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# Bacterial Distribution in the Epidermis and Mucus of the Coral *Euphyllia glabrescens* by CARD-FISH

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**Hsiu-Hui Chiu, Andreas Mette, Jia-Ho Shiu, and Sen-Lin Tang (2012)** Bacterial distribution in the epidermis and mucus of the coral *Euphyllia glabrescens* by CARD-FISH. *Zoological Studies* **51**(8): 1332-1342. The distribution of bacteria in coral mucus has long been poorly understood, although most coral-associated bacteria were suggested to dwell in the mucus and epidermis of corals. We hypothesized that different bacterial groups have different distribution patterns in the mucus and epidermis. To test this hypothesis, we overcame technical difficulties of mucus preservation during sample preparation and inspected the distributions of 2 dominant coral-associated bacterial groups, the alphaproteobacteria and gammaproteobacteria, in the mucus and epidermis of the coral, *Euphyllia glabrescens*, collected from Kenting and Ludao (also known as Green I.) in southern Taiwan. We used catalyzed reporter deposition-fluorescence *in situ* hybridization to detect the location of the bacteria in the mucus and epidermis, and results showed that the 2 bacterial groups had different distribution patterns in the coral. Alphaproteobacteria were frequently distributed at the interface between the mucus and epidermis, while gammaproteobacteria were only detected in the gastrodermis and rarely observed in the mucus or epidermis. This study provides the 1st direct evidence that different bacterial groups have habitat specificity in coral mucus. http://zoolstud.sinica.edu.tw/Journals/51.8/1332.pdf

**Key words:** Coral mucus-associated bacteria, CARD-FISH.

Coral mucus is excreted by mucocytes in the epidermis of corals and consists of major monosaccharides of fucose, *N*-acetylglucosamine, and glucosamine, and major amino acids of serine, aspartic acid, glutamic acid, and threonine (Brown and Bythell 2005, Klaus et al. 2007). The mucus often releases soluble and insoluble carbon, nitrogen, and phosphorous at 90.9, 7.6, and 1.3, and 27.7, 1.9, and 0.3 kmol/day, respectively (Wild et al. 2004). Notably, the N-content and organic compounds in the mucus constitute 20%-45% of the photosynthetic products fixed by symbiotic algae (Wild et al. 2008, Wang et al. 2012). Functionally speaking, coral mucus protects corals by filtering out intruding foreign matter and serves as an exchange interface between coral tissues and seawater that allows organic and inorganic materials to enter and leave (Wild et al. 2004 2008). Because of the functional characteristics of the mucus which contribute to its own small food chain (Johannes 1967, Coles and Strathma 1973, Benson and Muscatine 1974, Ducklow and Mitchell 1979, Krupp 1984), many organisms were discovered in the mucus including animals (mollusks, crustaceans, cnidarians, and nematodes), protozoa (ciliates and foraminifera), diatoms, microbes, and carbonate particles (Huettel et al. 2006).

Mucus-associated bacteria are some of the important organisms in the mucus, with likely

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functional roles in nutrient supply, health, and disease resistance of corals (Rohwer and Kelley 2004). The population structure of mucus-associated bacteria has been intensively studied in recent years (Ritchie 2006). However, no study focused on the specific distributions of different bacteria in the coral mucus and correlations with bacterial ecological functions. One of the main reasons was a failure to preserve coral mucus structures when detecting bacterial distributions using *in situ* molecular methods, such as fluorescence *in situ* hybridization (FISH). Hence, several basic and important questions are still unknown and urgently need to be answered, such as: How are bacteria distributed in coral mucus?; Is there a specific distribution pattern for a specific bacterial group?; and, If yes, why does it specifically dwell in a particular niche in the mucus in terms of coral physiology and ecology?

In this study, we overcome technical difficulties of mucus preservation and tested the hypothesis of whether different bacterial groups are specifically distributed in the coral mucus. We detected the distribution of 2 dominant bacterial groups, alphaproteobacteria and gammaproteobacteria, in the mucus and epidermis of *Euphyllia glabrescens* using catalyzed reporter deposition (CARD)-FISH. Our results show that the bacteria were distributed non-randomly in the epidermis and mucus. Alphaproteobacteria commonly appeared in the epidermis and mucus; however, gammaproteobacteria were mostly found in the gastrodermis. This study for the 1st time provides direct evidence of distribution specificity by coral-associated bacteria in the mucus.

## MATERIALS AND METHODS

### Sampling

Coral samples were collected from Tanzi Bay in Kenting National Park (21°57'04"N, 120°46'15"E) and Shi-Lang on Ludao (also known as Green I.; 22°35'04"N, 121°28'15"E) at 10 m in depth in Nov. 2008. A small healthy fragment of *Euphyllia glabrescens* was separated with a hammer and chisel. One liter of seawater was also collected near the coral. To anesthetize the coral tentacles to prevent contraction, we treated them with sterilized $MgCl_2$ (at a final concentration of 4% in seawater). Each tentacle was cut with micro-scissors (Albert Heiss, Tuttlingen, Germany), embedded in optical cutting temperature compound (OCT) (Sakura Finetek, California, USA) on a base mold, and frozen in a freezer at -20°C. All anatomic processes were completed in 1 h in the laboratory.

### Frozen sectioning and histochemical staining

The embedded coral tentacles were sectioned with a Leica CM 1900-Cryostat microtome (Leica Microsystems, Nussloch, Germany). The chamber temperature was -17°C, and the specimen head temperature was adjusted to -25°C. The microtome blades at 35/75 mm (HP35, Thermo Shandon, Yokohama, Japan) were selected for sectioning. Each slide contained 3-5 pieces of 5-7-μm frozen sections of 1 tentacle. Ten tentacles of each coral were observed. The section slides were stored at -20°C before further experiments.

Sections on the slides were histochemically stained with hematoxylin and eosin (H&E). Concentrations of the stains and procedures followed the method of Ainsworth et al. (2007).

### CARD-FISH

The ingredients of the buffers for the CARD-FISH analysis were described in details by Dijk et al. (2008). The hybridization buffer was composed by 0.9 M NaCl, 0.02 M Tris-HCl (pH 8.0), 30% v/v formamide, 0.02% w/v sodium dodecylsulfate (SDS), and 1% w/v blocking reagent. The wash buffer was composed by 0.06 M NaCl, 0.02 M Tris-HCl, 0.005 M EDTA, and 0.01% w/v SDS. The amplification buffer contained paraformaldehyde-phosphate-buffered saline (PBS: 0.089 M NaCl, 4.76 mM $Na_2HPO_4$, and 2.04 mM $NaH_2PO_4$) with a formaldehyde concentration of 30%, 1.33 M NaCl, 0.067% w/v blocking reagent, and 0.006% w/v dextran sulfate. To detect the bacterial distribution, we used EUB 338 I-III (for bacteria) as the universal probe, ALF 968 (for alphaproteobacteria), and GAM 42a (for gammaproteobacteria) (Amann and Fuchs 2008), and tested the probe specificity using several known standard bacterial strains. These probes were designed by an ARB software program that included all sequences from the database of bacterial sequences, and were chosen to match most target groups.

