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A microbial community analysis of the octocoral *Eunicea fusca*

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Abstract While there is a significant and growing body of knowledge describing the microbial communities of marine invertebrates such as sponges, there are very few such studies focused on octocorals. The octocoral *Eunicea fusca* is common on reefs in various regions of the Caribbean and has been the subject of natural product investigations. As part of an effort to describe the microbial community associated with octocorals, a culture-independent analysis of the bacterial community of *E. fusca* was conducted. Specifically, a 16S rDNA clone library analysis was performed to provide baseline data. A total of 40 bacteria members from 11 groups were found. In general, Proteobacteria were the dominant group with a total of 24 species and α-Proteobacteria represented the highest percentage of bacteria associated with *E. fusca* (27.5%). Other prominent groups observed were Acidobacteria, Actinobacteria, Cyanobacteria, Planctomycetes, δ-Proteobacteria, Lentisphaerae and Nitrospirae. This is the first analysis of bacterial populations associated with the gorgonian *E. fusca*.

Keywords: 16S rDNA, bacteria, culture-independent analysis, *Eunicea fusca*, microbial community, octocoral

INTRODUCTION

The octocoral *Eunicea fusca* (Subclass Octocorallia, Order Gorgonacea, Family Plexauridae) is the source of anti-inflammatory compounds such as fuscol and the fuscosides A and B. These act as selective inhibitors of leukotriene synthesis (Shin and Fenical, 1991; Jacobson and Jacobs, 1992a; Jacobson and Jacobs, 1992b). As exemplified by *E. fusca*, a variety of structurally unique, bioactive compounds have been isolated from octocorals (Faulkner, 2002). It has been suggested that bacteria living in symbiosis with these organisms have an important role in the production of these bioactive compounds (Radjasa and Sabdono, 2009). For instance, a *Vibrio* sp. isolated from the surface of the soft coral *Sinularia polydactyla* is the producer of Aqabamycins A-G, novel metabolites with antibacterial and cytotoxic activities (Al-Zereini et al. 2010). Even though octocorals are well known for their secondary metabolites, their associated microbial communities are relatively understudied in comparison to other well-studied marine invertebrates such as sponges and scleractinian corals (Enticknap et al. 2006; Littman et al. 2010).

In addition to their possible involvement in secondary metabolite production, symbiotic bacteria may also contribute to the coral's physiology and health (Rosenberg et al. 2007; Shnit-Orland and Kushmaro, 2009). Since corals have no adaptive immune system, bacteria associated with healthy corals are believed to play an important role in innate immunity (Reshef et al. 2006). For instance, it has been hypothesized that the microbial community of the mucopolysaccharide surface layer of *Gorgonia ventalina* plays a role in protection against disease by occupying niches that otherwise might be inhabited by pathogens, and also by producing antimicrobial compounds (Gil-Agudelo et al. 2006).

Bacterial biofilms have been implicated in promoting larvae metamorphosis of the scleractinian corals (Webster et al. 2004). It is possible that some of these bacteria can enter into a symbiotic relationship with the developing coral. Some of the most exciting and recent studies are examining the relationship between coral disease and bacterial population changes. For instance, microbial communities associated with *Acropora millepora* were shown to change during bleaching events shifting to *Vibrio*-dominated communities just prior to visual bleaching signs (Bourne et al. 2008). Changes in bacterial populations associated with bleached or azooxanthellate corals (Koren and Rosenberg, 2006) and with diseased corals have also been reported (Sunagawa et al. 2009). Therefore, an understanding of microbial communities in the relatively unstudied octocorals is of great importance.

The small number of studies that have examined the microbial assemblages of soft corals have reported a predominance of γ-Proteobacteria (Brück et al. 2007; Santiago-Vázquez et al. 2007). Studies that have examined the culturable bacteria of soft corals have also observed an abundance of γ-Proteobacteria followed closely by α-Proteobacteria (Ivanova et al. 2005) in addition to a minor presence of Bacteroidetes in the soft coral *Paragorgia arborea* (Nedashkovskaya et al. 2005). Interestingly, an examination of bacteria associated with *Plexaura fusifera* showed that unbleached corals were dominated by low G+C Firmicutes whereas bleached corals were dominated by γ-Proteobacteria demonstrating the specificity of these relationships (Frenz-Ross et al. 2008). While little is known about bacterial communities in octocorals, scleractinian coral-bacteria associations have been found to be specific (Rohwer et al. 2002; Lema et al. 2012).

