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Diversity of Coral *Eunicea fusca* Associated Bacteria Using Culture Dependent Techniques

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Abstract: Invertebrates are known to be associated with diverse bacterial population, however, very little is known about the structure, composition and maintenance of these bacterial communities. In the current study, we characterize the culturable bacterial community associated with gorgorian coral *Eunicea fusca*. This was achieved using culture-based methods and molecular techniques for the identification of the bacterial isolates. The culturable heterotrophic bacterial community of this coral is composed mainly of the bacterial groups Alphaproteobacteria (65.5%), Gammaproteobacteria (20.7%), Betaproteobacteria (6.9%), Cytophaga-Flexibacter Bacteroids (3.4%) and Firmicutes (3.4%). This study provides evidence of specific bacterial association with the coral in comparison to bacterial community in the coral surrounding seawater. Furthermore, bacterial isolation using oligotrophic conditions,. at slightly alkaline pH, was found to increase the culturability and diversity of *Eunicea fusca* associated bacteria.

Key words: Biodiversity, 16S rDNA, BOX-PCR, phylogenetic, marine

INTRODUCTION

Coral reefs are the most biodiverse of all marine ecosystems; however, very little is known about the prokaryotic diversity in these systems. Our understanding of the role of bacteria in coral reef ecosystems is still evolving. Bacteria are known to be abundant and active in seawater around corals, in coral tissues and within their surface micro layer. Many researchers as Santavy (1995), Kushmaro *et al*. (1996), Koh (1997), Kushmaro *et al*. (1997) Gast *et al*. (1998), Gili and Coma (1998), Lyons *et al*. (1998) and Toren *et al*. (1998) have examined the interactions between corals and microbes. These studies have shown that there is a dynamic microbiota living on the surface and possibly within the tissue of corals and in the surrounding reef waters. However, it is still not known whether microbes play specific roles in coral biology or if the observed associations are merely opportunistic interactions of the coral animal with water-column bacteria. Early studies using culturing methods demonstrated the importance of coral-associated bacteria in coral nutrition and in response to stress. During the last three decades, most of the studies on coral-associated bacteria have linked the presence of bacteria in corals to several diseases (Ben-Haim *et al*., 2003; Sutherland *et al*., 2004). It was shown that the bacterial community changes when corals are bleached (Ritchie *et al*., 1994) or exhibited white-band disease or exposed to an environmental stress (Ritchie and Smith 1995). Our primary goal in this study was to characterize the culturable natural bacterial community associated with *Enicea fusca* coral using culture-based methods in comparison with that of the coral surrounding water column.

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MATERIALS AND METHODS

Coral Samples Collection and Preparation

Eunicea fusca was collected in sterile bags at Hillsboro ledge in Florida, August 2006 by SCUBA diving at depth of approximate 30 feet. Samples were kept fresh in seawater in and processed upon arrival to the laboratory. Control seawater was collected in the area surrounding the coral. In this case, the water sample was collected in sterile bottle that was opened adjacent to the coral *in situ*.

Isolation and Enumeration of Coral Associated Bacteria

The collected corals (Three samples) were washed under aseptic condition using filtered seawater (FSW) to remove the loosely attached bacteria, weighed, minced with razor blade and homogenized using a sterile blender. The homogenate was filtered through sterile cheesecloth to remove the large particles and debris. The homogenate was serially diluted up to 10^{-5} with FSW. A 100 μL aliquot of each dilution was plated in triplicates on different agar media. Nutrient rich and. nutrient poor media, Marine agar and 1/10th strength Marine agar media, respectively were used at different pH 5, 7 and 9 (Difco™, Sparks, MD). The plates were incubated at 30°C for 2-3 weeks. Bacterial isolation from the collected seawater was done at the same conditions. Representatives of each colony morphotype were serially streak-plated onto fresh media to obtain pure cultures and stored in 20% glycerol at -80°C.

Box-PCR Genomic Fingerprint

The bacterial isolates were grown overnight in 5 mL Marine broth. Total DNA was extracted with the Ultra Clean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's procedure. BOX PCR was carried out using BOX-AIR primer (5'-CTACGGCAAGGCGACGCTGACG-3') (Nick *et al*., 1999). Cycling conditions included an initial denaturation step of 95°C for 2 min followed by 35 cycles of 94°C for 3 sec, 92°C for 30 sec, 50°C for 1 min, 65°C for 8 min. A final extension step at 65°C for 8 min was added. The amplified fragments were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide and ran at 60 V for 3.5 h.