The 2 specific probes were separately labeled by 2 kits carrying different fluorescences and then mixed together to allow them to hybridize with our coral samples, which were freshly sectioned and mounted on slides. Probe labeling followed the procedures of the Platinum

Bright™ Nucleic Acid Labeling Kit (KREATECH Biotechnology, Amsterdam, the Netherlands). To avoid interference from coral autofluorescence, we evaded the field of the strongest autofluorescence and used labeling dyes with different emission peaks from the peak autofluorescence according to an analysis by confocal microscopy. Two fluorescent dyes, Platinum Bright 415 blue and 547 red/orange (KREATECH Biotechnology), were chosen. An oligonucleotide probe at 1 μg, 2 μl of ULS label dye, and 2 μl of a labeling solution (total 20 μl) were mixed, incubated at 85°C for 30 min, and then immediately placed on ice. The labeled probe was purified using KREApure columns (KREATECH Biotechnology).

The sample was hybridized with 2 probes labeled with 2 stains and pictured twice at 2 excitation wavelengths. The 2 photos were then superimposed. For CARD-FISH, *Escherichia coli* was used as a positive bacterial control. The strain was embedded in OCT and frozen-sectioned, and then the same procedures were followed as with the coral samples. The slide was pre-hybridized in hybridization buffer (0.9 M NaCl, 0.02 M Tris-HCl, 0.6 ml formamide, 0.02% w/v SDS, and 1% w/v blocking reagent) at 35°C for 1 h. Hybridization was carried out in a hybridizing bag including 300 μl of hybridization buffer and 3 μg of probes at 35°C for 2.5 h. The slide was washed in washing buffer (0.064 M NaCl, 0.02 M Tris-HCl, 0.005 M EDTA, and 0.01% w/v SDS) for 10 min, incubated with amplification buffer (0.13 M NaCl, 7 mM $Na_2HPO_4$, 3 mM $NaH_2PO_4$, 2 M NaCl, 0.1% w/v SDS, 1% w/v blocking reagent, and 0.01% w/v dextran sulfate) for 15 min, washed in 0.2 M PBS for 5 min, briefly rinsed twice in MilliQ $H_2O$ (Millipore, Billerica, MA, USA), and stored in approximately 100% ethanol. The slide was air-dried, detected with an epifluorescence microscope (Nikon Eclipse 90i, Tokyo, Japan), and photographed with a charge-coupled device imaging camera (COOLSNAP $HQ^2$, Photometrics, Tucson, AZ, USA). Bacterial cells were enumerated using the NIS-Elements Advanced program vers. 3.0 (Nikon). For better resolution to distinguish target cells, all digital pictures were deconvoluted by the image program, AutoQuantX vers. 2.0.1 (MediaCybernetics, Silver Spring, MD, USA). Only slides containing the complete tissue structure (including the mucus, epidermis, and a part of the gastrodermis) were chosen for the image analysis and cell counting. Every count was carried out using 30 different fields of view. The thicknesses of the epidermis and mucus were measured using a Java program developed by one of the authors (Andreas Mette), which calculates a circle by 3 points and the distance between the point and the circle. Emission spectra of the target bacterial cells hybridized with specific probes were also examined with a confocal laser scanning microscope (Zeiss LSM 510, Carl Zeiss, Jena, Germany). Finally, all counts, and the width and area of both the epidermis and mucus were analyzed by an one-way analysis of variance (ANOVA; Microsoft® Office Excel 2003).

**Confocal microscopy**

All sections on the slides hybridized with specific fluorescent probes were analyzed and confirmed by confocal laser-scanning microscopy (Zeiss LSM 510), with ultraviolet (UV) (405 nm) and visible wavelength (543 nm) lasers and a photomultiplier instrument (Carl Zeiss, Jena, Germany). The fluorescence of the dyes and the autofluorescence of the coral tissues and symbiotic algae were also defined using the same instruments.

**DNA extraction, purification, and multiple displacement amplification**

To isolate the total DNA of the coral, frozen coral samples in microcentrifuge tubes were ground up in a mortar and pestle in 300 ml of sterile seawater for 20 min. The ground-up sample was transferred to 2-ml bead solution tubes provided in the Ultra Clean™ Soil Kit (MO BIO, Carlsbad, CA, USA). All procedures were carried out on a laminar flow bench and followed the protocols recommended by the manufacturer. To isolate total DNA in seawater, the collected seawater was filtered through a 0.2-μm membrane (Whatman, Maidstone, UK). The membrane containing bacteria was cut into small pieces with sterile scissors, and total DNA was extracted using an Ultra Clean™ Soil Kit.

The total DNA extracted from the coral or seawater was purified following the method of Hong et al. (2009). Total coral DNA was subsequently amplified using a Genomiphi vers. 2 DNA Amplification Kit (Amersham Biosciences, Sunnyvale, CA, USA). The DNA quality and concentration were determined with an ND-1000 NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

### Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) analysis

Bacterial 16S ribosomal DNA was amplified by a PCR, with the reaction mixture consisting of PCR buffer, 0.2 mM dNTP, 0.5 M of the universal primers 341F (5'-CCTACGGGAGGCAGCAG-3') with GC clamps and 907R (5'-CCGTCAATTCCTTTRAG TTT-3') (Schabereiter-Gurtner et al. 2003), 0.1 units/μl Taq DNA polymerase (Takara Bio, Shiga, Japan), and 50 ng total DNA. The amplification program consisted of an initial denaturation step at 94°C for 5 min; 50 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min; with a final extension step at 72°C for 10 min. The PCR product was checked by agarose electrophoresis. The target band of 550 bp was determined in a UV transilluminator. DGGE was performed with a 7% acrylamide gel and a denaturing gradient from 22.5% to 45%. The total running time was 16 h at 65 V. Bands on the gel were visualized by silver staining (Radojkovic and Kušic 2000). Four bands per lane were chosen and cut from the DGGE gel. Each band was placed in a clean tube with 50 μl sterile MilliQ water to elute the DNA out of the gel. The selected DNAs were re-amplified by a PCR with the same primers (i.e., 341F and 905R) without the GC clamps under the same PCR conditions. The PCR products were sent out for sequencing (Mission Biotech, Taipei, Taiwan). The identity of each sequence was confirmed by a BLAST search on the NCBI website (http://www.ncbi.nlm.nih.gov/).

