

Evaluating the Potential for Somatic Coliphage Replication in Environmental Waters

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Fecal indicator bacteria (e.g. *Escherichia coli*, fecal coliforms) are used to assess the presence of disease causing fecal bacterial/viral contamination in recreational waters. Recent research has suggested that these bacteria may have the ability to replicate in the environment, thus limiting their usefulness as indicators of recent pollution. In current epidemiological studies, somatic coliphages have shown promise as a potential replacement for current indicator bacteria. However, publications including U.S. Environmental Protection Agency (USEPA) documents, have suggested that coliphages are also capable of replicating in natural waters. Since coliphages cannot replicate outside a host cell, their proliferation in the environment is dependent on their ability to infect *E. coli* cells. This study focuses on whether *E. coli* isolated from sites in South Carolina and California can effectively serve as hosts for somatic coliphages isolated from the same locations. The major hypothesis is that only a small percentage of environmental *E. coli* will support somatic coliphage replication; and these percentages will not differ significantly when analyzing only coliphages and *E. coli* isolated from a single water sample.

Initially, somatic coliphages will be obtained from water samples using the USEPA Single Agar Layer technique, and *E. coli* will be isolated by membrane filtration and growth on mFC agar. Somatic coliphages will be spotted onto pour plates; each containing a single environmentally-collected *E. coli* strain. The formation of plaques where the coliphage was spotted indicates that *E. coli* cells in that area have been lysed as a result of coliphage infection and replication. The percentage of *E. coli* able to support replication of the somatic coliphages will be evaluated. Failure of the majority of *E. coli* strains to effectively serve as somatic coliphage hosts would weaken arguments that somatic coliphages would make poor fecal indicators as a result of environmental replication.

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Isolation and Structural Characterization of a Novel Glycoside Pigment from *Euglena* sanguinea

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Euglena sanguinea is a ubiquitous algal species found in many shallow, eutrophic freshwater systems. Originally considered benign, *Euglena sanguinea* blooms have now recently been observed to result in fish kills. The isolation and structural characterization of a toxic alkaloid, euglenophycin, has precipitated the need for a rapid method of assessing harmful *Euglena* blooms. One possible method to accomplish this assessment is analysis of differentially expressed pigments between toxic and benign algae. Here we report the extraction of a novel pigment from cultured *Euglena* cell mass that is expressed at high levels in toxic algae. The pigment was purified through chromatographic methods and analyzed using gradient elution and photo-diode array detection. Nuclear magnetic resonance spectra (¹H, ¹³C, APT, COSY, HSQC, and HMBC) and mass spectra are currently being analyzed for the completion of the chemical structure. Data to date corresponds to no known pigment providing evidence that this is a novel compound. A completed structure will provide a biomarker for the rapid identification of toxic *Euglena*.

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Post-transcriptional regulation of the DNA Replication Fork Protein, PCNA, in the Florida Red Tide dinoflagellate, *Karenia brevis*

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The dinoflagellate, *Karenia brevis*, is responsible for harmful algal blooms in the Gulf of Mexico that cause extensive marine mortalities and human illness on a nearly annual basis. The molecular mechanisms controlling the cell cycle in this dinoflagellate are important because bloom development occurs through vegetative cell division. Microarray and qPCR studies have demonstrated that, unlike typical eukaryotes, dinoflagellate cell cycle genes are not regulated at the transcriptional level, including replication fork proteins that are typically activated by the E2F transcription factor at the restriction point, which regulates entry into S-phase. Post-transcriptional control of these genes is further suggested by the presence of a trans-spliced leader sequence on their transcripts. The current study investigated whether the expression of the replication fork protein, PCNA, is regulated at the translational or post-translational level. Immunolocalization using a peptide antibody developed for K. brevis PCNA showed that PCNA is constitutively present in the nucleus throughout the cell cycle. However, its distribution within the nucleus changes, with prominent staining of chromatin-bound PCNA during S-phase, whereas it is present in a peripheral pool during the rest of the cell A similar pattern is observed in the trypanosome, a protist that also possesses a cycle. spliced leader sequence and controls gene expression at the post-transcriptional level. Western blot data similarly show that PCNA is present during the entire cell cycle, although it appears to be more highly expressed during S-phase than G1. However, there appears to be an increase in the size of PCNA from 28 kDa during G1 to a ~37 kDa band dominating during S-phase, and both forms present during G2 and M. We hypothesize that this change in size may be due to sumovlation of the protein concurrent with its chromatin association, as has been observed in yeast. We have identified a ~9kDa sequence in our K. brevis cDNA library with homology to SUMO (small ubiquitin like modifier), indicating that dinoflagellates possess the SUMO mechanism for posttranslational protein modification. Furthermore, immunoprecipitated K. brevis PCNA probed with an antibody to SUMO revealed an immunoreactive band at ~37 kDa. Together, our results suggest that the expression and activity of PCNA is controlled at both the translational and post-translational levels in this dinoflagellate, in the absence of transcriptional regulation.

