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The Microbiome of the Red Sea Coral *Stylophora pistillata* Is Dominated by Tissue-Associated *Endozoicomonas* Bacteria

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***Endozoicomonas* bacteria were found highly associated with the coral *Stylophora pistillata*, and these bacteria are also ubiquitously associated with diverse corals worldwide. Novel *Endozoicomonas*-specific probes revealed that *Endozoicomonas* bacteria were abundant in the endodermal tissues of *S. pistillata* and appear to have an intimate relationship with the coral.**

Reef-building corals are "metaorganisms"; i.e., the coral animal lives in a mutualistic relationship with photosynthetic, endosymbiotic dinoflagellates of the genus *Symbiodinium* along with microorganisms including bacteria, archaea, fungi, and viruses. The significance of the bacterial assemblage to the coral animal is not well understood, although coral bacteria have been characterized as species specific (1) and may have roles in nitrogen fixation, carbon fixation, antibiotic production, and other features that enable their health and functioning (1–5). A substantial component of the coral bacterial community resides within the mucus layer (6, 7), and there is little understanding of which microbial partners are actually in residence within the coral tissues and potentially interacting with the coral. Here, we address this lack of knowledge by examining if and how a dominant group of bacteria frequently recovered in sequencing-based studies is located internally within the coral tissues of the Red Sea coral *Stylophora pistillata*. This study used Sanger and 454 pyrosequencing of the bacterial 16S rRNA gene to document the bacterial community of *S. pistillata* and a fluorescence *in situ* hybridization (FISH) approach to examine if the dominant bacteria (*Endozoicomonas*) associated with *S. pistillata* reside within the coral. Additional analyses of other Red Sea corals, as well as an *in silico* analysis of worldwide corals, were used to examine the prominence of *Endozoicomonas* in other coral species.

Five *S. pistillata* samples (Sp1 to Sp5) were collected in the southern Red Sea in June 2009 by scuba diving at depths between 2 and 5 m at five sites (see Fig. S1 in the supplemental material). DNA was extracted from airbrushed tissue with the PowerPlant DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA), with some modifications (8). Several primer pairs were tested prior to 454 pyrosequencing to ensure specificity to bacterial DNA (see the supplemental material), and primers 784F and 1061R (9), which include variable regions 5 and 6 of the 16S rRNA gene, were used for this analysis. Libraries were generated with the GS FLX Titanium emPCR kit (Lib-A; Roche, Branford, CT) and sequenced by Titanium FLX chemistry. Data analysis was conducted with the mothur software v.1.24.1 (10). Sequencing resulted in 287,488 reads, of which 131,421 remained (median length, 250 bp) after cleaning (Table 1). The sequences were clustered into operational taxonomic units (OTUs) at 97% similarity and classified against the 2011 version of the GreenGenes database (11) as described by Wang et al. (12), with a bootstrap cutoff of 60%. Rarefaction curves demonstrate that for samples Sp4 and Sp5, most of the diversity has been sampled but curves did not plateau for the other samples (see Fig. S2 in the supplemental material). The evenness was very low, indicating that few bacterial OTUs make up the majority of the microbiome (Table 1). In fact, two OTUs dominated the bacterial community in *S. pistillata*, and they were classified in the genus *Endozoicomonas* of the order *Oceanospirillales* and the genus *Burkholderia* of the order *Burkholderiales* (Fig. 1A).

Full-length sequences were also obtained from each sample (Sp1 to Sp5, 86 to 94 clones per sample after chimera removal; see the supplemental material) and yielded bacterial community composition results similar to those of the 454 data (Fig. 1A). Phylogenetic analyses indicated that the *Endozoicomonas* OTUs obtained in this study all clustered with cultivated species (*Endozoicomonas numazuensis*, *E. montiporae*, and *E. elysicola*), as well as other *Endozoicomonas* sequences from a diverse range of marine invertebrates, including many species of reef-building and gorgonian corals (see Fig. S3 in the supplemental material). The closest relative of the *Burkholderia* sequences was isolated from a white-rot fungus (see Fig. S4 in the supplemental material), suggesting that the *Burkholderia* bacteria found on *S. pistillata* were associated with a fungus on the coral.