## RESULTS

Bacteria in the epidermis and mucus of *Euphyllia glabrescens* were detected and enumerated according to microscopic observations, and the structure of the tentacles was separated into 3 main layers: the gastrodermis, mesoglea, and epidermis. Most zooxanthellae existed in the gastrodermis. The mesoglea between the gastrodermis and epidermis had no obvious cells or tissue. The outer layer of the epidermis contained dense coral epidermal cells and nematocysts. Semi-transparent mucus covered the surface of the epidermis (Fig. 1A). The bacterial density in the mucus was determined using the software, NIS-Elements Advanced program vers. 3.0 (Nikon). The area occupied by mucus, calculated by 30 different microscopic fields, was approximately 6000 μm$^2$ which was larger than the epidermis which ranged 1976.4-

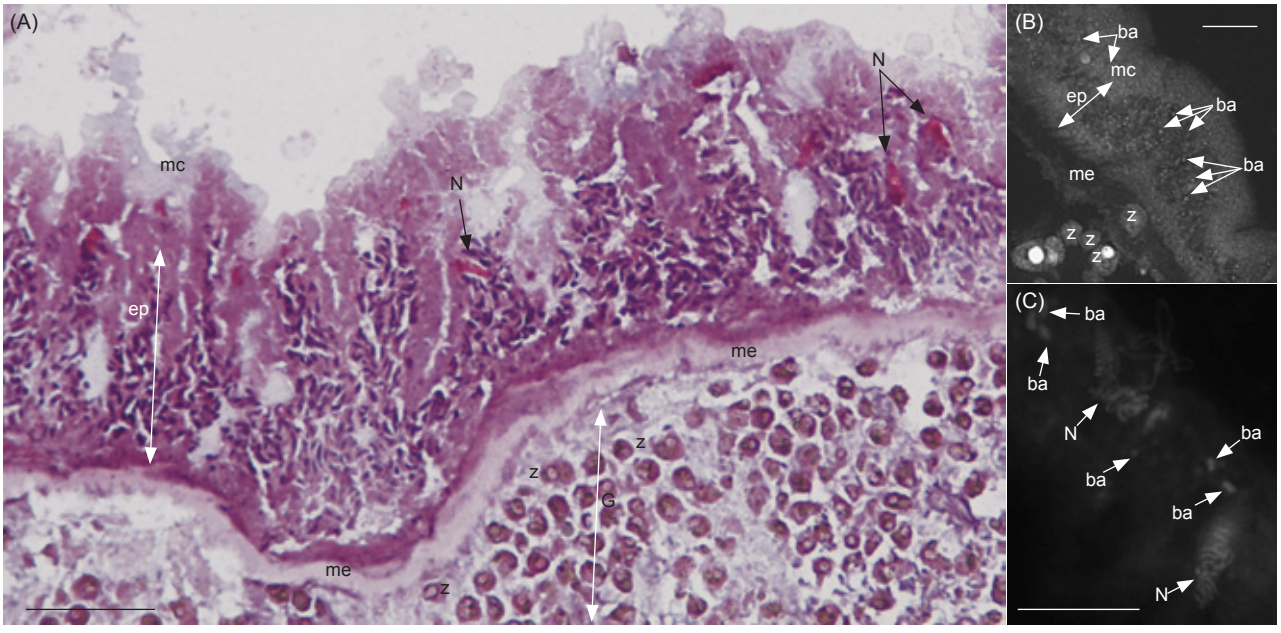


**Fig. 1.** Anatomy of the tentacles of *E. glabrescens*. (A) Most zooxanthellae (z) were gathered in the gastrodermis (G). Most bacteria (ba) were observed in the mucus and epidermis of *E. glabrescens* from Kenting (B) and Ludao (C). The tissue section was stained with hematoxylin and eosin. From inside to outside, the section is composed of the gastrodermis, mesoglea (me), epidermis (ep), and mucus (mc). N, nematocysts. Scale bars = 20 μm. Bacteria were detected by an EUB 338 I-III probe in (B) and (C).

2859 μm² in this study. The bacterial density in the Kenting samples was 2.5 cells/100 μm² in both the epidermis and mucus. In the Ludao samples, the bacterial density ranged 0-0.2 cells/100 μm² (Table 1). The results of the one-way ANOVA showed that neither the bacterial cell number nor cell density had a significant correlation with the area of the epidermis or mucus layers (data not shown).

The distribution of specific bacterial groups was confirmed using the exact emission wavelength on the confocal microscope that could distinguish the dye signals of the specific probes, ALF 968 and GAM 42a, from the coral's autofluorescence. At an excitation wavelength of 405 nm, the dye-labeled alphaproteobacteria emitted at 450-460 nm, the coral's epidermis emitted at 510 nm, and zooxanthellae emitted at 680 nm (Fig. 2). At an excitation wavelength of