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Influence of selenium and mercury chemistries on the progression of cardiomyopathy: impacts on oxidative stress and selenoprotein profiles in pygmy sperm whales

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Pygmy sperm whales (Kogia breviceps) are the second most frequently stranded toothed whale along the U.S. Atlantic and Gulf coasts. More than half of documented cases exhibit signs of cardiomyopathy (CMP). Many factors may contribute to the development of idiopathic CMP in K. breviceps, including genetics, infectious agents, contaminants, biotoxins, and dietary intake (vitamins, selenium, mercury, and prooxidants). This study assesses trace elements in K. breviceps at various stages of CMP progression using fresh frozen liver and heart samples collected from stranded individuals. The K. breviceps diet, consisting mainly of squid, imparts a high dose of mercury (Hg), which requires detoxification, and polyunsaturated fatty acids (PUFAs) that require effective antioxidant biochemistry to regulate free radical formation. Standard addition calibration and collision cell ICP-MS were employed for total Se analysis and pyrolysis atomic absorption (AA) was utilized for total Hg analysis to examine if the Se/Hg detoxification pathway inhibits the bioavailability of Se. Double spike speciated isotope dilution GC/ICP-MS was utilized to measure MeHg and iHg. Due to the important role Se can play in antioxidant biochemistry and protein formation, selenoprotein and small molecule Se species profiles were examined by multi-dimension LC/UV/ICP-MS detection, and selenoprotein identification was performed by LC-ESI-MS/MS. Immunoblot detection and colorimetric assays were used to assess overall protein oxidation status. Data collected on trace elements, selenoproteins, and oxidative status were evaluated in the context of animal life history, disease state markers, and other complementary histological information to gain insight into the biochemical pathways contributing to the development of CMP in K. breviceps.

Funding for this study is provided by the National Institute of Standards and Technology and through interagency agreements established with the National Oceanographic and Atmospheric Administration (NOAA) National Marine Fisheries Service. This research is being performed in the context of a larger study on Kogia species in partnership with NOAA's Center for Coastal Environmental Health and Biomolecular Research and HBOI Division of Marine Mammal Research and Conservation.

Perfluorinated Compounds in Plasma of Northern Fur Seals (Callorhinus ursinus)

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Perfluorinated compounds (PFCs) are contaminants of emerging concern with worldwide distribution. PFCs exhibit toxicological effects in laboratory animals and may pose a risk of adverse effects in marine mammals. There have been several studies examining PFCs in marine mammals; however, to date perfluorooctane sulfonate (PFOS) is the only compound that has been analyzed in northern fur seals (*Callorhinus ursinus*). In this study we report concentrations of thirteen perfluorinated compounds measured in northern fur seal plasma. Samples were collected from animals harvested on St. Paul Island, Alaska in 2006 and 2007. Liquid chromatography/tandem mass spectroscopy (LC/MS/MS) was used to perform the analysis. In plasma, perfluoroundecanoic acid (PFUnA) was the most abundant compound with a median concentration of 5.4 ng/granging from < the limit of detection (LOD) to 18.0 ng/g, followed by perfluorononanoic acid (PFNA) at 3.4 ng/g (1.2 to 9.7 ng/g) and PFOS at 2.8 ng/g (<LOD to 18.6 ng/g). Interestingly, PFOS is not the most abundant compound as it is in most environmental studies, suggesting a different source or preferential metabolism of the C11 and C9 carboxylic acid compounds. The results reported here demonstrate that several perfluorinated compounds are at measurable quantities in the northern fur seal, some PFCs being measured for the first time in this species.