In order to examine the ubiquity of *Endozoicomonas* bacteria in other coral species from the Red Sea, healthy specimens of *Acropora humilis* and *Pocillopora damicornis* (three of each species) were obtained from the same area as the *S. pistillata* samples (see Fig. S1 in the supplemental material). Bacterial small-subunit (SSU) rRNA genes were examined by cloning and sequencing ($n = 20$ to 102 per sample after chimera check and contaminant removal; see the supplemental material). *Endozoicomonas* bacteria

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TABLE 1 Summary statistics for 454 sequencing of SSU rRNA genes from *S. pistillata*[a]

Sample	No. of reads	No. of reads subsampled	No. of OTUs observed	Inverse Simpson	Shannon index	Chao1 richness	ACE richness	Simpson evenness	Sampling site
Sp1	18,676	18,676	418	2.51	1.88	845.5	1,250.5	0.006	5
Sp2	29,292	18,676	465	3.59	2.02	895.8	1,361.8	0.0077	12
Sp3	22,954	18,676	276	2.57	1.62	578.8	790.9	0.0093	14
Sp4	35,588	18,676	139	1.66	0.80	387.1	837.6	0.012	17
Sp5	24,911	18,676	147	1.54	0.90	218.9	205.9	0.0105	15

[a] Statistics are based on 18,676 subsampled reads.

accounted for 70 to 95% of the bacterial abundance in *P. damicornis* and *A. humilis* (Fig. 1B). Each coral species was associated with one to three *Endozoicomonas* OTUs (see Fig. S3 in the supplemental material).

To determine how prevalent *Endozoicomonas* bacteria are in other corals, we performed an *in silico* analysis of full- and partial-length *Endozoicomonas* SSU rRNA gene sequences, which revealed that these bacteria associate with 14 species of scleractinian corals (Table 2) (8, 13–19). The fact that sequences were recovered from corals over vast geographic regions suggests that *Endozoicomonas* bacteria probably have an important relationship with corals. Interestingly, none of the *Endozoicomonas* OTUs were detected in more than one species, suggesting that each coral harbors its own unique *Endozoicomonas* strain. Additionally, closely related OTUs may associate with the same coral species in different geographic regions. For example, *Endozoicomonas* sequences recovered from *P. damicornis* in the Red Sea (this study) formed a clade with sequences from *P. damicornis* from the Great Barrier Reef, Australia (13) (see Fig. S3 in the supplemental material). One of the *Endozoicomonas* OTUs from *A. humilis* (this study) also clustered with an *Endozoicomonas* isolate from an acroporid in the Caribbean (8) (see Fig. S3). These results suggest coevolution of *Endozoicomonas* bacteria and coral, an important strategy to maintain their association over time and space.

To further investigate the relationship of *Endozoicomonas* bacteria and *S. pistillata*, FISH experiments were conducted with