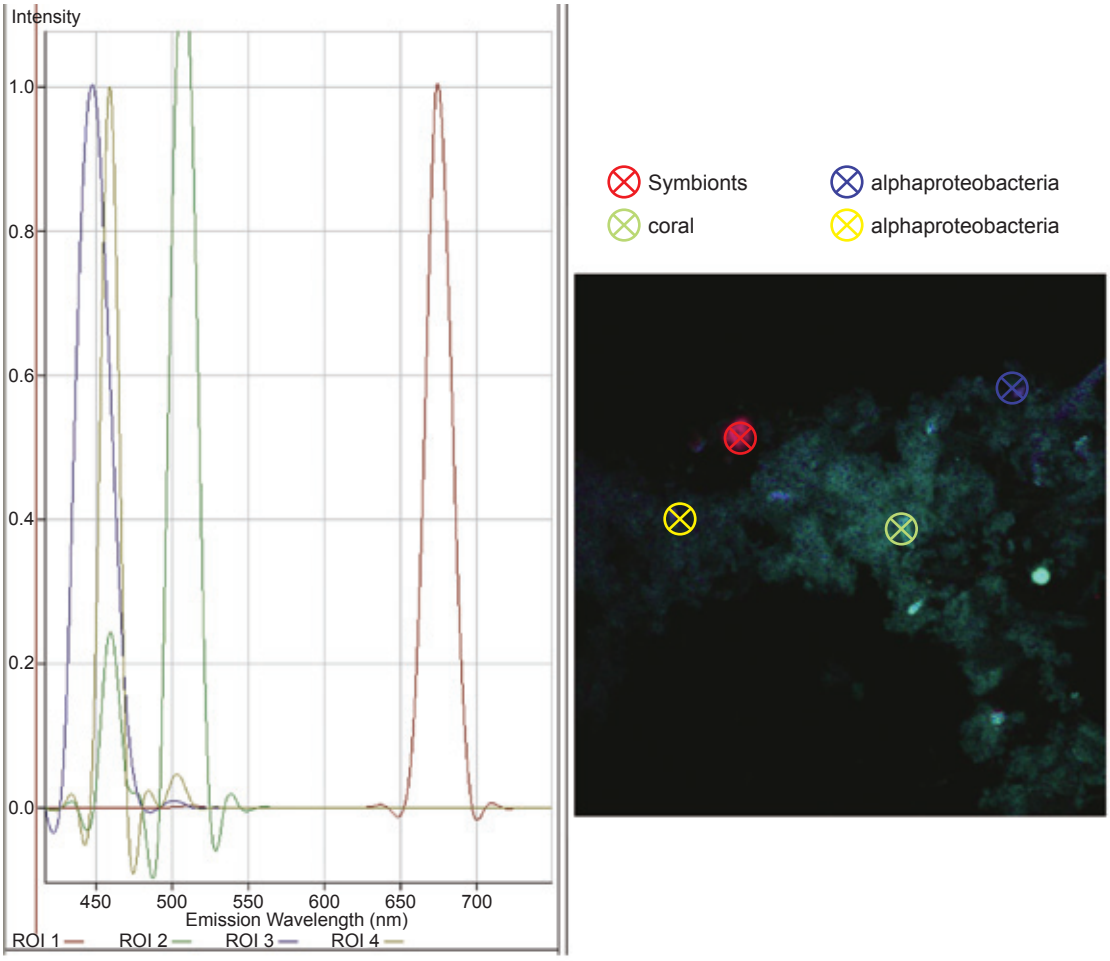


**Fig. 2.** Respective emission spectra of zooxanthellae, coral tissue, and Alphaproteobacteria were 680, 510, and 460 nm. The excitation wavelength was 405 nm.

**Table 1.** Bacterial cell number and density in the coral epidermis and mucus collected from Kenting and Ludao

| | Epidermis | | | Mucus | | |
|---|---|---|---|---|---|---|
| | No. of cells[a] | Area (μm²) | Density (cells/100 μm²) | No. of cells | Area (μm²) | Density (cells/100 μm²) |
| Kenting | 49.2 ± 20.3 | 1976.4 ± 533.7 | 2.5 | 104.9 ± 23.4 | 5998.7 ± 2606.7 | 2.5 |
| Ludao | 8.6 ± 4.6 | 2859.4 ± 1542.1 | 0.4 | 17.0 ± 6.3 | 5925.9 ± 1929.8 | 0.3 |

[a]Cell numbers for each sample were obtained by averaging cell counts from at least 20 microscopic fields of view ($n$ > 20).

543 nm, dye-labeled gammaproteobacteria were detected at 570 nm and zooxanthellae at 675 nm (Fig. 3).

The epifluorescence microscopic observations clearly showed that the alphaproteobacteria and gammaproteobacteria appeared in different microenvironments in corals from both sites (Fig. 4). Alphaproteobacteria were widely distributed in the epidermis and mucus, while gammaproteobacteria were mostly found in the space between the gastrodermis and the interface of the epidermis and mesoglea.

**Identification of dominant bacterial groups in the samples**

To verify the identities of the dominant bacterial species of alphaproteobacteria and gammaproteobacteria in the collected samples, the bacterial profiles in coral and seawater were revealed using DGGE (Fig. 5). According to the DGGE profile of 16S rRNA PCR products from the bacteria in corals and seawater, 37 bands were cut from the DGGE gel for sequencing to identify these bacterial species.

From Ludao, 4 bands from seawater samples were identified as SAR11 of the *Alphaproteobacteria* and *Oscillatoriales* sp. of cyanobacteria (Table 2). In Kenting seawater, 3 bands were identified as SAR11 and another band was *Nodularia* sp. of the cyanobacteria. Corals from Ludao hosted alphaproteobacteria and gammaproteobacteria which differed from the bacterial community in seawater (Table 2). However, Kenting's coral-associated bacteria were mainly betaproteobacteria and actinobacteria in