The goal of this study is to examine the bacterial communities closely associated with *E*. *fusca* by culture-independent techniques (CIT; 16S rDNA clone library) to provide a baseline for other studies. CIT approaches are known to provide a broader analysis of bacterial populations when compared to culture dependent studies. It has been estimated that only 0.1% to 1% of microorganisms can be cultured in the laboratory (Jannasch and Jones, 1959). The DNA of the bacteria was amplified by universal eukaryotic 16S rDNA primers and a clone library was constructed. Restriction fragment length polymorphism (RFLP) analysis was used as a dereplication tool. Subsequently, PCR amplicons of interest were sequenced and analyzed. In accordance with previous observations from other soft corals, it was shown that γ-Proteobacteria were the most prominent bacteria observed. A study of the microbial ecology of this coral will contribute to the identification of potential producers of secondary metabolites and will provide a preliminary baseline of the bacterial community of the coral in its healthy state.

MATERIALS AND METHODS

Supplies

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). General laboratory supplies were obtained from Fisher Scientific (Suwannee, GA, USA). Pre-formulated bacterial broths and agars were supplied by Difco Laboratories (Detroit, MI, USA).

Coral sample collection

Eunicea fusca was collected at Hillsboro Ledge, Deerfield Beach, Florida, USA, on April 2005 by SCUBA diving at depths of approximately 10 m. Coral colonies were handled with gloves and stored in individual plastic bags. Individual colonies were rinsed several times with sterile seawater, immediately flash frozen in liquid nitrogen, and stored at -80ºC until DNA extraction was performed.

DNA extraction

Genomic DNA (gDNA) was extracted from 100 mg of flash frozen *E. fusca* using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). *E. fusca* was ground with liquid nitrogen using a sterile mortar and pestle. The coral slurry was transferred to a sterile 1.5 mL centrifuge tube and DNA was extracted according to manufacturer's instructions. Samples were eluted 2X in a total of 60 µL of ddH_2O (double distilled water). Extracted DNA was analyzed by gel electrophoresis. Samples were loaded, along with the appropriate molecular weight markers, on a 1.5% (w/v) agarose gel, 1X TBE (Tris-Borate EDTA) buffer, pH 8.3 and 1 µg/µL ethidium bromide. Electrophoresis was performed at 100V for 60 min. DNA

bands were visualized in a Typhoon 9410 Imager (GE Healthcare, Piscataway, NJ, USA). DNA concentration was quantified by reading the absorbance at 260 nm on a SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA, USA).

Amplification and purification of 16S rDNA

Purified DNA from coral tissue was diluted to 1:10, 1:100, and 1:1000 of the original concentration with ddH_2O. These dilutions were used for 16S rDNA amplification with universal eubacterial primers FC27 (5'- AGAGTTTGATCCTGGCTCAG-3') and RC1492 (5'TACGGYTACCTTGTTACGACTT3'; Mincer et al. 2004). Each 50 µL PCR reaction consisted of 1 µL template DNA at the different concentrations, 25 µL Go Taq Green Master Mix (Promega, Valencia, CA, USA), 1 µL of each primer, and 22 µL of ddH_2O. PCR cycling conditions were as follows: initial denaturation at 95ºC for 5 min, followed by 35 cycles of 95ºC for 30 sec, 55ºC for 30 sec, 72ºC for 1.5 min. A final extension of 7 min at 72ºC was added. PCR products were confirmed by gel electrophoresis. PCR amplicons of the anticipated size (~1.5kb) were selected for cloning. The PCR products were eluted from the gel with the MinElute Gel Extraction Kit (Qiagen) following manufacturer's instructions. Samples were eluted twice in a total of approximately 10 µL. Two µL of the eluted product was loaded on a 1.2% agarose gel alongside a quantifying ladder 1 kb Plus DNA Ladder E-Gel® DNA markers (Invitrogen, Carlsbad, CA) to estimate quantity of the eluted PCR amplicon in preparation for cloning.