Bacterial Identification by 16S rRNA Gene Sequence Analysis

The bacterial strains were identified by 16S rRNA gene sequence analysis. The bacterial isolates were grown overnight in 5 mL Marine Broth. Total DNA was extracted with the Ultra Clean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's procedure. Eubacterial-specific primers, forward primer 16F27 (5′- AGA GTT TGA TCC TGG CTC AG-3′) and. reverse primer. 16R1525 (5′- AAG GAG GTG ATC CAG CCG CA-3′) derived from *E. coli* 16S-rDNA sequence (Lane, 1991) were used to amplify 16S rDNA gene. The reaction mixture of 50 μL contained at least 100 ng of genomic DNA (in 10 mM Tris-HCl, pH 8), 0.2 μM of each primer and PCR Supermix High fidelity (Taq and Go, Promega, CA). PCR fragments were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and sequenced on an ABI 377 automated sequencer using the PRISM Ready Reaction Kit (Applied Bio Systems, Foster City, CA). Sequence data were analyzed by comparison with 16S rRNA genes in the GenBank database. The nearest relatives of each organism were obtained by BLAST searches (Altschul *et al*., 1997).

RESULTS AND DISCUSSION

The total colony forming unit (cfu) of the coral associated bacteria in different media is shown in Table 1. The total CFU of the coral associated bacteria was found to be five fold more in nutrient poor media than in nutrient rich media. In nutrient rich media, the ration of the cfu at different pH

Table 1: Colony forming unit count (cfu) of the *Eunicea fusca* associated bacteria and seawater on nutrient rich and nutrient poor media at different pH values

	Rich media				Poor media			
Media	pH 5	pH 7	pH 9	Total	pH 5	pH 7	pH 9	Total
cfu g^{-1} coral	1043	2696	3304	7043	16000	11087	9304	36391
cfu mL^{-1} sea water	1720	3240	2880	7840	1710	3190	1650	6550

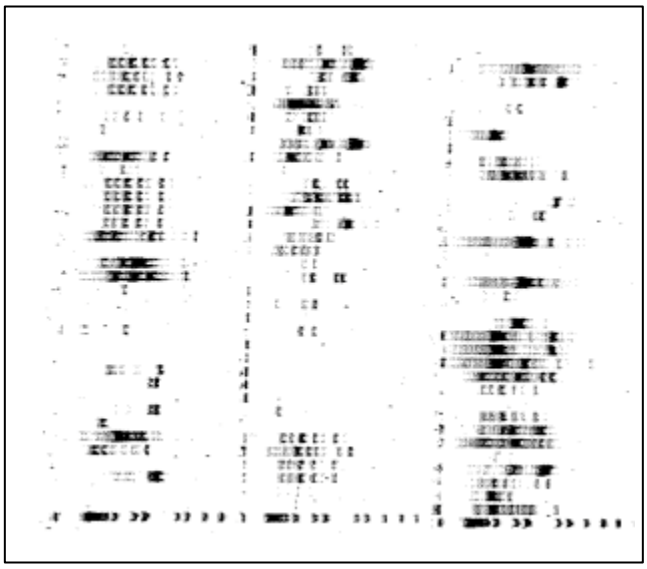

Fig. 1: DNA fingerprint of the *Eunicea fusca* associated bacteria generated with primers BOX-A IR. Lane 1 is a standard DNA marker of 1 Kb ladder

was 14.6, 37.8. and. 47.6 at pH 5, 7, 9, respectively. In nutrient poor media, the ratio of the total cfu at different pH was 44, 30.5 and 25.5 at pH 5, 7 and 9, respectively. The total cfu g^{-1} coral was about 4.8 fold more than cfu mL^{-1} of the seawater in nutrient poor media. In case of seawater, in nutrient rich media, the ration of the cfu at different pH was 22, 41.3 and 36.7% at pH 5, 7 and 9, respectively. In nutrient poor media the ratio of the total cfu at different pH was 26.1, 48.7 and 25.2% at pH 5, 7 and 9, respectively.