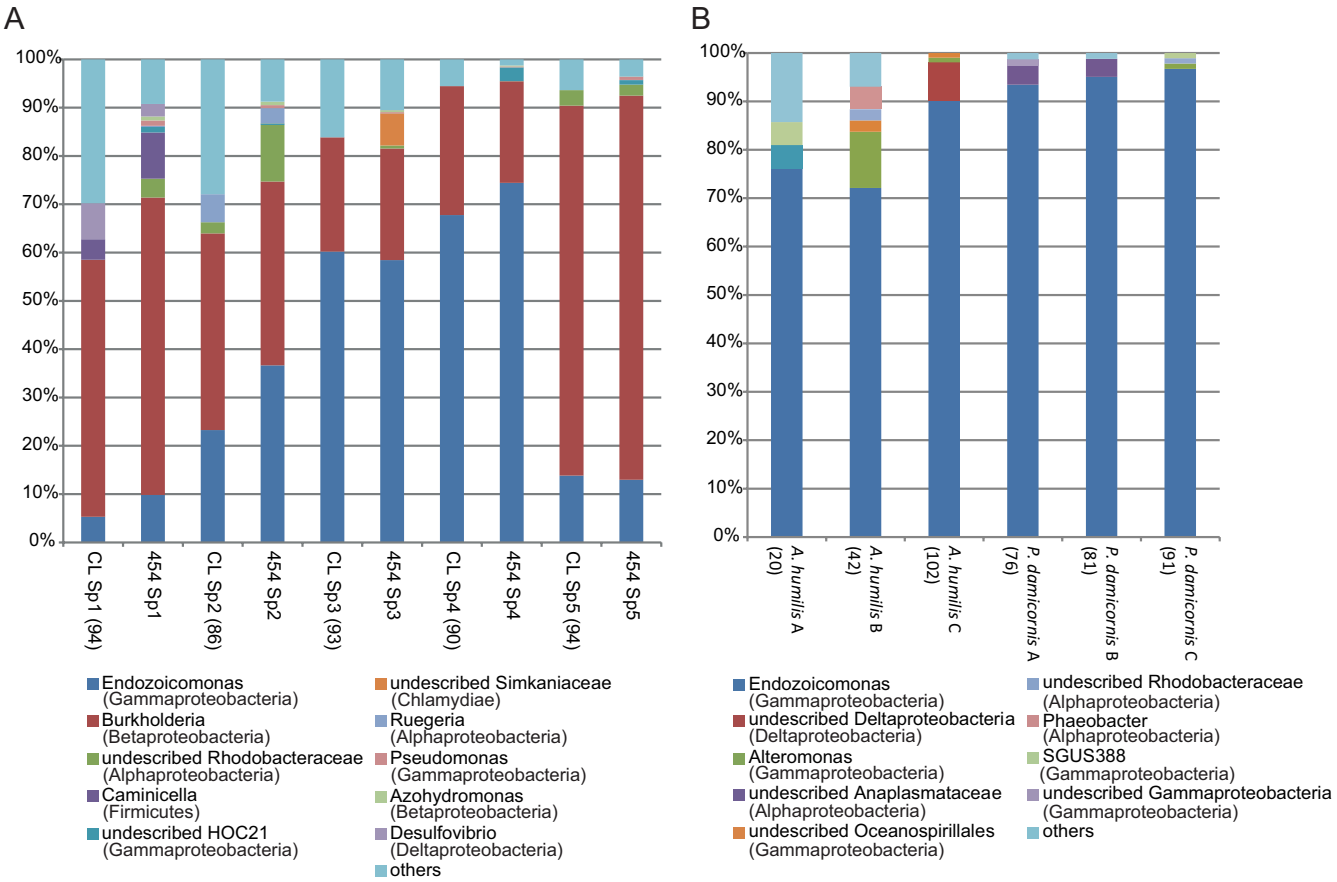

FIG 1 Phylogenetic distribution of coral-associated bacteria. Sequences were classified to the genus level with a minimum bootstrap value of 60%. Read counts from genera other than the 10 most common ones are summarized in the "others" category. The number of clones analyzed is shown in parentheses, and the number of reads analyzed by 454 sequencing is shown in Table 1. (A) *S. pistillata* (Sp1 to Sp5; $n = 5$)-associated bacteria detected by cloning and sequencing (CL) and 454 sequencing (454). (B) *A. humilis* (A to C; $n = 3$)- and *P. damicornis* (A to C; $n = 3$)-associated bacteria detected by cloning and sequencing.

TABLE 2 Summary of *Endozoicomonas* sequences recovered from corals

Coral species	Location(s)	Reference(s)
Acropora palmata	Panama	8
Acropora hemprichii	Red Sea	14
Acropora humilis	Red Sea	This study
Ctenactis echinata	Red Sea	C. Roder et al.,[b] unpublished data
Ctenactis crassa	Red Sea	16
Diploria strigosa	Panama, Curacao	8, 18
Herpolithia limax	Red Sea	16
Montastrea faveolata	Panama, Puerto Rico	8, 15
Montastrea franksi	Panama	8
Montipora aequituberculata	Taiwan	17
Pocillopora damicornis	GBR,[a] Red Sea	13, this study
Porites asteroidetes	Panama	8
Porites compressa	Hawaii	19
Stylophora pistillata	Red Sea	This study

[a] GBR, Great Barrier Reef.
[b] C. Roder, T. Bayer, M. Aranda, M. Kruse, C. Walcher, and C. R. Voolstra.

novel *Endozoicomonas*-specific oligonucleotide probes (see the supplemental material). Five or six specimens from colonies Sp1 to Sp5 were examined by imaging in three or four areas of each specimen and compared with control specimens (no probe, NON338 nonsense probe) of each respective colony. In all of the colonies examined, the *Endozoicomonas* probes were found to hybridize to cells located in close proximity to *Symbiodinium* cells (Fig. 2A and C) and within the autofluorescent coral tissues, suggesting that they reside within the coral endoderm. *Endozoicomonas* cells were arranged in multiple dense aggregates generally containing 10 to 50 cells and represented the majority of the cells that hybridized to the general bacterial probe (Fig. 2B and D). These are the first observations of *Endozoicomonas* in association with corals, but the aggregations do resemble the "ovoid bacterial clusters" that have been previously identified with corals (20–22). Hybridization of the *S. pistillata* samples with the NON338 probe resulted in the probe hybridizing to aggregates similar to the *Endozoicomonas* probed cells (Fig. 2E), but the intensity and abundance of the apparent nonspecific probe binding were much lower than those of the specific probes (Fig. 2A to D). The nonspecific binding to portions of the aggregates, as well as to coral nematocysts (Fig. 2F), suggests that an adhesive-type substance may surround the aggregates. Aggregates were also dimly visible in the no-probe controls at the same intensity as the autofluorescent coral tissues (not shown), and this suggests that the cells are embedded within the host endoderm.