16S rDNA cloning

The 16S rDNA fragments were cloned using the TOPO TA Cloning Kit for Sequencing according to the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA, USA). A 100 µL aliquot from each transformation was plated onto pre-warmed selective LB plates containing Ampicillin (50 mg/mL) and incubated over night at 37ºC. A minimum of 96 clones from each cloning plate were randomly selected and PCR-amplified using M13F (GTAAAACGACGGCCAG) and M13R (GTAAAACGACGGCCAGT) primers following manufacturer's cycling conditions. PCR products were separated by 1.5% (w/v) agarose gel electrophoresis and visualized on a Typhoon. Clones with the correct amplicon showed a single band at approximately 1700 bp. The number of clones ultimately picked was determined by the subsequent diversity observed by RFLP.

Restriction Fragment Length Polymorphism (RFLP)

The 16S rDNA PCR clones were digested with the restriction enzyme *Hha*I (Promega, Madison, WI, USA). The restriction digest reaction contained 5 µL PCR product, 0.1 µL *Hha*I (2 U/µL), 1 µL reaction buffer, 0.1 µL BSA, and 3.8 µL sterile ddH_2O and was incubated at 37ºC for 1 hr and at 65ºC for 10 min. The resulting fragments were loaded on a 1.2% agarose gel and analyzed by electrophoresis as detailed above. Restriction patterns and OTUs (operational taxonomic units) were compared and 2-3 clones that displayed different patterns were chosen for sequencing analysis.

DNA sequencing and analyses

Clones with unique RFLP patterns were sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Forster City, CA, USA) on an ABI 3100 automated sequencer (Applied Biosystems) according to the manufacturer's instructions. Alternatively, sequences were sent to BioAnalytical Services Laboratory, Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD, USA.

Sequences were viewed and edited using Chromas Lite 2000 (Technelysium, Tewantin, Australia; www.technelysium.com.au/chromas.html) and EditSeq (Lasergene, DNASTAR, Madison, WI, USA). Contiguous (contigs) sequences were built by Seqman (Lasergene).

16S rDNA sequence comparison

The 16S rDNA sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis at the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov). The

nucleotide sequences of the 16S rDNA gene sequences reported are available in GenBank under accession numbers EF657844 to EF657883.

Phylogenetic analysis

The 16S rDNA sequences of the clones were aligned and a using MegAlign (Lasergene) and the ClustalW algorithm. Subsequently, a phylogenetic tree of the 16S rDNA sequences was constructed using Phylip and the neighbour-joining method. Each reconstructed group was statistically evaluated by bootstrapping with a 1000 replicates (Nei and Kumar, 2000).

RESULTS AND DISCUSSION

This study analyzed the microbial community of bacteria associated with *Eunicea fusca*. There is evidence demonstrating that bacteria may be the source of biologically active natural products initially isolated from octocorals and in other marine organisms (Unson et al. 1994; Radjasa and Sabdono, 2009; Al-Zereini et al. 2010; Donia et al. 2011) and thus it has become a priority to gain a detailed understanding of the microbial communities in such systems. This knowledge may lead to an efficient and sustainable supply of these compounds that does not rely on harvesting coral tissue from the environment. In addition, the physiological role of these bacteria in the octocoral host is not understood.

A 16S rDNA clone library was constructed from an *E. fusca* genomic DNA sample. The resulting transformants were screened by RFLP analysis with *Hha*I as the restriction endonucleases that allowed for the clustering of similar clones. This analysis analyzed a total of 310 clones with the presence of 40 unique OTUs. Representatives of these clones were chosen for sequence analysis.

