Marine Biotechnology Research in Florida Sea Grant 1996-2003: An Outreach and Communication Foundation

Mark Schrope

Florida Sea Grant College Program

March 2004

Technical Paper 134

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Foreword

Purpose

This document contains summaries of the 24 marine biotechnology research projects funded by Florida Sea Grant since 1996. Florida Sea Grant has one of the largest marine biotechnology research theme areas in the U.S. Sea Grant network. Its long-range plan calls for development of a complementary outreach component. The database in this document creates the foundation for that outreach.

FSG is in regular contact with active and potential users of science-based technical information who are seeking to stimulate Florida's biotechnology sector. These interests include business and government, who have advised development of such material as a means of aiding science-based decisions concerning research, policy and investment. The information herein, both on the FSG website and excerpted for other communications, also is offered to scientists, science writers and the informed lay readership seeking current research findings relevant to coastal resource conservation and development.

Format

The narratives are arranged roughly according to subject matter, including medicine-related and bioactive materials, detection and analytical procedures, and genetics. Each includes information about project results and scientific advancement, publications, student training and impacts. Findings from Florida Sea Grant research serve applications in fisheries, aquaculture, seafood quality, medicine and environmental management.

Acknowledgements

Kim Wagner maintained the database of reports and assisted in preparation of this report, which was put in final format by Jackie Whitehouse. (Images obtained from some investigators and also taken by Tom Wright are stored in a separate file by Florida Sea Grant.)

Contents

Production of the Ecteinascidins, Biomedically Important Marine Natural Products, Through Cell Culture of *Ecteinascidia turbinata*

Project Number: R/LR-MB-1
September 1, 1994 through August 31, 1997

Principal Investigator: Shirley Pomponi, Harbor Branch Oceanographic Institution
Co-principal Investigator: Amy Wright, Harbor Branch Oceanographic Institution

AND

Marine Invertebrate Cell Culture for *In Vitro* Production of Compounds with Therapeutic Potential

Project Number: R/LR-MB-5
February 1, 1998 through April 30, 2000

Principal Investigator: Shirley Pomponi, Harbor Branch Oceanographic Institution
Associate Investigator: Jose Lopez, Harbor Branch Oceanographic Institution

AND

Application of DNA Microarray Technology for Marine Invertebrate Cell Culture and Marine Natural Products Production

Project Number: R/LR-MB-13
October 1, 1999 through March 31, 2001

Principal Investigator: Shirley Pomponi, Harbor Branch Oceanographic Institution
Co-Principal Investigator: Robin Willoughby, Indian River Community College
Associate Investigator: C. Russell, Research Genetics

AND

Molecular Basis of Marine Natural Product Function and Production for Improved Utilization of Bioactive Compounds

Project Number: R/LR-MB-19
February 1, 2002 through January 31, 2004

Principal Investigator: Shirley Pomponi, Harbor Branch Oceanographic Institution
Associate Investigator: Robin Willoughby, Indian River Community College

OVERVIEW

One of the greatest challenges in exploiting the enormous potential benefits of marine natural products is the difficulty in finding sustainable means of production for compounds of interest. Having sustainable supplies is not only critical if a chemical is to be marketed as a drug or other product, reliable production is also a necessity to support the research needed to study and understand novel compounds before commercial potential can even be evaluated.

In general, wild collection of the organisms that produce potentially beneficial compounds is not a long-term supply option not only because such collection could pose a threat to marine habitats, but also because wild collection is generally too costly. The three potential sustainable means of producing compounds of interest in quantity are chemical synthesis in the laboratory, aquaculture of producing organisms, and maintenance of cell cultures of producing organisms. These four projects, which collectively encompass the longest-running marine biotechnology program funded by Florida Sea Grant, focused on the latter two options.

In broad terms, the goals of these four projects, all led by Dr. Shirley Pomponi of the Harbor Branch Oceanographic Institution, were to improve techniques for maintaining cell cultures and to increase production of biomedically important compounds by cell cultures. Later projects also focused on understanding how certain marine natural products affect human cells and their function in the organisms that produce these metabolites. Overall the projects have led to substantial improvements to marine invertebrate cell culture techniques and the generation of other information that may prove critical to the development of new drugs.

PROJECT 1

The first project focused on development of techniques to allow successful culture of cells from *Ecteinascidia turbinata*, a sea squirt or tunicate, which produces potent anti-tumor compounds called ecteinascidins. In fact, ecteinascidin is in development by PharmaMar S.A. and Ortho Pharmaceuticals (under the trade name Yondelis®) for the treatment of cancer. Pomponi's group was able to maintain cultures of a variety of cell types including embryonic and heart for periods of weeks to months (heart cells would actually beat in culture!) and, through addition of epidermal growth factor and insulin, two growth regulating compounds, to stimulate cell division in cultures, but not continuous division. The work was severely hampered by the difficulty of getting enough cells to work with from the tunicates, which are only about the size of a grape.