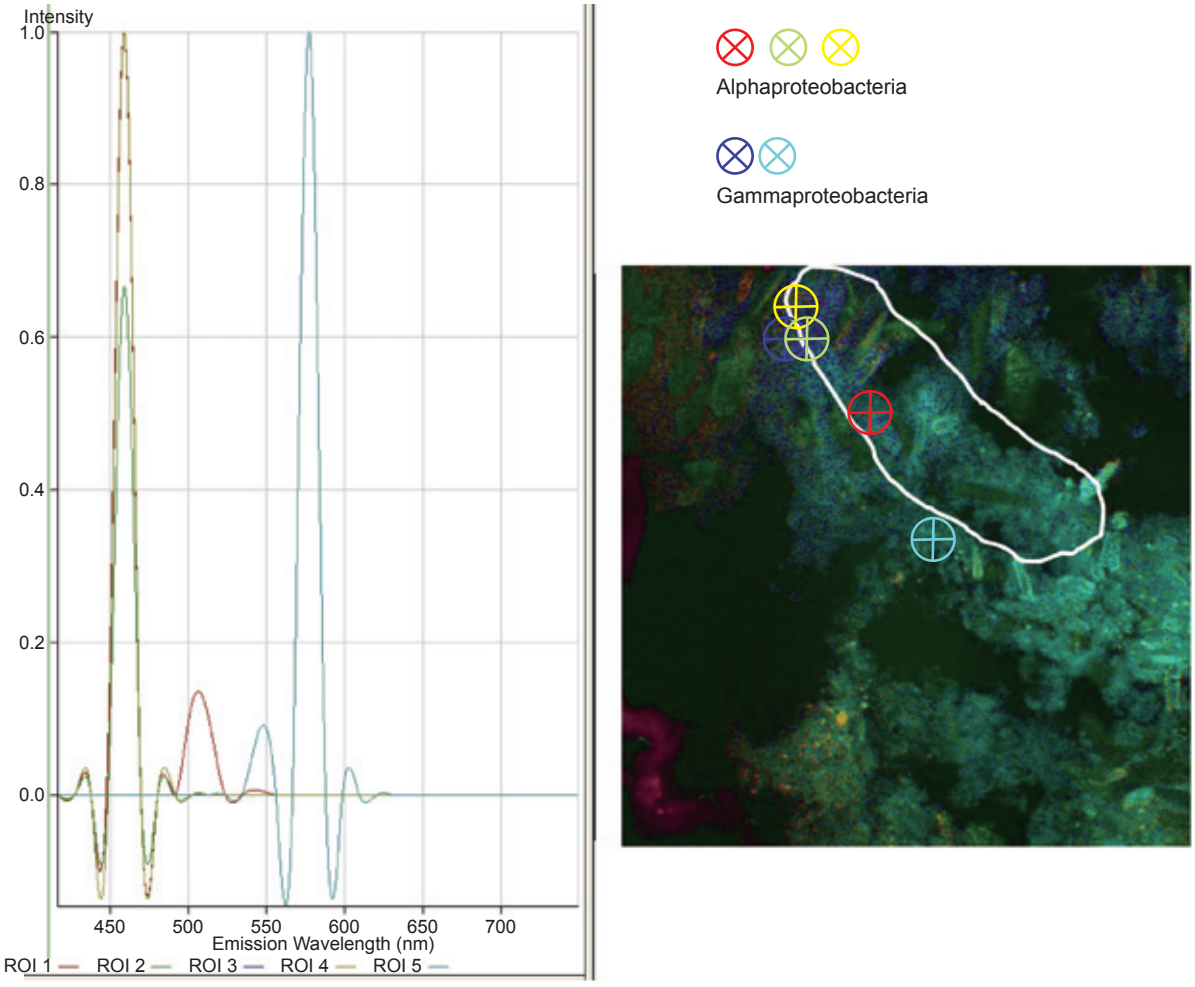


**Fig. 3.** Analysis of the confocal microscopic spectrum. Alphaproteobacteria were hybridized by the probes labeled with blue dye with absorption at 405 nm and emission at 470 nm. Gammaproteobacteria were hybridized by probes labeled with red dye with absorption at 547 nm and emission at 570 nm.

isolated bands (Table 2).

## DISCUSSION

This study provides the 1st direct evidence of the distribution of coral-associated bacteria in the mucus and epidermis of *Euphyllia glabrescens* by CARD-FISH with an improved frozen embedding method. We used the frozen embedding method to keep both living cells and the entire organization of the coral intact. CARD-FISH is recommended as a useful method for detecting bacterial groups in coral research. The present study is the 1st report applying a combined approach of the frozen embedding method and CARD-FISH to coral specimens. Our results show that 2 dominant coral-associated bacteria had dissimilar distribution patterns in the mucus and epidermis of the coral, suggesting that coral-associated bacteria have specific preferences for habitat niches inside corals.

### Distribution of 2 bacterial groups among *E. glabrescens*-associated bacteria and their potential roles

Like other coral-associated bacterial profiles, alphaproteobacteria and gammaproteobacteria were also two of the frequent bacteria in *E. glabrescens* and seawater as verified by the DGGE-16S rRNA analysis and FISH.

Alphaproteobacteria: Our results showed that alphaproteobacteria were widely distributed in both the epidermis and mucus without particular agglomeration at certain sites in *E. glabrescens*. These alphaproteobacteria, mainly *Phyllobacterium myrsinacearum*, in the coral differed from the alphaproteobacteria, SAR 11, in seawater, suggesting that there was a selective effect of coral specificity and a low exchange rate between the 2 communities of corals and seawater. This observation differs from results reported by Kooperman et al. (2007), who showed that the overlap of alphaproteobacteria in the 2 communities of corals and seawater indicated an interaction between the water and mucus. However, we found differences in alphaproteobacterial members between the coral and seawater. This inconsistency could have been caused by experimental limitations, as only a few bacterial sequences were identified by the DGGE analysis, or by a high selection for seawater bacteria to enter the epidermis and mucus of coral species (Brown and Bythell 2005, Bythell and Wild 2011); only certain alphaproteobacteria could inhabit, grow, and further participate in metabolism within coral mucus (Rohwer and Kelley 2004).

In addition, *P. myrsinacearum* might be involved in the metabolism of nitrogen-containing

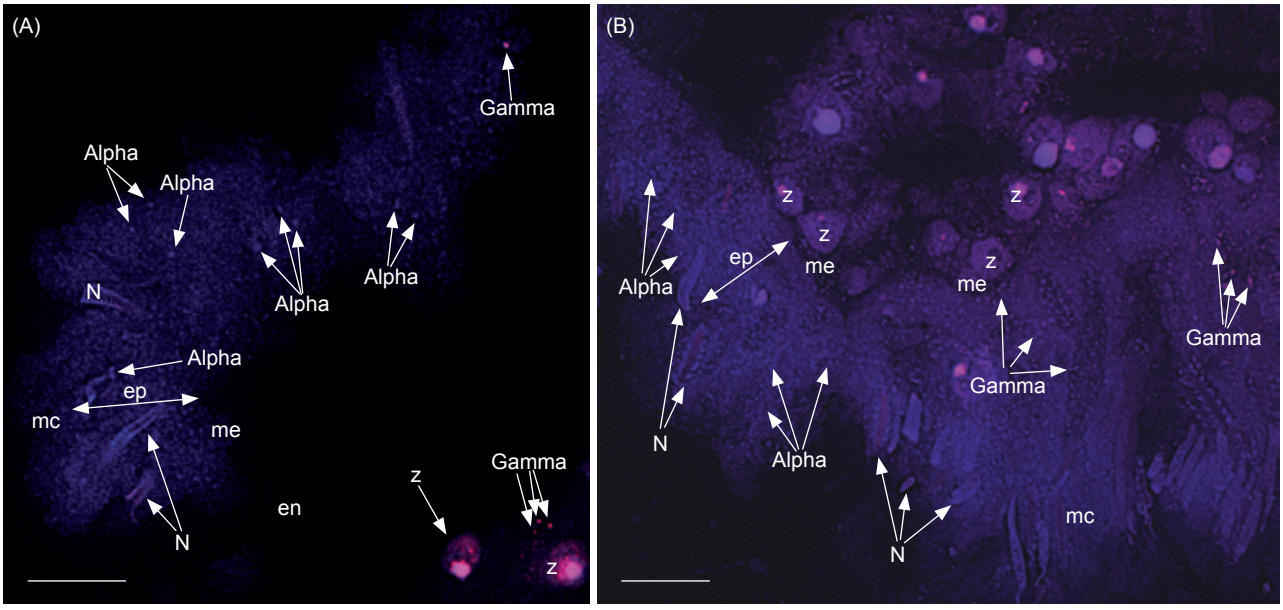


**Fig. 4.** Distributions of alphaproteobacteria and gammaproteobacteria in corals isolated from Ludao and Kenting. Alphaproteobacteria (Alpha, blue) existed in the ectoderm (ec) and mucus (mc). Many gammaproteobacteria (Gamma, red) were observed in the endoderm (en, gastrodermis), but seldom in the ectoderm. Ludao, A; Kenting, B. Scale bars = 20 μm.

compounds in the mucus, because this bacterium can symbiotically reduce nitrate and nitrite in its hosts (Mergaert et al. 2002). *Phyllobacterium myrsinacearum* was first found in plants. Yet in recent reports, this bacterium was associated with microalgae and cyanobacteria in corals. Furthermore, *P. myrsinacearum* is a nitrogen fixer and may play a role in supplying nitrogen to zooxanthellate corals which often experience a status of inorganic nitrogen limitation (Gonzalez-Bashan et al. 2000, Lesser et al. 2004).