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The effects of 9-cis retinoic acid on 1,25-dihydroxyvitamin-mediated transcriptional activation in Atlantic bottlenose dolphin (*Tursiops truncatus*) skin cells

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The vitamin D pathway, mediated by the bioactive form of vitamin D3, has been well characterized in terrestrial animals. Vitamin D intake via diet or exposure to UVB radiation triggers the synthesis of 1,25-dihydroxyvitamin (1,25D3), the biologically active metabolite of vitamin D3, within the skin. 1,25D3 binds to the nuclear vitamin D receptor (VDR), which is a ligand-activated transcription factor regulating a large suite of genes. VDR's activation requires heterodimerization with another nuclear receptor: the retinoid X receptor (RXR). The RXR/VDR formation identifies and binds to vitamin D response elements (VDRE) within the promoters of certain genes to induce their expression. We are interested in whether 9-cis retinoic acid (9-cis RA), RXR's ligand, acts negatively or synergistically with 1,25D3 to induce the vitamin D pathway. The effect that 9-cis RA has on the vitamin D pathway is controversial and not well studied. We are using dolphin skin cells as our model because neither vitamin A nor vitamin D pathways have been well-studied in marine mammals, and each may serve as a potential innate immune mechanism within dolphin skin. Preliminary results show that 9-cis RA moderately activates transcription of a vitamin D sensitive promoter, albeit not nearly as strongly as that by 1,25D3. Combined exposure to 1,25D3 and 9-cis RA produces similar transactivity of this promoter as 1,25D3 alone, suggesting that 9-cis RA, if anything, exerts a positive effect on 1,25D3-mediated transcription. We propose to further test the differential effects of the two ligands on VDR and RXR protein levels and on the expression of dolphin-specific vitamin target genes using Western blot analysis and real-time PCR. Because dolphins may be appropriate models for humans, elucidating the effects that 9-cis RA has on the vitamin D pathway in dolphin skin cells provides information for crosstalk between the two pathways and for the appropriate vitamin A supplementary intake with respect to vitamin D in humans.

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The Microbial Functional Structure associated with Healthy and Yellow Band Diseased *Montastraea faveolata*, a Caribbean Reef-building Coral

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Microbial communities are ubiquitous across ecosystems and often live symbiotically within eukaryotic organisms. For example, the human gut houses an abundant and diverse population of microorganisms essential for digestion and metabolism. Moreover, variations in the human gut microbiome are thought to play a role in human health by influencing disease etiology. Likewise, coral-associated microbial communities are increasingly recognized as important components of the coral holobiont that influence coral health; however, few studies directly address the functional role of these communities. We hypothesize that variation between the phylogenetic structure of coralassociated microbial communities found in healthy and diseased corals result in a shift of functional potential associated with the microbiome. In our study, we examined the functional potential of the coral-associated microbial community found in the surface mucopolysaccharide layer (SML) and tissue of a Caribbean coral, Monastrea faveolata. The samples were collected from healthy and yellow band diseased (YBD) colonies off the coast of La Parguera, Puerto Rico. The GeoChip 2.0, a functional gene array, was used to investigate the presence of over 10,000 biogeochemical cycling genes. We detected 6728 genes present in the microbial communities associated with M. faveolata. The relative percentages of genes found in each biogeochemical process surveyed are as follows: carbon cycling (19%), sulfur cycling (7.5%), nitrogen cycling (21.5%), metal homeostasis (19%), and xenobiotic degradation (33%). Our analysis revealed a significant difference in functional structure of healthy and YBD M. faveolata colonies. Furthermore, those differences were specific to the physical niche examined. This study is the first broad screening of functional genes in coral-associated microbial communities and provides insights regarding their biogeochemical cycling capacity in healthy and diseased states.