Box-PCR

All the isolated, were subjected to Box-PCR analysis to provide indication of the unique fingerprints (Fig. 1). A total of 56 unique patterns resulted from the BOX-PCR. All the strains with unique patterns were identified with sequence analysis of the 16S rDNA.

Phylogenetic Analysis of the Isolates

Isolates with unique patterns from the BOX-PCR were subjected to 16S rDNA sequence analysis. The 16S rDNA of 29 strains, isolated from the *Eunicea fusca* and 29 strains, isolated from the seawater, were sequenced. The resulted sequences were compared to the sequences of known bacteria in the GenBank. The sequences of the isolates possess 96-99% similarity compared to sequences of previously known bacteria in the GenBank (Table 2). The coral associated bacteria were dominated by Alphaproteobacteria with 65.5% of the isolates falling within this group. This was followed by 20.7% of Gammaproteobacteria, Firmicutes 6.9%, Betaproteobacteria 3.4 and 3.4%

Table 2: Characterization of culturable bacteria associated with *E. fusca* isolated in different media

Isolate*	Phylogenetic Association	Closest Gen Bank Match Bank Match	Gen Bank No.
5PC-3	Gammaproteobacteria	*Vibrio harveyi*	DQ146935
5PC-12	Gammaproteobacteria	*Vibrio* sp.	DQ005910
5PC-22	Gammaproteobacteria	*Vibrio harveyi*	DQ146936
9PC_1	Gammaproteobacteria	*Alteromonas* sp.	DQ097237
9PC_8	Gammaproteobacteria	*Psychrobacter* sp.	DQ396354
5RC-3	Gammaproteobacteria	*Alteromonas* sp.	AB015135
9RC-10	Betaproteobacteria	*Ralstonia* sp.	AY216798
5PC-18	Alphaproteobacteria	Alpha proteobacterium	DQ416557
5PC-32	Alphaproteobacteria	Alpha proteobacterium	DQ227656
7PC-1	Alphaproteobacteria	*Silicibacter* sp.	AF201086
7PC_2	Alphaproteobacteria	Alpha proteobacterium	DQ227657
7PC_3	Alphaproteobacteria	*Silicibacter* sp.	AF201086
7PC_4	Alphaproteobacteria	Alpha proteobacterium	DQ227656
7PC-5	Alphaproteobacteria	*Bacterium* s1 cb33	DQ416557
7PC-6	Alphaproteobacteria	Alpha proteobacterium	DQ399712
7PC-7	Alphaproteobacteria	*Bacterium* s1 cb33	DQ416557
7PC_8	Alphaproteobacteria	Alpha proteobacterium	DQ227657
7PC=9	Alphaproteobacteria	*Bacterium* s1 cb33	DQ227657
7PC-10	Alphaproteobacteria	*Silicibacter* sp.	AF201086
7PC-16	Alphaproteobacteria	Alpha proteobacterium	DQ097238
9PC-2	Alphaproteobacteria	Alpha proteobacterium	DQ097262
9PC_7	Alphaproteobacteria	Alpha proteobacterium	DQ097237
9PC-12	Alphaproteobacteria	*Bacterium* s1 cb33	DQ097262
9PC_14	Alphaproteobacteria	Alpha proteobacterium	DQ227657
9PC-20	Alphaproteobacteria	*Silicibacter* sp.	AF201086
9PC-21	Alphaproteobacteria	Uncultured alpha proteobacterium	DQ446160
7PPC-22	Sphingobacteria	*Cytophaga* sp.	AB073588
7PC-23	Firmicutes	*Bacillus aquimaris*	EF089472
7PC-24	Firmicutes	*Bacillus marisflavi*	AF453507

*The acronyms given to the isolates read as a follow: e.g. PC: Nutrient poor (coral), RC: Nutrient rich media (coral). The first digit is referring to the pH value

of Cytophaga-Flexibacter Bacteroids (CFB) (Table 4). Gammaproteobacteria has been reported to be the major bacterial group in many other soft coral using culturing technique (Ritchie and Smith, 1995; Rohwer *et al*., 2001). By comparing the composition of culturable bacterial isolates of the coral *Fungia scutaria* to the present study it is found that while *F. scutaria* isolates were dominated by an almost equal distribution of Alphproteobacteria and Gammaprotebacteria (Lampert *et al*., 2006), *E. fusca* was dominated mainly by Alphaproteoacteria.