Gammaproteobacteria: In contrast to alphaproteobacteria, gammaproteobacteria were only distributed in the gastrodermis and rarely in the internal epidermis. There were fewer gammaproteobacteria than alphaproteobacteria in the coral. This shows a difference from the common idea that gammaproteobacteria are more dominant than alphaproteobacteria in coral-associated microbial communities (Hong et al. 2009). The discrepancy might be explained by at least 2 reasons. One is the higher copy numbers of 16S rRNA genes in genomes of gammaproteobacteria compared to those of alphaproteobacteria, so that the actual population size of gammaproteobacteria is smaller (Campbell et al. 2011, Dikow 2011). The other is that many gammaproteobacteria dwell in other tissues of corals that were not evaluated in this study.

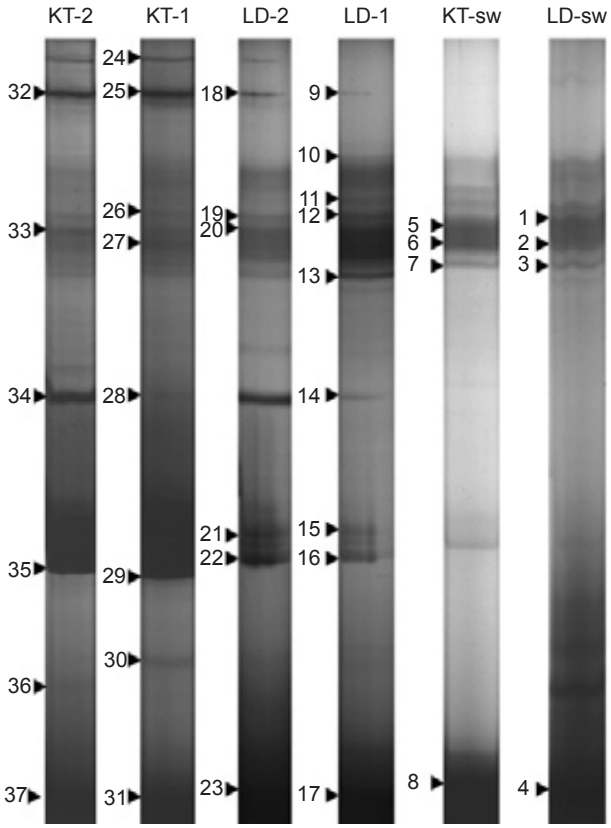


**Fig. 5.** DGGE profile of bacterial 16S rRNA genes of coral and seawater samples. Six taxonomic bacterial groups were identified from the selected DNA bands, including alphaproteobacteria (1-3, 5, 7, and 12); unclassified bacteria (4, 8, 17, 20, and 24); cyanobacteria (6); bacteroidetes (10 and 13-16); actinobacteria (9, 11, 18, and 19); and betaproetobacteria (21-23). sw, seawater; KT, Kenting; LD, Ludao.

Why did these gammaproteobacteria only appear inside the coral? While this is an interesting observation, the answer is yet unknown. However, previous studies indicated that coral-associated gammaproteobacteria seem to have a closer relationship with corals than seawater because the gammaproteobacteria community is relatively more abundant than that in the surrounding seawater (Kooperman et al. 2007, Shnit-Orland and Kushmaro 2009). Although lacking similar evidence in the comparison between seawater and corals in this study, we suggest that the interaction between coral-associated gammaproteobacteria and the coral might be close but is unlikely to be directly related to metabolism in the probiotic function of the mucus.

The community of *E. glabrescens*-associated gammaproteobacteria was composed of *Serratia marcescens* of the *Enterobacteriaceae* as the main species with a 98% similarity in its 16S rRNA sequence. These *S. marcescens*-like bacteria might contribute to fermentation or degradation of carbon-containing compounds in the anaerobic microenvironment inside corals, particularly at night (Grimont and Grimont 1994, Kühl et al. 1995). In addition, we noted that *S. marcescens* was suggested to be a pathogen of the white pox coral disease (Patterson et al. 2002). The *S. marcescens*-like bacteria detected in *E. glabrescens* could be non-pathogenic according to the coral's apparent good health.

## Geographic variations in bacterial communities in *E. glabrescens*

Population sizes of *E. glabrescens*-associated bacteria differed between the 2 sampling sites. Although the reason remains unknown, we speculated that it was caused by differences in environmental physiochemical factors. For example, the nutrient content in the seawater in the Ludao region is poorer than that at Kenting. Ludao is situated in the Kuroshio Current, the nutrient content of which is as only 1/2 that of ordinary

seawater (Baek et al. 2008). The sampling site at Kenting is located within a Kuroshio Branch Current and an upwelling region, where nutrients, inorganic phosphorus, and the primary production capability are known to be richer than at Ludao (Chen et al. 2004a b, Chen et al. 2005, Hsiao et al. 2011).

In addition, we noted that the DGGE results showed that the dominant bacteria in Kenting were dissimilar to those at Ludao. In the Kenting samples, we found no representative bands of alphaproteobacteria or gammaproteobacteria that would have been caused by an over-dominance of actinobacteria. Gammaproteobacteria and alphaproteobacteria should become relatively smaller groups; nonetheless, the results of CARD-FISH showed that they were present.

Obviously, the effect of host specificity was weak in driving the population structure of coral-associated bacteria compared to that of

**Table 2.** Identification of selected bands in the DGGE profile of 16S rRNA which was isolated from seawater and corals. Relatedness was based on a BLAST search in NCBI's nucleotide collection database and ribosomal database project. Codes of the bands are shown in figure 5