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Tools to study the metabolism and elimination of brevetoxin metabolites

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Brevetoxins are potent polyketide neurotoxins produced during harmful algal blooms by the dinoflagellate *Karenia brevis*. Food web accumulation of these toxins are responsible for marine mammal mortality events, economic losses in fisheries and tourism, and risks to human health resulting from the consumption of contaminated shellfish and the inhalation of toxic aerosols. Metabolic transformation of brevetoxin after exposure results in the production of brevetoxin intermediates of varying potencies and concentrations. Studies of metabolic transformation have been hampered by the lack of suitable detection methods for the study of brevetoxin metabolites. Here we describe suitable extraction and detection methods for the analysis of these important metabolites, and the production of radiolabeled analogues of these metabolites. These tools are necessary to conduct *in vivo* exposure studies and measure brevetoxin metabolite distribution and elimination. This research will provide a better understanding into the adverse effects of exposure to brevetoxin and its metabolites in marine animals and humans.

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Induction of metacaspase expression and caspase-like activities during senescence in the toxic dinoflagellate, *Karenia brevis*

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Karenia brevis, a toxic dinoflagellate, is responsible for near annual harmful algal blooms (HABs) in the Gulf of Mexico causing extensive ecological and economic losses. Evaluation of bloom termination in other bloom forming phytoplankton has implicated a role for metacaspases, metazoan caspase orthologs, in regulating programmed cell death (PCD). Molecular mechanisms governing K. brevis bloom demise have remained largely uninvestigated; therefore identification and characterization of K. brevis metacaspases may lead to possible targets for bloom control strategies. We have identified five metacaspases (KbMC 1-5) in K. brevis all containing the well-conserved caspase catalytic diad and p20 domain previously identified in other unicellular organisms. Metacaspase expression over a growth curve, characterized by immunoblot with a polyclonal antibody raised against a recombinant *Emiliania huxleyi* metacaspase protein, revealed an induction of both metacaspase type and quantity in stationary phase/senescence. Metacaspase expression was further characterized in logarithmic and stationary phase cultures using a peptide antibody developed against the histidine active site of KbMC 1 and 2. Preliminary results indicate an induction of fulllength KbMC1 and 2 in stationary phase cultures, as well as activated p20 domain cleavage products, suggesting both an increase in metacaspase protein expression and activation in senescent cells. Caspase-like activities over a growth curve were characterized by quantifying the cleavage of fluorogenic canonical caspase tetrapeptides, and demonstrated that K. brevis exhibits a significant increase in activity during the transition into stationary phase and peaks in late stationary. Together these data indicate that K. brevis may be upregulating and utilizing PCD machinery during late senescence prior to culture demise. Further characterization of the involvement of metacaspases in cell death may lead to the identification of molecular biomarkers for K. brevis bloom termination.

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Assessing Oxidative Stress in the Sea-ice Diatom Fragilariopsis cylindrus

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Environmental factors have been shown to increase reactive oxygen species (ROS) in phytoplankton, including light, salinity, metal and viral exposure. Excessive buildup of ROS can cause widespread oxidative damage to a cell, thus organisms have developed various antioxidant mechanisms including the sulfur compound glutathione. Certain marine phytoplankton species produce another sulfur compound, dimethylsulphonioproprionate (DMSP), which plays important climate and biogeochemical roles. Cellular concentrations of DMSP are responsive to various environmental variables but its physiological role is still poorly understood. Recently it has been hypothesized to serve as a cellular antioxidant due to its ability to readily react with hydroxyl radicals. In order to better understand associations between environmental stressors, DMSP accumulation, and oxidative stress, the purpose of the current work was to optimize an *in vivo* method to quantify cellular ROS levels. The membrane permeable compound 2',7'-dichloro-dihydrofluorescein (DCFH-DA) was selected because following esterase conversion and oxidation by ROS within the cell, DCFH-DA produces the green-fluorescent compound 2',7'-dichlorofluorescein (DCF) which is retained within the cell and its emitting fluorescence has minimal overlap with chloroplast autofluorescence. Hydrogen peroxide (HO) and methyl viologen (MV) treatments, both known to increase intracellular ROS, were used to optimize assay parameters. Intracellular green fluorescence was measured at 520nm by flow cytometry. Sixty minutes was found to be a suitable stain incubation time. At the highest stain concentration tested the mean intracellular green fluorescence was elevated in non-treated controls similarly to HO and MV treatment groups, suggesting at high concentrations the stain generates ROS. However at lower stain concentrations HO and MV treatments both showed increased green fluorescence relative to untreated controls. These results demonstrate the utility of this method to assess ROS in a sea-ice diatom despite certain assay limitations. Future experiments will look at salinity and light stress effects on DMSP and ROS accumulation, as well as global proteome changes.