None of the methods explored ever led to a continuously dividing culture, and indeed the group has long since abandoned attempts to maintain *E. turbinata* cell cultures because of the difficulties discovered, in favor of focusing on cell cultures of sponges, which have proven much better suited to long-term culture. For *E. turbinata*, aquaculture now appears to be the best option, and PharmaMar is farming the species as well as working on chemical synthesis as it co-develops this drug with Ortho.

Pomponi says that though the group was not successful in establishing long-term *E. turbinata* cell lines, the work did allow substantial improvements in general invertebrate cell culture techniques. For instance, they learned while working with the tunicates that density changes in growth medium, for instance those brought about by introducing additives, could adversely affect cultures, even leading to decrease in production of bioactive compounds. "It was very helpful," she says of the project, "it allowed us to refine our techniques."

The group also faced problems in its attempts to pinpoint the types of cells that produce the ecteinascidins. For instance, concentrations of the ecteinascidins were widely dispersed, under most conditions, preventing localization of production zones. During regression, there was concentration in the stolons, but stolon cells proved prohibitively challenging to collect in quantities sufficient for experimentation.

A secondary line of research for the project was to test the hypothesis that harvesting the flatworm *Pseudoceros crozeri*, which feeds on *E. turbinata*, would lead to higher yields of the ecteinascidins than

working with the tunicates themselves. The group did in fact show that both the flatworms and their eggs were high in ecteinascidins.

PROJECT 2

For the second project, the main goal was to develop techniques that would maximize growth of *Axinella corrugata* (= *Teichaxinella morchella*) (which produces the anti-tumor agent stevensine) and *E. turbinata* cells in culture as well as their production of bioactive metabolites. This was to be accomplished mainly through development of techniques to down-regulate genes that retard cell growth and up-regulate genes responsible for production. The group made substantial progress toward both ends, and was most successful with down-regulation efforts.

The general plan for this research was to test the effects on the invertebrates of compounds known to regulate growth in mammalian cell cultures. Next, probes derived from other organisms for the genes that produce those compounds that appeared to regulate growth in *A. corrugata* and *E. turbinata* would be used to search for similar growth-regulating genes in these two species. Finally, these growth-regulating genes would be cloned and expressed to produce growth-regulating supplements for cell cultures of the two species. This experimental scheme had never before been applied to a marine system.

The team found that the invertebrates would in fact respond to mammalian growth regulators. Specifically they detected responses to the IL-4 and IL-6 interleukins (a group of protein factors), fibroblast growth factor and fetal bovine serum. For *A. corrugata*, measured effects included increases in DNA and protein synthesis as well as in the activity of esterase, an enzyme involved in the breakdown of certain organic compounds. For *E. turbinata*, the only measured effect from any of the compounds tested was increased protein synthesis in response to fetal bovine serum.

For the second step of probing for growth-regulating genes, the group had difficulty obtaining enough DNA and RNA from *E. turbinata* to work with, so they focused strictly on the sponge, *A. corrugata*.

Though the original goal was to probe for genes that produce the compounds found to regulate growth in *A. corrugata*, there were at that time no sequences available for these genes from any invertebrates. For that reason, the team shifted its focus to searching for other known growth-regulating genes for which there was greater representation in sequence libraries and that exhibit a high degree of evolutionary conservation. These included genes for p53, insulin-like growth factor, and CDC-cyclin. Though that particular work was not successful, as the project progressed two probes for growth-regulating genes in marine invertebrates became available. One of those genes, for a sponge apoptosis inducer called MA-3, was present in *A. corrugata*. Later, a gene for ß-actin was also found.

As an offshoot of this work, the group developed a molecular marker that can be used to positively identify *A. corrugata* in cultures. The marker, a short DNA sequence unique to *A. corrugata*, is now an important tool that allows researchers to ensure that cultures contain *A. corrugata* rather than symbionts or contaminants.

PROJECT 3

The third project in this research series was also aimed at developing techniques to down-regulate production of factors that prevent cell division and growth and to up-regulate production of stevensine, but this time the group was working to exploit commercially available DNA microarrays. Microarrays are one of the most powerful tools in the field of genomics and had never been applied to marine

invertebrate studies. Because *E. turbinata* had proven so difficult to work with in culture during past projects, that species was not used as a model for this project and the researchers instead focused strictly on *A. corrugata*.

Microarrays are small nylon membranes containing thousands of DNA segments, or probes, and are commercially available for “model” organisms (like humans, mice, fruit flies, etc.) . For this project, the group used microarrays containing DNA from various organisms ranging from yeast to humans. The goal was to determine which genes expressed by sponges might match genes from these organisms. Because the sequence and function of the genes that make up the microarray probes are typically already known, if an expressed sponge gene matches, researchers can infer that that gene serves roughly the same purpose in the sponge. So, each match, or hybridization, can allow identification of a sponge gene and its function.