The taxonomic relatedness of the bacteria associated with *E*. *fusca* clones was deduced by the construction of a phylogenetic tree inferred from comparative sequence analyses of the 16S rDNA sequence from each clone (Figure 1 and Table 1). Based on this comparison, eleven distinct groups were observed with representatives from the sub-divisions α-Proteobacteria (27.5%), β-Proteobacteria (15.0%), γ-Proteobacteria (15.0%), Planctomycetes (12.5%), CFB (12.5%), Lentisphaerae (5.0%), Actinobacteria (2.5%), δ-Proteobacteria (2.5%), Acidobacteria (2.5%), Nitrospirae (2.5%), and Cyanobacteria (2.5%). Unpublished results from our group analyzing *E. fusca*'s culturable bacteria showed the collection of isolates was dominated by α-Proteobacteria (77%) followed by γ-Proteobacteria (13%), β-Proteobacteria (7%), and the division Cytophaga-Flexibacter-Bacteroidetes (CFB; 3%). In both cases bacterial populations were dominated by Proteobacteria in agreement with previous reports for other octocorals (Harder et al. 2003; Ivanova et al. 2005; Brück et al. 2007; Santiago-Vázquez et al. 2007; Webster and Bourne, 2007).

A survey of the identified taxa revealed candidates that hold promise as potential producers of bioactive secondary metabolites. These include the γ-Proteobacteria EF0605 (Accession no. EF657879). Evidence suggests that the related marine bacterium *Pseudomonas rhizosphaerae* may be the producer of potent antibacterial and antilarval secondary metabolites (Qi et al. 2009). Similarly, *Pseudomonas* spp. isolated from marine sources were found to be the source of potent antimicrobial compounds (Needham et al. 1994; Charyulu et al. 2009). A second candidate is the γ-Proteobacteria EF1505 (EF657861). This bacterium is related to *Candidatus* Endobugula sertula, the endosymbiont bacterium of the bryozoan *Bugula neritina* and also the known source of the bryostatins, a family of macrocyclic lactones with anticancer activity (Davidson et al. 2001). Finally, EF0705 (EF657868) and EF3305 (EF657846) showed a close phylogenetic association to actinobacteria. Marine actinomycetes are well known prolific sources of secondary metabolites (Lam, 2006).

Coral bacteria are believed to play an important role in the host's health. Corals are known to have symbiotic relationships with nitrogen-fixing bacteria (diazotrophs; Lema et al. 2012) therefore bacteria were examined for their potential involvement in the nitrogen cycle. A group of five α-Proteobacteria, EF2205 (EF657848), EF2405 (EF657850), EF2505 (EF657870), EF2605 (EF657871), and EF3905 (EF657852) were found to be related to *Pseudovibrio denitrificans* (AY486423). This coastal seawater organism is a facultative anaerobe capable of denitrification (Shieh et al. 2004). This process may allow bacteria to contribute to the health of the host by removing excess nitrate, and therefore controlling the population of the symbiotic dinoflagellate *Symbiodinium*. Excess *Symbiodinium* and the

subsequent production of harmful reactive oxygen species (ROS) are contributing factors to coral bleaching (Downs et al. 2002; Merle et al. 2007). Another isolate potentially involved in the Nitrogen cycle of *E. fusca* is the Nitrospirae EF0705 (EF657868). This bacterium is related to *Nitrospira marina*, a chemolithotrophic nitrite-oxidizing bacterium (Watson et al. 1986). In addition to nitrogen, bacteria involved in the carbon cycle are known to be associated with corals (Wegley et al. 2007). The species EF0505 (EF657862), part of the CFB group, is closely related to CFB found in coastal ocean bacterioplankton known to express transporter genes in response to DOC and therefore contribute to carbon turnover (Poretsky et al. 2010). Excess dissolved organic carbon (DOC) is detrimental to the health of corals (Kline et al. 2006).

