Flower Garden Banks National Marine Sanctuary:
Antimicrobial Properties of Bacteria Associated with the Boulder Star Coral, *Montastrea faveolata* and a Plague-like disease outbreak in 2005

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Overview

Corals have been shown to harbor unique microbial communities associated with their surface mucopolysaccharide layer, which is hypothesized to play an important role in defense against invading pathogens (Ritchie and Smith, 2004). In this study, bacterial associates of *Montastraea faveolata* surface mucus were compared between the healthy, disease-free reefs of the Flower Garden Banks National Marine Sanctuary and the disease-prone reefs of the Florida Keys National Marine Sanctuary. Bacteria isolated from the surface mucus of healthy *M. faveolata* colonies from both locations were subcultured to purification and screened for antibacterial activity against a range of human pathogens. Twelve bacterial isolates from the Flower Gardens West Bank displayed varying degrees of antibacterial activity against *Bacillus subtilis*. Genetic identification of antibiotic-producing bacteria places five of these isolates within the genus *Vibrio*. A *Pseudoalteromonas sp*. isolated from a *M. faveolata* colony located in the Flower Gardens East Bank displayed antibacterial activity against all strains tested, providing a potentially novel source for the identification of broad-spectrum antibiotics. No antibacterial activity was detected from bacterial associates of *M. faveolata* colonies in the Florida Keys, suggesting that *M. faveolata* may harbor a higher percentage of beneficial bacterial associates in healthy reef systems. Eleven of the twelve isolates from the Flower Gardens West Bank displaying antibacterial activity were isolated from subcultures grown on mucus-treated growth media. This result implicates a bacterial contribution to antibiotic production in coral mucus and suggests a novel symbiotic mechanism between corals and bacteria.

One hypothesis is that corals harbor antibiotic-producing bacteria in their SML as a defense against harmful pathogens and this may be a significant factor in disease resistance. In a Spring FGBNMS Research Cruise in 2005, three *Montastrea faveolata* colonies displaying a plaque-like disease outbreak were sampled at the disease interface as well as in adjacent apparently healthy regions of the coral colony. Libraries consisting of 192 bacterial strains were isolated from diseased and healthy tissue and from the water column. Initial antibiotic testing of these strains showed no antibiotic activity present in healthy or disease isolates. Genetic dereplication and identification of cultured isolates is currently being undertaken to identify anomalous bacteria associated with this disease outbreak.

Methods

Sampling

Samples of SMLs were collected from healthy *Montastrea faveolata* heads in shallow water reefs in the Flower Gardens National Marine Sanctuary (FGNMS; Table 1), ~60-90 ft depth, and the Florida Keys National Marine Sanctuary (FKNMS), ~10-30 ft depth, using 30.0 ml needleless sterile syringes after slight agitation of the coral surface to stimulate mucus production. Coral heads were sampled from each of two locations at each collection site: East Bank and West Bank at FGNMS, and Western Sambo and Looe Key at FKNMS. Water samples were taken ~1 meter from each coral head for comparison. Samples were kept on ice or incubated at 4°C until further analysis was conducted. In addition, two M. faveolata colonies and one M. franksii colony displaying a plaque-like disease outbreak were sampled (Table 1) at the disease interface as well as in adjacent apparently healthy regions of the coral colony and the water column.

Bacterial Isolations and Library Production

Serial dilutions of each sample were conducted and 100 µl of each dilution was spread plated onto glycerol artificial seawater agar (GASWA) media. Individual colonies from these plates were then streaked to purification on GASWA media for further analysis. Libraries of the bacterial communities from each sample were constructed using 96 well plates containing 100 µl GASW liquid media in each well. Each well was inoculated with a different bacterial isolate by using a sterile toothpick and a single colony from one of the pure cultures. In addition, libraries consisting of 192 bacterial strains were isolated from diseased and healthy tissue and from the water column.

Antibiotic Testing

Rectangular GASWA replica plates were made from each library for antibacterial testing. Replica plates were grown and challenged against a host of tester strains including Methicillin sensitive *Staphylococcus auraeus,* Methicillin resistant *Staphylococcus auraeus*, VRE (Vancomycin resistant *Enterococcus faecalis*), *Escherichia coli, Bacillus subtilis, Salmonella sp., Seratia marcescens, ATCC Seratia marcescens,* and *Enterococcus faecalis.* Antibacterial activity was as the zone of growth inhibition by the coral bacterium against the human tester strain and was recorded as the diameter in mm of the zone of inhibition from the colony edge to clearing zone interface.

Bacterial identification

Genomic DNA was extracted from pure colonies corresponding to the bacteria that produced antibacterial compounds and PCR Amplification of the 16SrRNA gene was performed followed by DNA sequencing (Macrogen, Inc) and analysis via NCBI for closest match in the GenBank database (Altschul, et al. 1997).

Preliminary Results

Twelve of sixty-three isolates from the FGBNMS, West Bank displayed antibacterial activity toward the Gram-positive human opportunistic pathogen, Bacillus *subtilis,* in varying degrees. Nine of the thirteen isolates displaying antibacterial activity were genetically identified as *Vibrio sp.*, and four were identified as *Pseudomonas sp.* One of

137 isolates from coral mucus collected from FGBNMS, East Bank, displayed antibacterial activity toward all strains tested (Table 1) and was genetically identified as a *Pseudoalteromonas sp.* No antibacterial activity was detected for samples obtained from the FKNMS of fifty-six isolates tested.

Initial antibiotic testing of isolates from diseased corals showed no antibiotic activity present in the White Plague-like disease isolates. Genetic dereplication and identification of cultured isolates from diseased corals at the same site is currently ongoing to identify anomalous bacteria associated with the 2005 FGBNMS disease outbreak.