To perform the work, the group first isolated messenger RNA, which is produced when a gene is expressed as the first step toward production of a protein, from sponge samples using a modified version of the commercial Qiagen Rneasy® protocol. This RNA was then used as template material for the production of complementary DNA, or cDNA using radioactively labeled nucleotide building blocks. This labeled cDNA was then applied to the microarrays, to determine at which spots this cDNA matched, or hybridized, with the microarray probes. When that occurred, sponge cDNA was held in place, and a hybridization could then be detected by imaging of the radioactivity from the sponge cDNA at that spot.

No such comparisons between a sponge genome and that of higher organisms has ever been performed, so the group had no set expectations for experimental results. To their surprise, team members discovered that a large number of sponge genes were analogous to genes from humans and other organisms. "It was a very cool project because it was a longshot," says Pomponi, "Low and behold, it worked. Apparently there's a fair amount of conservation of DNA."

That surprising initial success paved the way for the group to home in on sponge genes that play roles in either prolonging cell life or controlling the production of stevensine. For this next phase of research, the group again applied sponge cDNA to microarrays, but this time after first treating sponge cells with phytohemagglutinin, a mitogen or compound known to regulate cell growth. The researchers reasoned that genes expressed after mitogen application that had not been expressed in cells cultured without the mitogen were involved in upregulating cell growth, while those previously hybridized but not expressed after mitogen were down-regulated. Over 100 up- or down-regulated genes were identified.

The team plans to continue studying these genes in hopes of developing methods for increasing production of stevensine or other bioproducts. Understanding the proteins coded for by these genes might, for instance, lead to the identification of specific chemicals that could be added to sponge cell cultures to enhance growth or the production of compounds of interest.

Pomponi says work is also underway, thanks in part to new funding for the group as part of the state of Florida's new Center of Excellence in Biomedical and Marine Biotechnology, to create sponge microarrays so that genes unique to sponges can be discovered. Such work would involve first creation of a library of sponge DNA segments. These segments, most coding unknown genes, would be used as probes on the new microarrays. Experiments with different treatments and different sponge species could lead to identification of new and important genes through the process of elimination comparing results of experiments with different treatments and different sponge species. Sponge microarrays could allow identification of the genes responsible for the actual production of sponge-specific products such

as stevensine, unlike the work with human and other microarrays, which can only help in identification of more highly conserved genes. Ultimately, if such genes could be identified, they might lead to economical and sustainable production of important pharmaceutical compounds using recombinant DNA techniques, where genes are inserted into organisms that are easier to grow and maintain in culture so that they can produce the compounds. "That to me is the most exciting thing in all of the work," says Pomponi. "It's all built up to this."

PROJECT 4

The Harbor Branch group's fourth Sea Grant project in this cluster involves expansion of the human microarray work to study lasonolide, a promising anti-tumor compound isolated from the sponge *Forcepia* sp. Specifically, the project's main goal is to identify genes that are up or down-regulated in human pancreatic tumor cells and *Forcepia* cells in response to application of lasonolide. Already the group has had success working with the tumor cells.

The team found four pancreatic tumor cell genes that hybridized on the human microarray after treatment with lasonolide, but not without the treatment, suggesting that the compound regulates those genes. This is an important result because knowing the affected genes could allow researchers to identify the mechanism by which lasonolide blocks growth of cancer cells. The group will likely be applying for patents on some or all of these genes in the future.

A second goal for the project was to identify *Forcepia* genes regulated by addition of lasonolide. Such knowledge could offer valuable additional insight into lasonolide's effect on cells in general, as well as to understanding of the genetic basis for production of marine natural products. "We don't know what will happen," says Pomponi of the experiment, but she says it is possible that *Forcepia* possesses special defenses against the toxic lasonolide that it produces.

Early experiments to treat *Forcepia* cell cultures with lasonolide and monitor gene expression were unsuccessful because the group was unable to extract enough RNA from these samples to conduct the microarray experiments. More samples were collected in Fall 2003 and the experiments were repeated. Data are currently being analyzed.

The group also treated cells from another sponge species, *Tedania ignis* (which is closely related to *Forcepia* but does not produce lasonolide) with lasonolide and identified 15 genes that may be down-regulated. These data should prove valuable once experiments on *Forcepia* are done, because they will allow identification of those genes unique to *Forcepia* that are related to lasonolide's effects, allowing a better understanding of how the compound interacts with *Forcepia* cells and the role of lasonolide in *Forcepia* metabolism.

Resulting Publications:

(FROM R/LR-MB-1)

Pomponi, S.A., R. Willoughby, M.E. Kaighn, and A.E. Wright. 1997. Development of techniques for in vitro production of bioactive natural products from marine sponges. In: Proceedings of the 1996 World Congress on in vitro Biology. San Francisco, CA, June 22-27, 1996. Pp. 231-237.

Pomponi, S.A. 1997. Marine resources—sustainable use for drug discovery. Sea Technology 38(9):19-25.