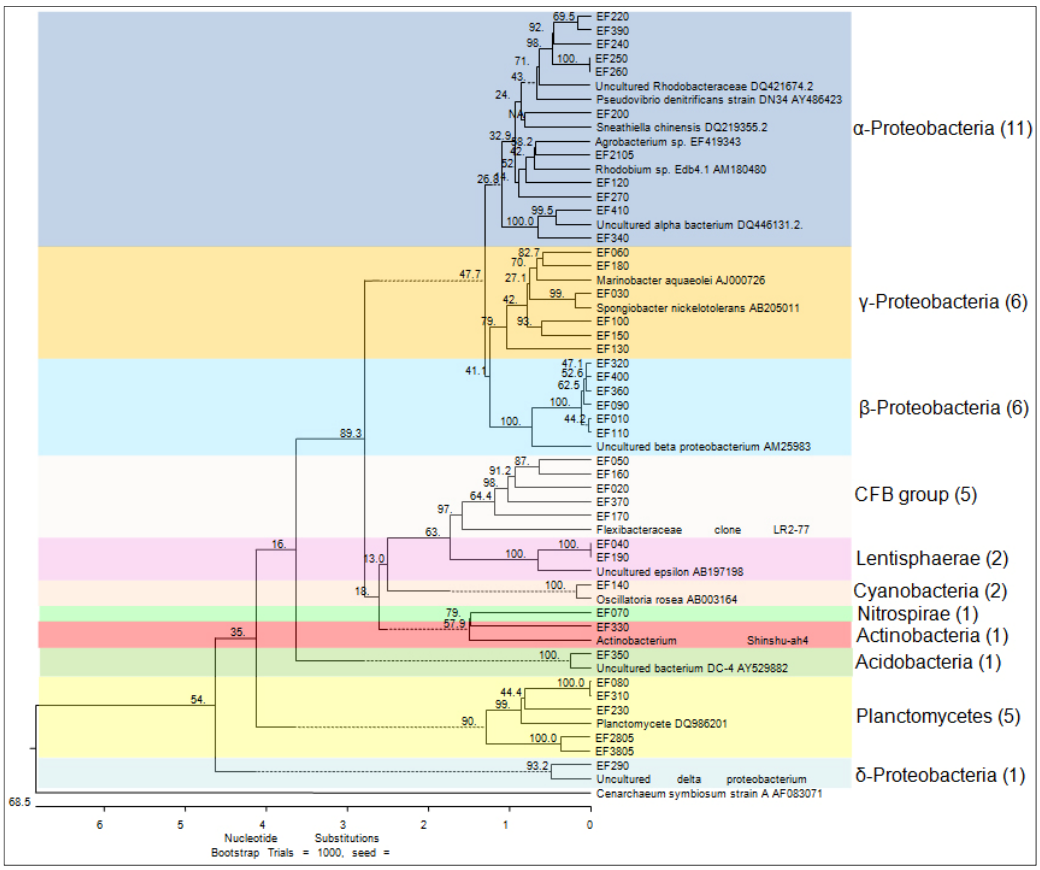

Fig. 1 Phylogenetic tree showing the phylogenetic relationships between *Eunicea fusca*'s associated bacteria as determined by 16S rDNA sequence analysis. Numbers at nodes are percentages indicating levels of bootstrap support, based on neighbour-joining analysis of 1000 re-sampled data sets. Reference sequences derived from GenBank entries are written with their corresponding accession numbers.

Our findings concur with data reported for other octocorals (Brück et al. 2007; Santiago-Vázquez et al. 2007) and suggest that a more in depth assessment using other techniques such as fluorescent *in situ* hybridization (FISH) and next generation sequencing is warranted. Such tools can be used to examine how these associations vary with geographical location and health state of *E. fusca*.

It can be concluded from this initial study that *E. fusca* hosts a diverse and complex assemblage of coral-associated bacteria dominated by Proteobacteria, many of which are only distantly related to previously described bacteria. This is the first study of the microbial community associated with the coral *E. fusca* which provides a baseline for further studies in the areas of coral health and natural products.

Table 1. *Eunicea fusca*'s bacterial associates.