The seawater was dominated with Alphaproteobacteria with 82% of the isolates falling within this group. This was followed by 9% of Gammaproteobacteria and 6% of Betaproteobacteria and 3% was falling within the division Cytophaga-Flexibacter Bacteroids (Table 4).

Differences were observed in the types of culturable bacteria between seawater and coral. The three groups α, β and γ proteobacteria were represented in both samples but with different species. The CFB and Firmicutes groups were only isolated from the coral samples. The coral associated bacteria are falling within nine genera with different species, *Vibrio* sp., *Alteromonas* sp., *Psychrobacter* sp., *Ralstonia* sp., *Silicibacter* sp., *Bacterium* s1 cb33, *Bacillus* sp., *Cytophaga* sp. and uncultured alpha proteobacterium.

Vibrio sp. has been isolated from other corals *Oculina patagonica* and *Montastraea franksi* (Koren and Rosenberg, 2006; Rohwer *et al*., 2001). *Alteromonas* sp. and *Silicibacter* sp. have been isolated from the Red Sea coral *Fungia scutaria* (Lampert *et al*., 2006).

Karen and Rosenberg (2006) found the *Bacterium* slcb33 associated with mucus and tissues from the coral *Oculina patagonica* in summer and winter of the cost Israel. This suggests a global distribution of *Bacterium* slcb33 as well as a possible coral specific association.

Table 3: Characterization of culturable bacteria isolated from sea water samples at different pH and nutrient media

Isolate*	Bacterial group	Identification	Gen bank accession No.
7PSW-3	Gammaproteobacteria	*Alteromonas* sp.	AB176405
9PSW-8	Gammaproteobacteria	*Alteromonas* sp.	EF061432
9PSW-10	Betaproteobacteria	Uncultured alpha proteobacterium	AY475203
5PSW-12	Alphaproteobacteria	*Thalassobius mediterraneus*	AJ878874
7PSW-2	Alphaproteobacteria	Uncultured alpha proteobacterium	AY663968
7PSW-5	Alphaproteobacteria	*Thalassobius mediterraneus*	AJ878874
7PSW-6	Alphaproteobacteria	*Thalassobius mediterraneus*	AJ878874
7PSW-7	Alphaproteobacteria	*Sulfitobacter* sp.	DQ985901
7PSW-18	Alphaproteobacteria	*Thalassobius mediterraneus*	AJ878874
7PSW-20	Alphaproteobacteria	*Thalassobius mediterraneus*	AJ878874
9PSW-1	Alphaproteobacteria	*Thalassobius mediterraneus*	AJ878874
9PSW-2	Alphaproteobacteria	*Thalassobius mediterraneus*	AJ878874
9PSW-3	Alphaproteobacteria	*Thalassobius mediterraneus*	AJ878874
9PSW-4	Alphaproteobacteria	*Ruegeria* sp.	AY568823
9PSW-5	Alphaproteobacteria	*Roseobacte*r sp.	AY745856
9PSW-6	Alphaproteobacteria	*Thalassobius mediterraneus*	AJ878874
9PSW-7	Alphaproteobacteria	Uncultured alpha proteobacterium	AM238614
9PSW_9	Alphaproteobacteria	Alpha proteobacterium S2-3 clone	DQ357746
7RSW-2	Alphaproteobacteria	*Sulfitobacter* sp.	DQ985901
7RSW-27	Alphaproteobacteria	Alpha proteobacterium	DQ227657
7RSW-35	Alphaproteobacteria	Alpha proteobacterium	DQ097263
9RSW-1	Alphaproteobacteria	*Furvibacter pelagius*	EF134718
9RSW-2	Alphaproteobacteria	*Furvibacter pelagius*	EF134718
9RSW-3	Alphaproteobacteria	*Furvibacter pelagius*	EF134718
9RSW_4	Alphaproteobacteria	*Roseobacter* sp.	DQ235574
9RSW_6	Alphaproteobacteria	*Roseobacter* sp.	DQ235574
9RSW_7	Alphaproteobacteria	*Roseobacter* sp.	AY745859
9RSW-10	Alphaproteobacteria	*Roseobacter* sp.	AY258077
9RSW_11	Alphaproteobacteria	*Roseobacter* sp.	AY258077