Pomponi, S.A., R. Willoughby, A.LE. Wright, C. Pecorella, S.H. Sennett, J. Lopez, and G. Samples. 1998. In vitro production of marine-derived antitumor compounds. In: New Developments in Marine Biotechnology. Pp. 73-76.

Scarpa, J., B. Weiss, E. Ruppert, J. Frick, A. Ford, and A. Wright. 1996. Direct evidence for planktotrophy in muller's larva of the tiger flatwork, pseudoceros crozeri. American Zoologist. 36(5):107A.

(FROM R/LR-MB-5)

Pomponi, S.A. and Willoughby, R. 2000. Development of sponge cell cultures for biomedical applications. In: Aquatic Invertebrate Cell Culture (C. Mothersill and B. Austin, eds). Springer-Praxis, Surrey, England. Pp. 323-336.

Willoughby, R. and Pomponi, S.A. 2000. Quantitative assessment of marine sponge cells in vitro: development of improved growth medium. In Vitro Cellular Development Biology (Animal) 36: 194-200.

Pomponi, S.A. 1999. The bioprocess-technological potential of the sea. Journal Biotechnology 70: 5-13.

Lopez, J.V., C.L Peterson, R. Willoughby, A.E. Wright, E. Enright, S. Zoladz, and S.A. Pomponi. 2002. Species specific genetic markers for in vitro cell line identification of the marine sponge, *Axinella corrugata*. Journal of Heredity 93(1): 27-36.

(FROM R/LR-MB-13)

Willoughby, R. 2002. In vitro gene expression in marine sponge cells stimulated by phytohemagglutinin. Doctoral Dissertation, Florida Institute of Technology, Melbourne, FL, 79 pp.

Resulting Presentations:

(FROM R/LR-MB-1 and also R/LR-MB-5)

"Development of Sponge Cell Culture Models for *In Vitro* Production", invited oral presentation by S. A. Pomponi at the Marine Natural Products Gordon Research Conference, Ventura, California, February 27 – March 2, 2000.

Four invited lectures which included results of this research were presented by Dr. S. Pomponi: BIO 98 (June 15, 1998, New York), BIO Florida (September 10, 1998, Bal Harbour, FL), Marine Bioprocessing Engineering Symposium, Opening Keynote Address (November 11, 1998, Amsterdam), NOAA workshop:Trends and Future Challenges for U.S. National Ocean and Coastal Policy (January 22, 1999, D.C.).

(FROM R/LR-MB-5)

"Marine Sponge Cell Culture: State of the Art", invited oral presentation by S.A. Pomponi at the Aquatic Invertebrate Tissue Culture workshop, Brest, France, September 4-5, 1999.

"Molecular Genetic Markers for In Vitro Cell Verification of the Marine Sponge, *Axinella corrugata*", oral presentation by J. Lopez at the Benthic Ecology 2000 meeting, Wilmington, North Carolina, March 10 -11, 2000.

"Molecular Characterization of Genetic Variation in Marine Invertebrates", oral presentations by J. Lopez to International Center for Living Aquatic Resources and Management, Makati, Philippines, July 13, 1998 and Institute of Biology, College of Science, University of the Philippines, July 14, 1998.

"Growth Medium Optimization for Marine Sponge Cells In Vitro", poster presentation by R. Willoughby at the annual meeting of The American Society for Cell Biology, San Francisco, December 12-16, 1998.

Students Involved in Projects:

(FROM R/LR-MB-5)

Enright, E. 2000. cDNA library construction in *Axinella corrugata (=T. morchella*) (non-thesis). Florida Institute of Technology, Melbourne, Florida.

Willoughby, R. 1998. Development of multiwell assays to monitor in vitro responses of dissociated marine sponge cells. Master's thesis. Florida Institute of Technology, Melbourne, Florida.

Zoladz, S. Internship Project: *T. morchella* geographic variation and genetic markers.

Lynch, J. High School - International Baccalaureate Extended Essay Project: *T. morchella* geographic variation and genetic markers.

(FROM R/LR-MB-13)

Willoughby, R. 2002. In vitro gene expression in marine sponge cells stimulated by phytohemagglutinin. Doctoral Dissertation.

Jelier, Rob. 2001. Wageningen Agricultural University, Netherlands (summer internship 2000). Master's thesis.

Biotechnological Development of Floridian Tunicate Products

Project Number: R/LR-MB-2
February 1, 1997 through October 31, 1998

Principal Investigator: Bill Baker, University of South Florida (Florida Institute of Technology during project)

Tunicates, such as sea squirts, are a common type of soft-bodied, sessile marine animal usually seen attached to rocks or dock pilings. Those of the genus *Eudistoma* produce an important series of compounds known as eudistomins. *Eudistoma olivaceum,* a Caribbean tunicate species, produces specific oxathiazepine-bearing eudistomins that act as powerful antiviral agents, but study of these compounds has been hindered by low availability. The same tunicate species is found in Florida, which would seem a ready source for the compounds, but for some reason the Florida tunicates do not appear to produce the eudistomins of interest.