Phylogenetic Association	Clone	Accession no.	Closest GenBank Homologue and Accession no.	% Similarity
α-Proteobacteria	EF2105	EF657847	Uncultured Rhizobiales bacterium **AB294974**	95
	EF2205	EF657848	Uncultured Alpha Proteobacterium clone Cobs2TisF11 **EU246824**	97
	EF2005	EF657849	Uncultured Alpha Proteobacterium **AJ581348**	95
	EF2405	EF657850	Uncultured Alpha Proteobacterium clone FII-AN025 **JQ579966**	93
	EF3405	EF657851	Uncultured Rhizobiales bacterium clone B255_A3 **EF092222**	93
	EF3905	EF657852	Uncultured Alpha Proteobacterium clone BBD_217_35 **DQ446160**	99
	EF4105	EF657853	Uncultured bacterium clone LX E63 **JF514279**	96
	EF2505	EF657870	Uncultured Rhodobacteraceae bacterium clone MD3.59 **FJ425647**	95
	EF2605	EF657871	Uncultured Rhodobacteraceae bacterium clone MD3.59 **FJ425647**	95
	EF2705	EF657881	Uncultured Alpha Proteobacterium **EU544719**	88
	EF1205	EF657882	Uncultured Alpha Proteobacterium clone LC1-25 **DQ289899**	94
β-Proteobacteria	EF0905	EF657857	Uncultured bacterium clone Mann16S_H10 **FJ952693**	91
	EF0105	EF657858	Uncultured bacterium clone Mann16S_H10 **FJ952693**	92
	EF1105	EF657859	Uncultured bacterium clone Mann16S_H10 **FJ952693**	94
	EF4005	EF657860	Uncultured bacterium clone transformant11 **GU799620**	92
	EF3205	EF657877	Uncultured bacterium clone **GU799620**	92
	EF3605	EF657878	Uncultured bacterium clone Mann16S_H10 **FJ952693**	93
γ-Proteobacteria	EF0305	EF657844	Uncultured *Spongiobacter* sp. clone EC79 **DQ889911**	96
	EF1505	EF657861	*Endobugula sertul*a strain BnSP **AF006606**	91
	EF1805	EF657865	Uncultured Gamma Proteobacterium clone XE2E10 **JN596612**	92
	EF0605	EF657879	Uncultured *Pseudomonas* sp. clone VHS-B4-54 **DQ395002**	91
	EF1005	EF657880	Uncultured bacterium clone QSW4 **JQ347469**	96
	EF1305	EF657883	Uncultured Gamma Proteobacterium clone RII-OX016 **JQ580103**	88
δ-Proteobacteria	EF2905	EF657863	Uncultured Delta Proteobacterium clone BME87 **DQ917833**	93
CFB group	EF0205	EF657854	Uncultured bacterium clone 5S54 **JF272214**	97
	EF1605	EF657855	Uncultured Bacteroidetes bacterium clone FII-TR116 **JQ579911**	95
	EF3705	EF657856	Uncultured Bacteroidetes bacterium clone d149 **GQ850585**	93
	EF0505	EF657862	Uncultured *Flavobacterium* sp. **FJ745113**	94
	EF1705	EF657864	Uncultured bacterium clone SHFH609 **FJ203538**	93
Planctomycetes	EF0805	EF657872	Uncultured Planctomycete clone XE2D09 **JN596618**	96
	EF3105	EF657873	Uncultured Planctomycete clone XE2D09 **JN596618**	98
	EF2805	EF657874	Uncultured Planctomycete clone RII-OX078 **JQ580165**	93
	EF2305	EF657875	Uncultured Planctomycete clone RII-AN084 **JQ580463**	92
	EF3805	EF657876	Uncultured Planctomycete clone RII-OX078 **JQ580165**	94
Lentisphaerae	EF1905	EF657866	Uncultured Lentisphaerae bacterium clone PRTBB8480 **HM798920**	98
	EF0405	EF657867	Uncultured Lentisphaerae bacterium clone PRTBB8480 **HM798920**	98
Acidobacteria	EF3505	EF657845	Uncultured Acidobacteria bacterium clone BME6 **DQ917814**	92
Actinobacteria	EF3305	EF657846	Uncultured Actinobacterium clone II10D **FJ205382**	95
Nitrospirae	EF0705	EF657868	*Nitrospira* sp. enrichment culture clone Aa01 **EU055608**	95
Cyanobacteria	EF1405	EF657869	*Oscillatoria rosea* IAM M-220 **AB003164**	95

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