Endozoicomonas bacteria appear to have an intimate and estab-

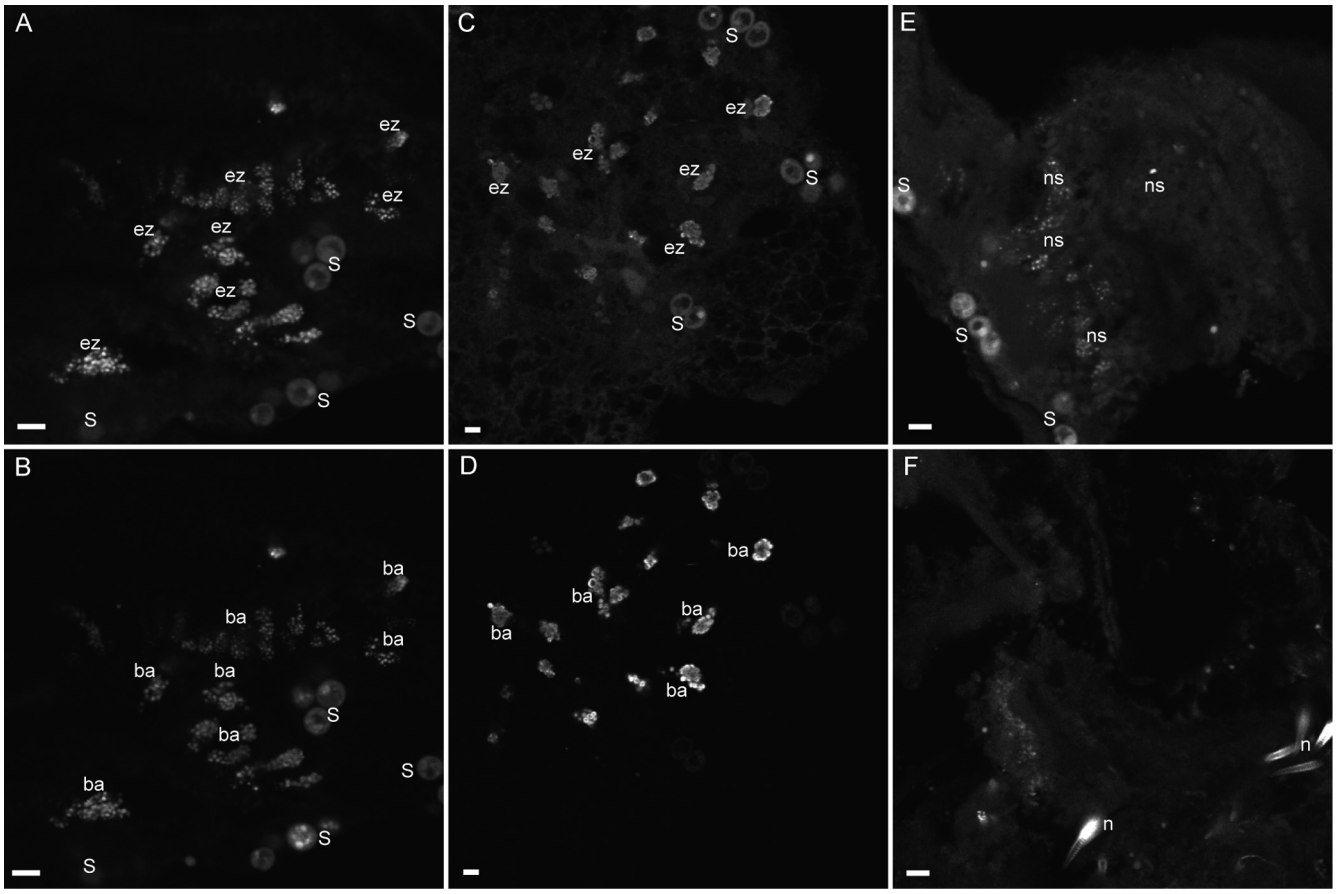

FIG 2 FISH analysis. The mixed *Endozoicomonas* probes (Cy3, selected aggregates designated "ez") within the tissues of *S. pistillata* (A, C) display a hybridization pattern similar to that of the general EUB338 bacterial probe (Cy5, selected aggregates designated "ba") (B, D). Some cells in aggregates from similar specimens were also illuminated by the nonspecific probe NONEUB (Cy3, selected nonspecificity labeled "ns") (E), and nematocyst cells (labeled "n") were also illuminated by the NONEUB probe (F). *Symbiodinium* cells (S) are autofluorescent at the imaging wavelengths used (except in panel D, where cells faded under imaging). Each image is a compilation of two to four optical slices. The scale bars are approximately 8 μm.

lished relationship with many Red Sea corals and other corals worldwide. One feature of *Endozoicomonas* bacteria associated with *S. pistillata* is that they may produce quorum-sensing molecules (reviewed in reference 23). The dense cell aggregations found suggest that reaching a critical mass may provide an advantage to the cells. Despite our limited knowledge of *Endozoicomonas* bacteria, they appear to be an important group of bacteria that require further investigation of their potential role in the functional system of the coral holobiont, as well as their interactions with other invertebrate associates.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the NCBI Sequence Read Archive under accession number PRJNA189184 and in the GenBank database under accession numbers KC668414 to KC669277.

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