For this project, headed by Dr. Bill Baker of the University of South Florida, the goal was to induce Florida tunicates to produce the oxathiazepine-bearing eudistomins by providing them with the necessary chemical precursors. "Our hypothesis," says Baker, "was that since they are the same species, perhaps the Florida tunicates have the capability and we just need to manipulate them one way or another to make them make the antiviral compounds." This goal was not accomplished during the span of the project, but Baker believes the research generated information that could make the goal achievable.

The research team hoped to induce production by the Florida tunicates of not only research quantities of the desired antiviral eudistomins, but also "unnatural" eudistomins, through experimentation with the precursors provided, in hopes of isolating new compounds with pharmaceutical potential.

Baker and his colleagues used two methods to introduce the eudistomin precursors, which were labeled with the stable isotope carbon-13. The first was an osmotic pump that periodically injected a tunicate with precursor for two to three weeks. The second was to inject tunicates with precursor every two to three days by hand.

To identify compounds produced as a result of the precursor additions, the research team used chromatography techniques to separate various fractions of the animals' products. These products were then analyzed to determine their Nuclear Magnetic Resonance (NMR) spectrums, which allowed identification of individual compounds of interest according to their characteristic NMR signals.

The main objective of the project, to induce Florida tunicates to produce oxathiazepine-bearing eudistomins in quantities sufficient for isolation, was not accomplished as the tunicates apparently did not metabolize the precursors. None of the compounds of interest were isolated. Nontheless, the research did produce benefits as it generated information about potential techniques for enhancing production of secondary metabolites as well as inducing production of new compounds.

Though this line of research is not currently being pursued in Baker's lab, he believes that with further work, the main objective could now be accomplished. Potential avenues to explore would be studying the tunicates more closely *in situ* and then using a variety of different techniques for introducing precursors, such as transferring tunicates from their natural environment into a container with precursors for a period of time and then returning them to their natural environment.

"I still think there's some potential there," says Baker of the general concept.

Resulting Presentation:

Baker, B.J. and K. Xie. 1998. “Biosynthesis of the Eudistomins in the Floridian Tunicate *Eudistoma olivacium*.” Poster presentation at the Marine Natural Products Gordon Research Conference, Ventura, CA.

Student Involved in Project:

Xie, K. 1997. Biosynthetic studies of eudistomins G, H and I using stable Isotopes. Master’s thesis.

Marine Invertebrate-Associated Microorganisms as a Source of Novel Agents for Biotechnology

Project Number:R/LR-MB-4
February 1, 1998 through March 15, 2000

and

Bioactive Agents Produced by Invertebrate-Associated Marine Microbes

Project Number: R/LR-MB-10
February 1, 2000 through May 15, 2002

Co-Principal Investigator: Bill Baker, University of South Florida (formerly at Florida Institute of Technology)
Co-Principal Investigator: Julia Grimwade, Florida Institute of Technology
Co-Principal Investigator: Alan Leonard, Florida Institute of Technology

These projects involved work with various marine organisms, especially tunicates and sponges, collected in Antarctica, Florida and Hawaii. The projects had two main goals, both of which were accomplished. The first was to isolate novel secondary metabolites with anticancer or antimicrobial potential from microbes associated with collected organisms. The second was to isolate and study plasmids from these organisms to develop new techniques that could be used for the production of important compounds such as promising secondary metabolites and for basic biology research.

The focus on culturing new marine microorganisms and isolating their secondary metabolites was motivated by recent declines in the rate of discovery of new compounds for pharmacological evaluation due to exhaustion of traditional, mainly terrestrial, sources. Marine microbes, especially those associated with invertebrate species, have remained a largely untapped source for microbes.

Baker says the group was attracted to Antarctica in its search for potential drugs by the very limited degree to which the region has been sampled for such work. "We need these products," says Baker, "and this is a largely understudied source for them." Antarctic samples included sponges and tunicates as well as molluscs, echinoderms and other invertebrates. Using standard laboratory methods the team was able to culture hundreds of new microbes associated with these organisms. Hundreds more microbes were cultured from invertebrates collected in Florida and Hawaii.

A large percentage of the thousands of isolated microbes were tested by the Grimwade group using competition assays to determine their antimicrobial potential. Species from temperate regions showed the most promise, with 43% of Floridian and Bahamian isolates showing antibiotic activity. In contrast, only 13% of the Antarctic isolates showed promise. Two hundred of the isolates were also tested by the group's commercial partners, Wyeth-Ayerst, for antimicrobial potential and by Ford Hospital for anticancer potential.

Of the isolates studied, six showed promising antimicrobial activity. One was closely analyzed and found to have little or no pharmaceutical potential because the mechanism of action determined, cell membrane disruption, is not specific enough. The team is still analyzing the remaining five candidates. Wyeth-Ayerst remains interested and is collaborating with the group on this work. A patent to cover several of these compounds was filed in 2002.