Discussion

Results suggest that coral bacteria are a novel source for the identification of antibiotics. The observed antibacterial activity of bacterial associates of corals suggests that bacteria may contribute to antibiotic production in the coral mucus intended to function in defense against invading pathogens for the coral. Another hypothesis is that the coral mucus may provide an enriching medium for certain bacteria, encouraging the production of antibacterial compounds for a niche advantage over competing bacteria trapped in the coral mucus.

It is noteworthy that the *Pseudoalteromonas* isolate EB4-2, 1-3 (Table 2) produces a broad spectrum antibiotic, suggesting that similar broad-spectrum antibiotic producers may be present among the bacterial associates of other healthy corals. This provides a promising new source of antibiotics from SML-associated bacteria.

FKNMS/FGBNMS Comparisons

The lack of antibacterial activity of bacterial associates of corals in the FKNMS suggests that *M. faveolata* may harbor a higher percentage of beneficial bacterial associates in healthy reef systems than disease-prone reef systems, thus inferring a correlation between coral health and the activity of the coral's bacterial associates. It should be mentioned that results of sampling from the FKNMS may be biased due to the lower number of bacterial isolates tested from the Florida Keys (104 total) as compared to isolates from the FGBNMS (200 total). Further testing in the Spring of 2006 will be conducted using a larger sample size and identical methodology.

Coral Disease Outbreak at FGBNMS 2005

One hypothesis is that corals harbor antibiotic-producing bacteria in their SML as a defense against harmful pathogens and this may be a significant factor in disease resistance. Initial antibiotic testing of isolates from diseased corals showed no antibiotic activity present in healthy or disease isolates. A potential explanation is that the microbial community has already shifted in corals harboring disease to a less beneficial/more opportunistic microbial consortium. Further dereplication and genetic identification in our libraries will reveal microbial population shifts and may pinpoint anomalous bacteria associated with this disease outbreak.

References

Altschul, S.F., T.L. Madden, A.A. Schaffer , J. Zhang, Z. Zhang, W. Miller, D.J. Lipman. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs, Nucleic Acids. Res. 25:3389-3402.

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Table 1: FGBNMS Sampling Log

Date	Location	Coral Head	Healthy/ Diseased	Depth	Notes
5/23/05	East Bank Buoy # 3	*M. faveolata*	Healthy	65-75 feet	Smooth surface, 2 zooxanthellae clade color morphs, 1% dead tissue
	East Bank Buoy # 3	*M. faveolata*	Healthy	65-75 feet	Smooth surface, no clade (color) variation, 0% dead tissue
	East Bank Buoy # 3	*M. faveolata*	Healthy	65-75 feet	Immediately adjacent to coring colony furthest NE, 0% dead tissue
5/24/05	East Bank Buoy # 4	*M. faveolata*	Healthy	65-75 feet	Within vicinity of Tagged colony #1
	East Bank Buoy # 4	*M. faveolata*	Healthy	65-75 feet	Within vicinity of Tagged colony #1
	East Bank Buoy # 4	*M. faveolata*	Healthy	65-75 feet	Within vicinity of Tagged colony #1
	East Bank Buoy # 4	*M. faveolata*	Diseased	65-75 feet	Photo by A. Bruchner Tagged colony # 6
5/24/05	West Bank Buoy #1	*M. faveolata*	Healthy	85-95 feet	
5/25/05	West Bank Buoy #2	*M. faveolata*	Diseased	80-90 feet	Tagged colony # 20
	West Bank Buoy #2	*M. franksii*	Diseased	80-90 feet	Tagged colony # 11
	West Bank Buoy #2	*M. faveolata*	Diseased	80-90 feet	Tagged colony # 12

Table 2: Antibacterial activity of bacterial associates of healthy *M. faveolata* surface mucus. Tester strain sensitivity is measured by zone of inhibition in the presence of coral bacterial isolate

Sample Location	Sensitive tester Strain	Coral Isolate	Zone of Inhibition (mm)	Genetic Identification
West Bank	*Bacillus subtilus*	WB1-3W2	3.0	*Pseudoalteromonas sp.*
West Bank	*Bacillus subtilus*	WB1-3M3	4.0	*Vibrio sp.*
West Bank	*Bacillus subtilus*	WB1-3M6	2.0	*Vibrio sp.*
West Bank	*Bacillus subtilus*	WB1-3M9	4.0	*Vibrio sp.*
West Bank	*Bacillus subtilus*	WB1-2T3	1.0	*Vibrio sp.*
West Bank	*Bacillus subtilus*	WB1-2T4	2.0	*Vibrio sp.*
West Bank	*Bacillus subtilus*	WB1-2T5	2.0	*Vibrio sp.*
West Bank	*Bacillus subtilus*	WB1-2T6	2.0	*Vibrio sp.*
West Bank	*Bacillus subtilus*	WB1-2T8	1.0	*Pseudoalteromonas sp.*
West Bank	*Bacillus subtilus*	WB1-1M4	2.0	*Pseudoalteromonas sp.*
West Bank	*Bacillus subtilus*	WB1-1M5	2.0	*Vibrio sp.*
West Bank	*Bacillus subtilus*	WB1-1C8	1.0	*Vibrio sp.*
East Bank	*Enterococcus faecalis*	EB 4-2,1-3	1.5	*Pseudoalteromonas sp.*
	Methicilin Resistant *Staphylococcus auraeus*		10.0	
	ATCC *Seratia marsescens*		1.5	
	Seratia marsescens White Pox isolate		6.0	
	Methicilin Sensitive *Staphylococcus auraeus*		8.0	
	Salmonella sp.		10.0	
	Shigella sp.		3.5	
	Escherichia coli		5.5	
	Bacillus subtilis		9.0	