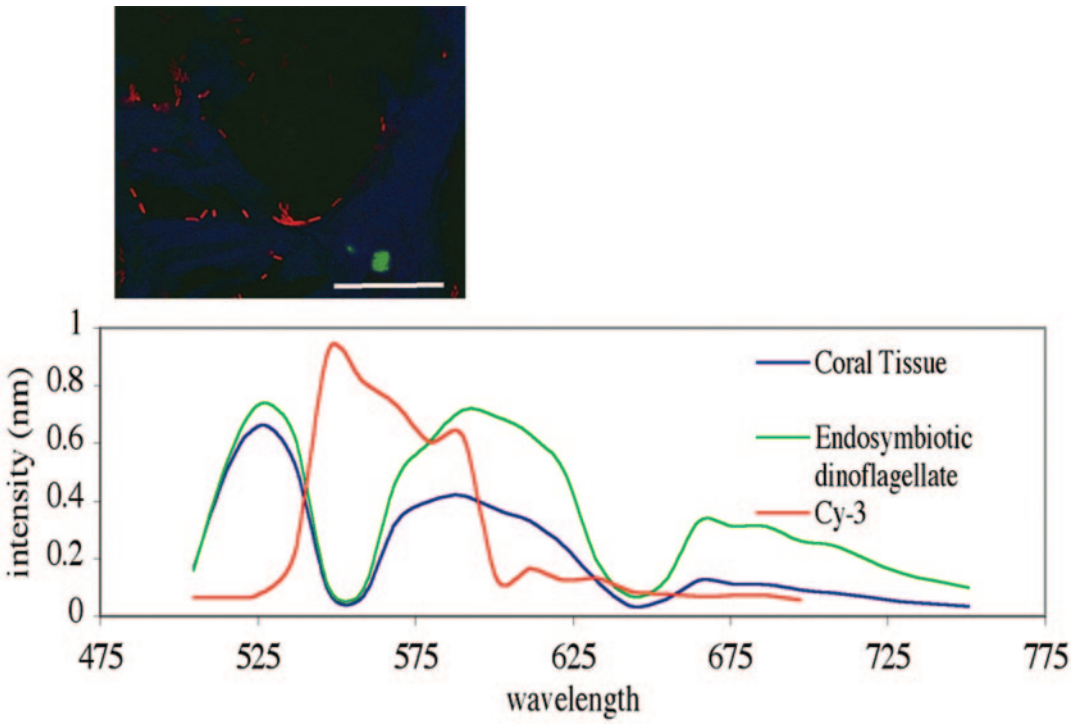

FIG. 1. Spectral imaging of *A. aspera* coral tissue, endosymbiotic dinoflagellate autofluoresence, and EUBmix-Cy3-labeled *E. coli*. Red, bacteria; blue, coral tissue; green, endosymbiotic dinoflagellates. Scale bar, 50 μm.