Beyond isolating microbes and their metabolites, a major component of the projects was to isolate plasmids from the bacteria cultured, work headed by Julia Grimwade at the Florida Institute of Technology. Plasmids are double-stranded units of DNA that replicate within a bacteria cell independent of the cell's main chromosomal DNA and are often commonly manipulated by researchers so that they will produce specific products of interest, such as a chemical with pharmaceutical potential. Regardless of the location of sampling, about 25% of the bacteria isolated contained plasmids. For as yet undetermined reasons, project researchers found that those bacteria with plasmids were about twice as likely to produce antimicrobial compounds.

A key goal for the plasmid work was to isolate a plasmid system that could be used for recombinant work involving non-standard bacteria such as those from cold environments. These could contain important chemicals that might be unstable at the standard temperature of 37°C where typical plasmids used for molecular biology work function. Hence, they could not be produced through recombinant technology using these plasmids.

The genetic sequences of several plasmids were partially analyzed, and a plasmid isolated from the microorganism that produced one of the promising compounds eventually became the focus of the work. The team's ultimate goal is to insert cloning sites for all the enzymes responsible for production of a single potentially beneficial chemical isolated from a microorganism. Having now inserted cloning sites in this plasmid, the group is approaching the point where its plasmid system will express the enzymes necessary for the first step in the biosynthetic production of a compound.

Resulting Publications:

Baker, B.J., Van Ert, M., Leonard, A.C. , and Grimwade, J.E. Cold-water marine invertebrate-associated microorganisms as sources of drug leads. In preparation for International Journal of Pharmacognosy.

Hahm C., Grimwade, J.E., Leonard, A.C., Frodyma, M.E., Van Ert, M., Singh, M.E., Janso, J.E., Maiese, W., and Baker, B.J. Chemical analysis of antibiotic activity in *Pseudoalteromonas clarkia* isolated from the Antarctic mollusc *Tritoniella belli*. In preparation for the Journal of Natural Products.

Frodyma, M.E., Van Ert, M., O'Connell, T., Leonard, A.C., Baker, B.J., and Grimwade, J.E. Diversity and bioactive metabolite production of bacteria associated with marine invertebrates in tropical, sub-tropical, and polar environments. In preparation for Applied and Environmental Microbiology.

Frodyma, M., Van Ert, M., Breitbart, M., Leonard, A., Baker, B., and Grimwade, J., Drug resistance and DNA topology in bacteria isolated from Antarctic and temperate marine invertebrates. In preparation for Journal of Bacteriology.

Van Ert., M.N., Frodyma, M., Baker, B.J., Leonard, A.C. and Grimwade, J.E. Characterization of bioactive metabolite production in bacteria associated with Antarctic and temperate marine invertebrates. In preparation for Applied and Environmental Microbiology.

Van Ert, M., Gancarz, B., Frodyma, M., Grimwade, J., Baker, B., and Leonard, A. Distribution, size, and host-range specificity of plasmids harbored by bacteria associated with Antarctic and temperate marine invertebrates. In preparation for Applied and Environmental Microbiology.

Resulting Presentations:

"In search of chemical diversity." Moffitt Cancer Center, Tampa, Florida, December 2000.

"Antarctic chemical ecology." Palmer Station, Antarctica, March 2000.

"*Pseudoalteromonas clarkii*, sp. nov., a psychrotrophic bacterium isolated from the Antarctic nudibranch *Tritoniellia belli*, produces an active secondary metabolite with potent antibacterial activity." 101st General Meeting of the American Society for Microbiology, May 2001.

"Chemical analysis of antibiotic activity in Antarctic Invertebrate-associated bacteria." 10th International Meeting on Natural Products, Okinawa, Japan, June 2001.

Students Supported by or Involved with Project:

Avancha, K. "Chemical investigation of Antarctic red algae." Doctoral Dissertation.

Hahm, C. Chemical investigation of Antarctic microorganisms. Received master's based mainly on work for this project. Master's thesis. University of South Florida.

Park, Y.C. chemist: spectroscopic analyses; current Ph.D. student, Department of Chemistry, University of South Florida.

Furrow, F. B. chemist: microbe plating, isolation of bacteria; Department of Chemistry, Florida Institute of Technology.

O'Connell, T. Identification and Genetic Analysis of Marine Invertebrate-Associated Bacteria. Master's thesis. Florida Institute of Technology, Melbourne, Florida.

Mayor, M. Gene sequencing, plasmid replication genes, Department of Biological Sciences, Florida Institute of Technology.

Larson, S. Bacterial isolations, bioactivity determinations, gene sequencing; Department of Biological Sciences, Florida Institute of Technology. (currently on medical leave of absence)

Schumacher, L. 2002. Bacterial isolations, bioactivity determinations, Department of Biological Sciences, Florida Institute of Technology. Master's thesis. Initial placement: Brevard Community College, Instructor; ZelTechnologies, Biological concept developer.

Calica, C. M.S. 2001. Extract preparation, bioactivity determination, Department of Biological Sciences, Florida Institute of Technology. Initial placement sales in health care industry.