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MEASURING PHENOTYPIC AND TRANSCRIPTOMIC CHANGES OF DOLPHIN LUNG IN A COMPROMISED MARINE ENVIRONMENT: DEVELOPMENT OF A CELL SENSOR TO STUDY HUMAN LUNG HEALTH.

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A primary target organ for pathogen assault is the lung. Every breath inhaled by mammalian lungs contains not only required oxygen, but also hundreds of microbes and a multitude of airborne contaminants. On the average the lung is exposed to more pathogens and airborne toxins than any other internal organ.

Several physiological parameters of the bottlenose dolphin (*Tursiops truncatus*) lung, in comparison to those of the human lung, make them an ideal model for assessment of immunological responses to water-borne pathogens on human lung health. In contrast to the human, the dolphin lungs are exposed to marine borne pathogens to a greater degree than the human lung.

We propose to combine cell and tissue-based screening with transcriptomic analysis of dolphin lung cells to establish the effect of a compromised marine environment on mammalian lungs, based on the hypothesis that dolphins could be good predictors of what may happen to human lung health after chronic or acute exposure to airborne pathogens and contaminants.

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Transcriptional Profile of *Vibrio* Injected *Litopenaeus vannamei* under Hypoxic and Hypercapnic Hypoxic conditions

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The Pacific whiteleg shrimp, *Litopenaeus vannamei*, is a valuable commercial and recreational crustacean species which lives in a microbially rich environment. However, in shrimp the relationship between environmental stressors and potential modifications to the immune response has largely been understudied. Marine organisms which inhabit coastal estuaries routinely experience diurnal, tidal and seasonal changes in dissolved oxygen (hypoxia). Hypoxia is typically accompanied by increased levels of carbon dioxide (hypercapnia), which decreases water pH. To elucidate the potential effects of hypoxia and hypercapnic hypoxia, custom oligonucleotide microarrays were used to identify the transcriptional profile of L. vannamei exposed to sublethal levels of hypoxia ($P_{O2} = 4.0$ kPa, $P_{CO2} < 0.06$ kPa) and hypercapnic hypoxia $(P_{O2} = 4.0 \text{ kPa}, P_{CO2} = 1.8 \text{ kPa})$ at 4 and 24 hours (normoxic $P_{O2} = 20 \text{ kPa}, P_{CO2} < 0.06 \text{ kPa})$. In separate studies, incorporating selective plating and quantitative PCR, we also observed that exposure to hypoxia and hypercapnia reduced the bacteriostatic activity of L. vannamei to Vibrio *campbellii*. Pursuant to the data obtained in the two above mentioned studies, we intend to use custom oligonucleotide microarrays containing 22,000 unigenes expressed in L. vannamei to further explore the mechanisms which underlie the host:pathogen relationship. We test the hypothesis that the unique transcriptional profile of L. vannamei under hypoxia and hypercapnic hypoxia changes following sublethal bacterial challenge. We predict that the additional stress of a bacterial challenge will further compromise L. vannamei's ability to maintain vital metabolic processes and to mount an effective immune response. Furthermore, we intend to use real-time RT-PCR to assess temporal changes in several immune-related genes at 0.5, 2, 4, 6, 12, 24, 48 and 72 hours to identify the timing and sequence of the immune response of L. vannamei stressed via bacterial challenge only compared to L. vannamei which are stressed by both bacterial challenge and hypoxia or hypercapnic hypoxia.

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FUNCTIONAL CHARACTERIZATION OF DOLPHIN (*Tursiops truncatus*) SURFACTANT PROTEIN B.

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Lung surfactant is a complex mixture of lipids and proteins that is essential for maintaining a large surface area for efficient oxygen/carbon dioxide exchange in the respiratory system of all mammals. The proteins in lung surfactant that function to reduce surface tension are surfactant protein B (SP-B) and surfactant protein C (SP-C). SP-B is our major focus due to its cardinal role in adjusting alveolar surface tension and preventing lung collapse. Our lab has previously found that the primary sequence of dolphin SP-B exhibits significant differences, including changes in charge, substitution and even added or deleted sequences. Because of dolphin's ability to survive lung collapse and extreme pressures encountered during deep dives, the dolphin respiratory system could be an important model for human lung therapy research. Understanding the structure and function of dolphin SP-B can lead to important advancements in treatment for lung collapse and respiratory distress syndrome.