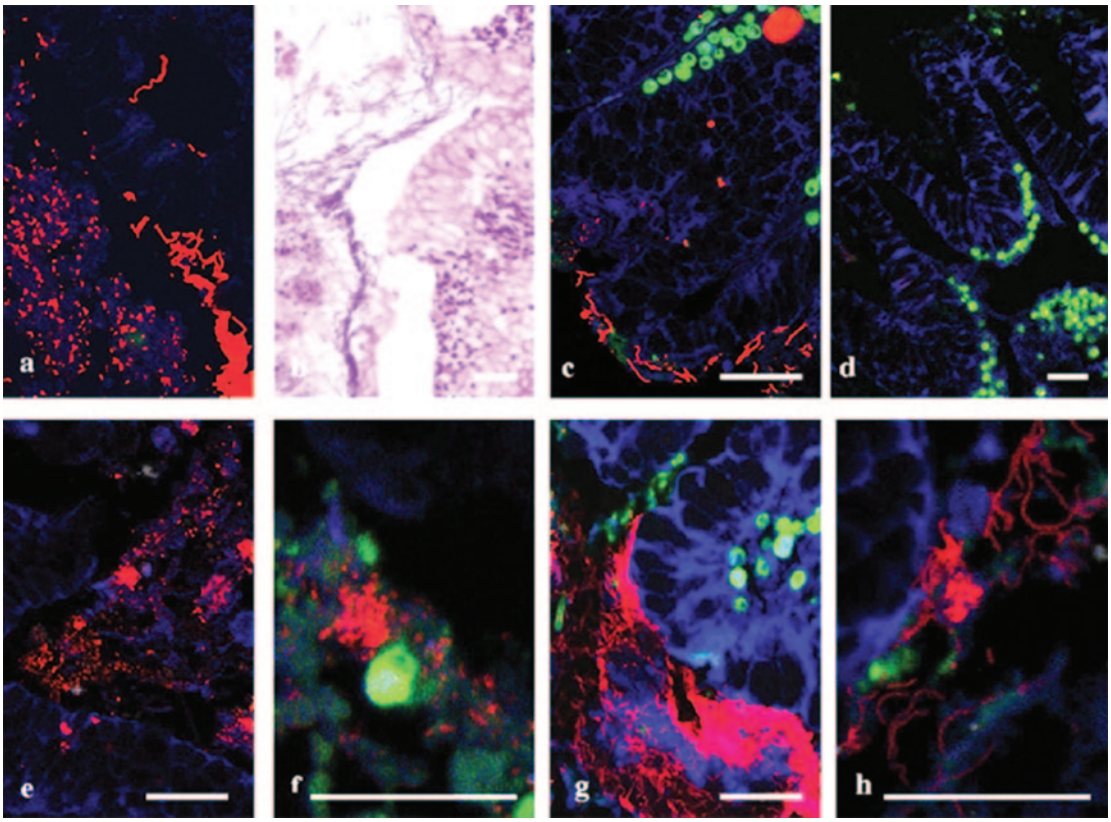

FIG. 2. Bacterial communities associated with the lesion created by predation of *A. formosa* by *Drupella* sp. FISH identified two bacterial morphologies using EUBmix-Cy3 (a) which were also evident in adjacent sections using hematoxylin and eosin staining (b) and coating the surface of adjacent coral tissues (c). Nonspecific staining of the FISH protocol was not evident (d), and the two bacterial morphologies were identified as a coccoid γ-proteobacterium using the probes GAM42a (e and f) and as a filamentous *Cytophaga-Flavobacterium* using the probe CF319 (g and h). Red, bacteria; blue, coral tissue; green, endosymbiotic dinoflagellates. Scale bar, 50 μm.

the predation lesion and identified as belonging to the γ-proteobacteria and *Cytophaga-Flavobacterium* (Fig. 2). A small population consisting of α-proteobacteria was identified associated with the surface of the coral tissue coating a region of epithelial tissue away from the lesion area. Nonspecific binding was not evident within this study, with no binding of the HGC69a, LGC354, or β-proteobacteria (BET42a) probes on sequential sections. This study also identified bacterial aggregates associated with coral tissues. EUBmix universal bacterial probes marked large ovoid inclusions associated with the gastroderm and mesenterial filaments of the coral tissues, and subsequently these were consistently identified as γ-proteobacteria (Fig. 3). Previously, bacterial aggregates within coral tissues have been described using standard histopathological techniques as large basophilic bodies of up to 40 μm or more associated with tissue layers (27, 28, 35). Interestingly, using 16S rRNA gene sequencing, Rowher et al. (33) consistently found a γ-proteobacterium that they designated PA1 in over 50 *Porites asteroides* colonies sampled and speculated that this bacterium was the one observed as ovoid aggregates by Santavy and Peters (35). The lack of available and reliable technology previously prevented detailed analysis of these aggregates within the context of the coral holobiont, but this problem has been overcome with this technique. This study's consistent identification of γ-proteobacterial aggregates in branching corals supports the speculation of Rowher et al. (33) and indicates the wider presence of γ-proteobacteria across scleractinian corals.