Muller, C. B.S. 2000, rRNA gene sequencing; Department of Biological Sciences, Florida Institute of Technology, Melbourne, Florida. Initial placement graduate school.

Truci, A. B.S. 2000, bacterial isolations; Department of Biological Sciences, Florida Institute of Technology, Melbourne, Florida. Initial placement graduate school.

Ferraz, S. B.S. 2000, bacterial isolations; Department of Biological Sciences, Florida Institute of Technology, Melbourne, Florida. Initial placement graduate school.

Hendirck, H. chemical tech: extract preparation, chromatographic isolation; Department of Chemistry, Florida Institute of Technology, Melbourne, Florida.

Hicken, N. B.S. 2000, chemical tech: extract preparation, bioassay; Department of Chemistry, Florida Institute of Technology, Melbourne, Florida.

Fuller, B. electroporation procedure, microbe characterization; Department of Biological Sciences, Florida Institute of Technology, Melbourne, Florida.

Bugge, A. B.S. 2001, bacterial isolations, bioactivity determinations; Department of Biological Sciences, Florida Institute of Technology, Melbourne, Florida. Initial placement-travel, then seeking employment.

Chapman, T. B.S. 2001, bacterial isolations, bioactivity determinations; Department of Biological Sciences, Florida Institute of Technology, Melbourne, Florida. Initial placement-travel, then seeking employment.

Moritz, N. B.S. 2001, bioactivity determinations; general lab preparation; Department of Biological Sciences, Florida Institute of Technology, Melbourne, Florida. Initial placement-travel, then seeking employment.

Van Ert, M. Analysis of Genetic Diversity and Antimicrobial Substance Production in Microorganisms Cultured from Antarctic and Subtropical Marine Invertebrates. Master's thesis.

Development and Optimization of *In Vitro* Production Methods of Bioactive Marine Alkaloids

Project Number: R/LR-MB-6
February 1, 1998 through June 30, 2000

Principal Investigator: Russell Kerr, Florida Atlantic University
Co-principal Investigator: Shirley Pomponi, Harbor Branch Oceanographic Institution

Though marine organisms have shown great promise as a source of compounds with pharmaceutical potential, research and clinical development with such compounds often either has been hindered by the lack of availability of source organisms, or has led to environmentally unsustainable over-collection of source organisms. For this reason, novel methods for producing supplies of bioactive marine natural products are urgently needed. This project addresses this problem. The goal of the project was to elucidate the biosynthetic pathway responsible for the production of the marine alkaloids the ecteinascidins and stevensine, and to develop and optimize *in vitro* production methods for these compounds. The group focused on these alkaloids because they were already working with cell cultures from the source organisms. In addition, the ecteinascidins have shown promise as cancer-fighting agents, and are already in human trials. The group had substantial success in identifying both the critical precursors in production of both the ecteinascidins and stevensine and potentially beneficial additions that could fuel *in vitro* production.

Much of the work involved in the project focused on cell cultures of the tunicate *Ecteinascidia turbinata* and the sponge *Teichaxinella morchella*, which produce the ecteinascidins and stevensine, respectively. To identify the chemical building blocks, or precursors, in the natural production of these alkaloids the researchers relied mainly on radioactive labeling of potential precursors. Individual radiolabelled compounds were added to cell cultures, and when the intermediate or final compounds produced in culture were also radioactive, it indicated that the initial compound was in fact used as a precursor.

For stevensine, the goal was to identify the most basic metabolic building blocks, namely, the amino acids required for its production. Likely amino acid building blocks were identified based on the structure of stevensine, then radioactively labeled and added to cell cultures of *T. morchella*. For the ecteinascidins, the goal was to identify advanced intermediate compounds required for production of the final alkaloids. Again the likely precursors were determined based on the structure of the ecteinascidins. These were then radiolabeled and tested as precursors. This particular work was conducted using a cell lysate of the tunicate rather than with a cell culture. Three of four potential precursors tested were in fact efficiently transformed into ecteinascidins. This set of advanced intermediates, the first to ever be identified in the biosynthesis of a bioactive natural product from a tunicate, included the diketopiperazines of the amino acids tyrosine and DOPA as well as mercaptopyruvic acid.

The group's success in synthesizing diketopiperazine of DOPA was especially significant as the production of a DOPA peptide had never before been reported. This work has led to a journal manuscript and new research on the potential synthesis of other peptides containing residue from the amino acid DOPA.