| Band no. | Source | Closest match by NCBI | Class | Sequence identity (%) | Alignment length (bp) |
|---|---|---|---|---|---|
| 1 | LD[a]-sw[b] | *SAR11* | Alphaproteobacteria | 92 | 434 |
| 2 | LD-sw | *SAR11* | Alphaproteobacteria | 86 | 449 |
| 3 | LD-sw | *Oscillatoriales* sp. | Cyanobacteria | 89 | 453 |
| 4 | LD-sw | *SAR11* | Alphaproteobacteria | 93 | 437 |
| 5 | KT[c]-sw | *SAR11* | Alphaproteobacteria | 87 | 464 |
| 6 | KT-sw | *Nodularia* sp. | Cyanobacteria | 87 | 456 |
| 7 | KT-sw | uncultured bacteria | Alphaproteobacteria | 87 | 449 |
| 8 | KT-sw | *SAR11* | Alphaproteobacteria | 96 | 480 |
| 9 | LD-coral[d] | *Corynebacterium mucifaciens* | Actinobacteria | 97 | 527 |
| 10 | LD-coral | *Serratia marcescens* | Gammaproteobacteria | 94 | 288 |
| 11 | LD-coral | uncultured bacteria | Alphaproteobacteria | 96 | 250 |
| 12 | LD-coral | *Flavobacteria johnsoniae* | Cytophaga-Flavobacteria-Bacteroides | 95 | 526 |
| 13 | LD-coral | *Phyllobacterium myrsinacearum* | Alphaproteobacteria | 93 | 526 |
| 14 | LD-coral | *Phyllobacterium myrsinacearum* | Alphaproteobacteria | 97 | 315 |
| 15 | LD-coral | uncultured bacteria | Alphaproteobacteria | 92 | 279 |
| 16 | LD-coral | *Flavobacteria* sp. | Cytophaga-Flavobacteria-Bacteroides | 85 | 529 |
| 17 | LD-coral | *Flavobacterium johnsoniae* | Cytophaga-Flavobacteria-Bacteroides | 81 | 440 |
| 18 | LD-coral | *Corynebacterium mucifaciens* | Actinobacteria | 99 | 526 |
| 19 | LD-coral | uncultured bacteria | Alphaproteobacteria | 96 | 527 |
| 20 | LD-coral | *Corynebacterium* sp. | Actinobacteria | 91 | 474 |
| 21 | LD-coral | *Corynebacterium* sp. | Actinobacteria | 99 | 459 |
| 22 | LD-coral | *Phyllobacterium* sp. | Alphaproteobacteria | 98 | 494 |
| 23 | LD-coral | *Candidatus aquirestis calciphila* | Cytophaga-Flavobacteria-Bacteroides | 99 | 541 |
| 24 | KT-coral | *Corynebacterium* sp. | Actinobacteria | 95 | 367 |
| 25 | KT-coral | *Aquabacterium* sp. | Betaproteobacteria | 84 | 515 |
| 26 | KT-coral | *Corynebacterium* sp. | Actinobacteria | 87 | 432 |
| 27 | KT-coral | *Aquabacterium* sp. | Betaproteobacteria | 94 | 534 |
| 28 | KT-coral | *Arthrobacter* sp. | Actinobacteria | 94 | 366 |
| 29 | KT-coral | *Aquabacterium* sp. | Betaproteobacteria | 85 | 519 |
| 30 | KT-coral | *Arthrobacter* sp. | Actinobacteria | 94 | 413 |
| 31 | KT-coral | *Arthrobacter* sp. | Actinobacteria | 94 | 417 |
| 32 | KT-coral | *Aquabacterium* sp. | Betaproteobacteria | 75 | 536 |
| 33 | KT-coral | *Corynebacterium mucifaciens* | Actinobacteria | 94 | 492 |
| 34 | KT-coral | *Actinopolymorpha rutilus* | Actinobacteria | 75 | 416 |
| 35 | KT-coral | *Micrococcineae* | Actinobacteria | 98 | 510 |
| 36 | KT-coral | *Propionibacterium acnes* | Actinobacteria | 96 | 543 |
| 37 | KT-coral | Corynebacterium | Actinobacteria | 93 | 543 |

The sequence lengths ranged 434-543 bp. Similarities are shown in the percentage of sequence matches. [a]LD, Ludao; [b]sw, seawater samples; [c]KT, Kenting; [d]coral, coral samples.

environmental variations in this study. There are some similar reports; for example, the compositions of *Acropora*-associated bacteria were considerably distinct between the Magnetic and Orpheus Is. on the Great Barrier Reef, Australia (Hong et al. 2009, Littman et al. 2009).

### *In situ* observations that coral mucus-associated bacteria are critical for coral microbiology

Direct observations of coral-associated bacteria could provide important information on the distribution, density, and amount in investigating the functional roles of coral-associated bacteria. Variations in microenvironments in coral mucus were suggested to be closely associated with the growth of corals and changes in the microbial composition and distribution (Koren and Rosenberg 2006, Kooperman et al. 2007, Lampert et al. 2008, Apprill et al. 2009). In the past decade, researchers widely investigated the diversity and composition of mucus-associated bacteria using culture-dependent methods (Ducklow and Mitchell 1979, Koren and Rosenberg 2006, Klaus et al. 2007, Kooperman et al. 2007, Lampert et al. 2008, Sharon and Rosenberg 2008, Nissimov et al. 2009, Shnit-Orland and Kushmaro 2009), but the distribution of bacterial associates in the mucus is still mostly unknown. Nevertheless, a number of reports emphasized the importance of the in situ distribution of coral mucus-associated bacteria (Ainsworth et al. 2007 2008). By overcoming certain technical difficulties, this study for the 1st time presents a successful example of detecting the bacterial distributions in coral mucus. Furthermore, combining our method and other techniques, such as microlaser dissection, would allow us to verify how bacteria are distributed and also what the bacteria are at any specific location in corals.

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## REFERENCES

Ainsworth TD, M Fine, G Roff, O Hoegh-Guldberg. 2008. Bacteria are not the primary cause of bleaching in the Mediterranean coral *Oculina patagonica*. Int. Soc. Microb. Ecol. J. **2:** 67-73.

Ainsworth TD, E Kramasky-Winter, Y Loya, O Hoegh-Guldberg, M Fine. 2007. Coral disease diagnostics: What's between a plague and a band? Appl. Environ. Microbiol. **73:** 981-992.

Amann R, BM Fuchs. 2008. Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. Nat. Rev. **6:** 339-348.

Apprill A, HQ Marlow, MQ Martindale, MS Rappé. 2009. The onset of microbial associations in the coral *Pocillopora meandrina*. Int. Soc. Microb. Ecol. J. **3:** 685-699.

Baek SH, S Shimode, MS Han, T Kikuchi. 2008. The influence of nutrients concentration and the radio on phytoplankton community structure during late spring and early summer in Sagami Bay, Japan. Algae **23:** 277-288. (in Korean with English summary)

Benson A, L Muscatine. 1974. Wax in coral mucus - energy transfer from corals to reef fishes. Limnol. Oceanogr. **19:** 810-814.

Brown BE, JC Bythell. 2005. Perspectives on mucus secretion in reef corals. Mar. Ecol. Progr. Ser. **296:** 291-309.

Bythell JC, C Wild. 2011. Biology and ecology of coral mucus release. J. Exp. Mar. Biol. Ecol. **408:** 88-93.

Campbell BJ, L Yu, JF Heidelberg, DL Kirchman. 2011. Activity of abundant and rare bacteria in a coastal ocean. Proc. Natl. Acad. Sci. USA **108:** 12776-12781.

Chen CC, FK Shiah, HJ Lee, KY Lee, PJ Meng, SJ Kao et al. 2005. Short-termed differential responses of biomass, production and turnover rate of phytoplankton as well as bacterioplankton in a semi-enclosed embayment with spring tide induced upwelling. Mar. Ecol. Progr. Ser. **304:** 91-100.

Chen CTA, LY Hsing, CL Liu, SL Wang. 2004a. Degree of nutrient consumption of upwelled water in the Taiwan Strait based on dissolved organic phosphorus or nitrogen. Mar. Chem. **87:** 73-86.