FISH technology allows for spatial and temporal analysis of bacterial community variability (7), and its application to coral microbial ecology can begin to address questions on the stability and role of bacterial communities. Jones et al. (19) conducted FISH, limiting excitation wavelengths to the red/far-red regions of the spectrum, with coral tissue slurries for confirmation of 16S rRNA sequence analysis and quantification of coral disease-associated microbial communities but did not conduct image analysis, nor did the investigators address the structural arrangement of the microbial communities and interaction with coral tissues. Importantly, the authors noted a difficulty in differentiating disease-causing organisms from secondary invaders (19), a problem that could be addressed by investigating the structural environment of the disease lesions and adjacent regions. The bacterial populations identified within this study are some of the most abundant oceanic bacterial groups and are involved in a wide range of functions (10, 20, 21). Yet investigating the spatial arrangement of microbial communities around lesions and tissues can allow the differentiation of pathogens, lesion colonizers, and persistent microbial communities.

Previous studies utilizing FISH for investigating coral micro-

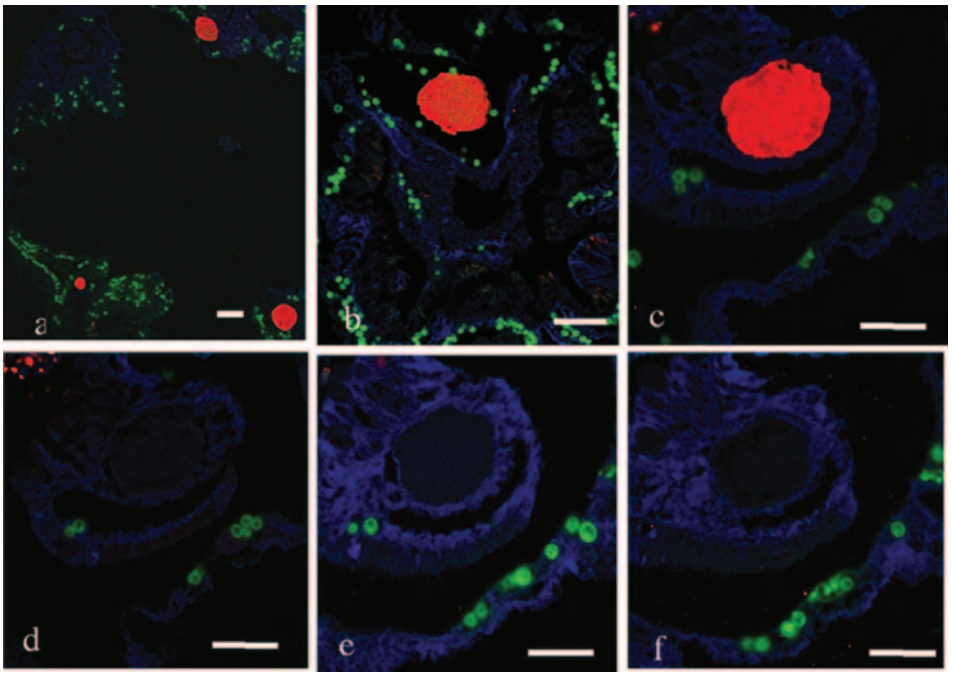

FIG. 3. FISH using universal bacterial probe EUBmix of large bacterial aggregates associated with coral tissues *S. pistillata* and *A. formosa* (a and b) and identification as γ-proteobacteria using GAM42a (c), with no binding of CF319 (d), HGC69a (e), or LGC 354 (f) probes. Bacterial aggregates, red; coral tissue, blue; endosymbiotic dinoflagellates, green. Scale bar, 50 μm.

bial ecology have been limited in determining community interactions by autofluorescence, poor image quality, and probe specificity. This study demonstrates that FISH combined with spectral imaging is a rapid and powerful tool to assess the microbial architecture of reef-building corals. With increasing disease and stress of scleractinian corals (17, 31, 38), the timely description of this technique allows for localization and identification of coral-associated bacterial community changes, suggesting a central role in the future analysis of microbial communities.

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