The long term goal of the project is that the variation observed between dolphin and land mammals in SP- B primary sequence produces an altered secondary structure and/or function. The hypothesis to be tested is that unique and enhanced surface-active properties of dolphin SP-B, relative to human SP-B, can be examined designing vectors for and production of recombinant dolphin SP-B mutants. This will be accomplished by generating constructs with wild type and mutant forms of dolphins SP-B constructs in bacteria, with mutations focusing on the amino acid residues of the dolphin N-terminus that differs significantly from land mammalian SP-B.

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CHARACTERIZATION OF SURFACTANT PROTEIN B IN PYGMY SPERM WHALE (Kogia breviceps): COMPARISON BETWEEN MARINE AND LAND MAMMALS.

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Pulmonary surfactant lipid-protein complex produced by type II alveolar cells. Water molecules rest on the surface of the alveolus and because each water molecule has a positive and negative charge distribution a strong attraction between molecules creates a high surface tension at the air/water interface. If the surface tension inside an alveolus is not decreased sufficiently the forces will become so great that the human alveolus will collapse thus not permitting air to enter the alveolus. When a human lung collapses it cannot be inflated without medical intervention. The lungs of all mammals have a complex lipid-protein mixture that is essential for lowering this surface tension and preventing lung collapse. The surfactant protein B (SP-B) is the primary component required for this functions. Deep diving marine mammals such as dolphins and whales will physically collapse their lungs when diving great depths and inflate them before coming back up to surface.

The focus of this research is to identify the amino acid sequence of SP-B in the pygmy sperm whale lung and compare it to the amino acid sequence of dolphins and terrestrial mammals. The central hypothesis is that the pygmy sperm whale SP-B sequence is more closely related to the sequence of the dolphin (another deep diving marine mammal) rather than to the sequence of land mammals.

To test this hypothesis, primers designed using the dolphin SP-B sequence (known) will be used to amplify the pigmy sperm whale SP-B from pygmy sperm whale lung cDNA. Lung mRNA was obtained from lung sections of a stranded pygmy sperm whale. RNA was successfully extracted from the lung tissue then converted into cDNA to use as a template for PCR to amplify portions of the pigmy sperm whale SP-B.

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Potential for Marine Antimicrobial Compounds from a Pseudovibrio sp.

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Increased emergence of antimicrobial-resistant pathogenic bacteria has prompted the search for novel antibiotics in marine ecosystems, including coral reefs and the diverse microbial community associated with the corals' surface mucopolysaccharide layer (SML). Since marine bacteria can produce compounds that are chemically distinct from their terrestrial counterparts there is potential for the discovery of novel antibiotics, which serve as chemical defenses against other bacteria, including marine pathogens. We have characterized the chemical ecology of the microbial community associated with the gorgonian coral *Pseudopterogorgia americana*, focusing on the hypothesis that P. americana SML-associated bacteria produce antibiotics against human and coral pathogens. For these studies, we first conducted an antimicrobial assay on 142 bacteria isolated from P. americana's SML using seven test strains known to be human or coral pathogens. Our results showed that 70% (99/142) of the coral isolates inhibited at least one test strain. Only one coral isolate, with 99% 16S rDNA similarity (1200 bp) to Pseudovibrio spp., inhibited all seven test strains. This isolate was mass cultured and extracted using dichloromethane and methanol. The methanol extract, which inhibited Gram-positive Bacillus subtilis and Gram-negative Vibrio harveyi and V. corallilyticus, was purified using high performance liquid chromatography. Using bioassay-guided fractionations, we detected at least two fractions with antibacterial activity, one inhibiting Gram-positive and the other inhibiting Gram-negative bacteria. Nuclear magnetic spectroscopy (NMR) and mass spectrometry suggests that one of the compounds is a small peptide (mass range 200-400). Pseudovibrios have been isolated from marine sponges, tunicates, and seawater and only one antibiotic has been characterized from the Genus. These results highlight the potential of Pseudovibrio spp. isolated from corals as sources of bioactive marine natural compounds.

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