Chen CTA, BJ Wang, LY Hsing. 2004b. Upwelling and degree of nutrient consumption in Nanwan Bay, southern Taiwan. J. Mar. Sci. Tech. **12:** 442-447.

Coles SL, R Strathma. 1973. Observations on coral mucus "flocs" and their potential trophic significance. Limnol. Oceanogr. **18:** 673-678.

Dijk JA, P Breugelmans, J Philips, PJ Haest, E Smolders, D Springael. 2008. Catalyzed reporter deposition-fluorescent *in situ* hybridization (CARD-FISH) detection of *Dehalococcoides*. J. Microbiol. Meth. **24:** 142-147.

Dikow RB. 2011. Genome-level homology and phylogeny of *Shewanella* (Gammaproteobacteria: Iteromonadales: Shewanellaceae). Biol. Med. Centra. Genomics **12:** 237.

Ducklow HW, R Mitchell. 1979. Bacterial populations and adaptations in the mucus layers on living corals. Limnol. Oceanogr. **24:** 715-725.

Gonzalez-Bashan LE, VK Lebsky, JP Hernandez, JJ Bustillos, Y Bashan. 2000. Changes in the metabolism of the microalga *Chlorella vulgaris* when coimmobilized in alginate with the nitrogen-fixing *Phyllobacterium myrsinacearum.* Can. J. Microbiol. **46:** 653-659.

Grimont PA, F Grimont. 1994. Genus: *Serratia*. *In* JG Holt, NR Krei, PHA Sneath, JT Staley, ST Williams eds. Bergey's manual of determinative bacteriology. Baltimore: Williams & Wilkins, pp.187.

Hong MJ, YT Yu, CA Chen, PW Chiang, SL Tang. 2009.

Influence of species specificity and other factors on bacteria associated with the coral *Stylophora pistillata* in Taiwan. Appl. Environ. Microbiol. **75:** 7797-7806.

Hsiao SH, TH Fang, CT Shih, JS Hwang. 2011. Effects of the Kuroshio Current on copepod assemblages in Taiwan. Zool. Stud. **50:** 475-490.

Huettel M, C Wild, S Gonelli. 2006. Mucus trap in coral reefs: formation and temporal evolution of particle aggregates caused by coral mucus. Mar. Ecol. Progr. Ser. **307:** 69-84.

Johannes R. 1967. Ecology of organic aggregates in the vicinity of a coral reef. Limnol. Oceanogr. **12:** 189-195.

Klaus JS, I Janse, JM Heikoop, RA Sanford, BW Fouke. 2007. Coral microbial communities, zooxanthellae and mucus along gradients of seawater depth and coastal pollution. Environ. Microbiol. **9:** 1291-1305.

Kooperman N, E Ben-Dov, E Kramarsky-Winter, Z Barak, A Kushmaro. 2007. Coral mucus-associated bacterial communities from natural and aquarium environments. FEMS Microbiol. Lett. **276:** 106-113.

Koren O, E Rosenberg. 2006. Bacteria associated with mucus and tissues of the coral *Oculina patagonica* in summer and winter. Appl. Environ. Microbiol. **72:** 5254-5259.

Krupp D. 1984. Mucus production by corals exposed during an extreme low tide. Pac. Sci. **38:** 1-11.

Kühl M, Y Cohen, T Dalsgaard, BB Jørgensen, NP Revsbech. 1995. The microenvironment and photosynthesis of zooxanthellae in scleractinian corals studied with microsensors for $O_2$, pH, and light. Mar. Ecol. Progr. Ser. **117:** 159-172.

Lampert Y, D Kelman, Y Nitzan, Z Dubinsky, A Behar, RT Hill. 2008. Phylogenetic diversity of bacteria associated with the mucus of Red Sea corals. FEMS Microbiol. Ecol. **64:** 187-198.

Lesser MP, CH Mazel, MY Gorbunov, PG Falkowski. 2004. Discovery of symbiotic nitrogen-fixing cyanobacteria in corals. Science **305:** 997-1000.

Littman RA, BL Willis, C Pfeffer, DG Bourne. 2009. Diversities of coral-associated bacteria differ with location, but not species, for three acroporid corals on the Great Barrier Reef. FEMS Microb. Ecol. **68:** 152-163.

Mergaert J, MC Cnockaert, J Swings. 2002. *Phyllobacterium myrsinacearum* (subjective synonym *Phyllobacterium rubiacearum*) emend. Int. J. Syst. Evol. Microbiol. **52:** 1821-1823.

Nissimov J, E Rosenberg, CB Munn. 2009. Antimicrobial properties of resident coral mucus bacteria of *Oculina patagonica*. FEMS Microbiol. Lett. **292:** 210-215.

Patterson KL, JW Porter, KB Ritchie, SW Polson, E Mueller, EC Peters et al. 2002. The etiology of white pox, a lethal disease of the Caribbean elkhorn coral, *Acropora palmata*. Proc. Natl. Acad. Sci. USA **99:** 8725-8730.

Radojkovic D, J Kušic. 2000. Silver staining of denaturing gradient gel electrophoresis gels. Clin. Chem. **46:** 883-884.

Ritchie KB. 2006. Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. Mar. Ecol. Progr. Ser. **322:** 1-14.

Rohwer F, S Kelley. 2004. Microbial communities of coral surface mucopolysaccharide layers. *In* E Rosenberg, Y Loya, eds. Coral health and disease. Berlin: Springer-Verlag, pp. 259-264.

Schabereiter-Gurtner C, W Lubitz, S Rölleke. 2003. Application of broad-range 16S rRNA PCR amplification and DGGE fingerprinting for detection of tick-infecting bacteria. J. Microb. Meth. **52:** 251-261.

Sharon G, E Rosenberg. 2008. Bacterial growth on coral mucus. Curr. Microbiol. **56:** 481-488.

Shnit-Orland M, A Kushmaro. 2009. Coral mucus-associated bacteria: a possible first line of defense. FEMS Microbiol. Ecol. **67:** 371-380.

Wang JT, PJ Meng, YY Chen, CA Chen. 2012. Determination of the thermal tolerance of *Symbiodinium* using the activation energy for inhibiting photosystem II activity. Zool. Stud. **51:** 137-142.

Wild C, M Huettel, A Klueter, SG Kremb, MYM Rasheed, BB Jørgensen. 2004. Coral mucus functions as an energy carrier and particle trap in the reef ecosystem. Nature **428:** 66-70.

Wild C, C Mayr, L Wehrmann, S Schöttner, M Naumann, F Hoffmann, HT Rapp. 2008. Organic matter release by cold water corals and its implication for fauna-microbe interaction. Mar. Ecol. Progr. Ser. **372:** 67-75.