*The acronyms given to the isolates read as a follow: e.g., PSW: Nutrient poor media (seawater). PSW: Nutrient rich nutrient media (seawater) (RC). The first digit is referring to the pH values

Table 4: Different bacterial groups in coral tissues and seawater sample

	Bacterial source	
Bacterial group	*Eunicea fusca* (%)	Seawater (%)
Gammaproteobacteria	20.7	6.9
Betaproteobacteria	3.4	3.4
Alphaproteobacteria	65.5	89.7
CFB*	3.4	0.0
Firmicutes	6.9	0.0

*Cytophaga-flexibacter bacteroids

The seawater strains, 29 isolate, were found to be more diverse that affiliated to nine genera with different species, *Alteromonas* sp., *Ralstonia* sp., Uncultured alpha proteobacterium, *Thalassobius*, *Sulfitobacter* sp., *Ruegeria* sp., *Roseobacter* sp., *Furvibacter*, *Rhodobacter* sp. (Table 3).

Most of the strains isolated from the *E. fusca* tissues were absent in the strains isolated from the seawater, only one strain, *Alteromonas* sp., has been found in both the coral and the surrounding seawater sample which provides evidence of specific bacterial association with the coral indicated a specific bacterial association with the coral tissues. Similar bacterial specific association with the coral has been reported in coral *Montastraea franksi* (Rohwer *et al*., 2001).

Analysis of The Nutrient Rich and Nutrient Poor Media Isolates

The total bacterial count (cfu) of the coral associated bacteria was found to be five fold more in nutrient poor media than in nutrient rich media. In nutrient rich media, while it was about 1.2 fold more in rich media in case of seawater. The results shown in Table 5 indicated that the nutrient poor

Table 5: Different bacterial groups isolated in nutrient poor and nutrient rich media

	Media	
Bacterial group	Nutrient poor (%)	Nutrient rich (%)
Gammaproteobacteria	14.0	18.0
Betaproteobacteria	5.0	12.0
Alphaproteobacteria	65.0	70.0
CFB*	5.0	0.0
Firmicutes	10.0	0.0

*Cytophaga-flexibacter bacteroids

media showed more bacterial groups diversity than nutrient rich media while both were dominated by Alphaproteobacteria. Furthermore, the number of the different strains was about two fold higher in nutrient poor media.

In addition to media types, the pH of the media was another important parameter. The results of bacterial isolation from the coral tissues and seawater, suggested that acidic pH was not the most optimal for bacterial growth as evidence by the many fewer strains obtained at pH 5 in comparison with pH 7 and 9. Overall, using slightly alkaline oligotrophic conditions, nutrient, increased the number and diversity of the culturable bacteria isolated from seawater and *Eunicea fusca*.

The term the great plate count anomaly was coined by Staley and Konopka in 1985 (Staley and Konopka, 1985) to describe the difference in orders of magnitude between the numbers of cells from natural environments that form colonies on agar media and the numbers countable by microscopic examination. Marine ecosystems are a well-studied example of this phenomenon: only 0.01 to 0.1% of oceanic marine bacterial cells produce colonies by standard plating techniques (Stephanie *et al*., 2002). There are numerous explanations for this anomaly. For example, species that would otherwise be culturable may fail to grow because their growth state in nature, such as dormancy, prevents adjustment to conditions found in the medium used for the plate counts (Deming and Baross, 2000). This hypothesis does not explain the substantial discrepancy between 16S rRNA genes recovered from seawater directly by cloning and those of the readily cultured marine taxa (Stephanie *et al*., 2002). Another explanation for the great plate count anomaly is that many of the microbial species that dominate in natural settings are not adapted for growth in media containing high concentrations of complex organic carbon. Many microorganisms may need oligotrophic or other fastidious conditions to be successfully cultured. There are many examples of microbial strains that are common in nature, but can only be cultivated by specialized techniques (Button *et al*., 1998; Partensky *et al*., 1999; Vancanneyt *et al*., 2001; Wirsen *et al*., 2002; Stephanie *et al*., 2002).

In summary, *Eunicea fusca* associated bacteria have been isolated and characterized. Comparison of the coral associated bacterial population with the bacterial community in the coral surrounding seawater provided evidence of specific bacterial association with the coral. Furthermore, investigation of different isolation conditions demonstrated that slightly alkaline oligotrophic conditions increased the culturability and diversity of *E. fusca* associated bacteria.

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