Because lipids such as sterols can often be limiting factors in cell cultures, the group also set out to identify those lipids important to the production of the ecteinascidins and stevensine. Lipids are often obtained through diet rather than production by source organisms, so they can be especially important as cell culture additions to enhance production. However, the researchers ended up with surprising results for this aspect of the project. "We had expected to find regular sterols and instead we saw some rather

unusual compounds," says Kerr. *T. morchella* appears to be quite primitive, using only "sterol-like" compounds, just as bacteria and other prokaryotes do. No other sponge has ever been found to lack the key lipid class sterols. This finding, while interesting, slowed progress to some extent because the sterol-like compounds were more difficult than common sterols to identify. The group was able to identify these compounds, a novel class of diepoxysteroids, which are also likely to be limiting factors in production and normally obtained through diet. Lipid work was not pursued for *E. turbinata.*

While potentially beneficial cell culture additions were identified through the project, the benefits of these additions in improving production were not tested as there were later problems maintaining healthy cell cultures, and because research within the labs involved shifted focus. However, the methods developed and improved for studying the biosynthesis of ecteinascidins and stevensine have advanced related work with other important compounds.

Another focus of the project was to concentrate the biosynthetic proteins from extracts of *T morchella*, identify the co-factors required for stevensine production and use the extract to produce stevensine chemoenzymatically. This technique offered an alternative system to cell cultures for studying the biosynthetic pathway of stevensine, which the groups involved have used as a model system in their research. The main method used to produce these extracts was a protein precipitation technique known as acetone powder formation, which leads to the production of a catalyst powder containing all the proteins found in a sample organism. "The acetone powder has the enzymatic machinery," says Kerr, "so we give it the raw materials." Those raw materials are simply amino acids that were mixed with the powder, ultimately leading to stevensine production.

This chemoenzymatic production is not a sustainable method of production because it relies on samples of the source organism. Instead it is a tool for elucidating the biosynthetic pathway that leads to stevensine to aid in the development of sustainable production methods. It also allows manipulation of various steps in the biosynthesis of a compound so that novel compounds can be produced. As with the cell cultures, by adding individual radiolabelled amino acids and then identifying radioactive products, Kerr and his colleagues were able to determine which amino acids were critical in stevensine production, and to identify intermediate compounds, allow further elucidation of the stevensine biosynthetic pathway.

Resulting Publications:

Andrade, P., R. Willoughby, S.A. Pomponi and R.G. Kerr. 1999. Biosynthetic studies of the marine alkaloid, stevensine, in a cell culture of the Marine Sponge *Teichaxinella morchella*. Submitted to Tetrahedron Letters, 40, 4775-4778.

Jeedigunta, S., J.M. Krenisky and R.G. Kerr. 2000. Diketopiperazines as advanced intermediates in the biosynthesis of ecteinascidins. Tetrahedron 56, 3303-3307.

Jeedigunta, S., H. Fawehinmi and R.G. Kerr. An enzymatic production of -mercaptopyruvic acid and its involvement in ecteinascidin biosynthesis. Manuscript in preparation.

Saleh, M. and R.G. Kerr. Oxidation of tyrosine diketopiperazine to DOPA diketopiperazine with tyrosine hydroxylase. Submitted to Tetrahedron Letters.

Resulting Presentation:

"Biosynthetic studies of stevensine using a cell culture of *Teichaxinella morchella*." P. Andrade, R. Willoughby, S.A. Pomponi and R.G. Kerr. Presented at the Annual meeting of the American Society of Pharmacognosy, Orlando, Florida, July 1998.

Students Involved in Project:

Andrade, P. 1999. In vitro biosynthetic studies of stevensine, a natural product from the sponge *Teichaxinella morchella*. Master's thesis. Department of Chemistry and Biochemistry, Florida Atlantic University. Currently enrolled as a PhD student at Penn State.

Fawehinmi, H. 1999. An efficient preparation of -mercaptopyruvic acid, a putative intermediate in ecteinascidin biosynthesis. Master's thesis. Department of Chemistry and Biochemistry, Florida Atlantic University. Currently enrolled as an MD student at Nova Southeastern University.

Fuhr, S.: assisted cell culture chemical analyses.

Jeedigunta, S. 1999. Synthesis and testing the intermediacy of diketopiperazines in the biosynthesis of ecteinascidins. Master's thesis. Department of Chemistry and Biochemistry, Florida Atlantic University. Currently working as a research lab manager at the University of Vermont.

Cellular Localization and Production of Bioactive Discodermia Metabolites

Project Number: R/LR-MB-7
February 1, 1998 through April 30, 2000

Principal Investigator: Susan Sennett, Harbor Branch Oceanographic Institution
Co-Principal Investigator: Peter McCarthy, Harbor Branch Oceanographic Institution
Co-Principal Investigator: Shirley Pomponi, Harbor Branch Oceanographic Institution
Co-Principal Investigator: Sarath Gunasekera, Harbor Branch Oceanographic Institution

Several compounds found in sponges from the genus *Discodermia* have shown great pharmaceutical potential, most notably discodermolide, a potent anti-tumor agent. But as with all bioactive products, the commercial potential of such products depends on availability of sustainable production methods. This project was designed to address that problem.

Though synthetic methods for producing discodermolide have now been devised, when this project began there was no sustainable option available. At that time there had been recent indications from ongoing research with *Discodermia* that the important compounds associated with it were actually produced by microorganisms harbored within the sponge. The goal of the project was to isolate these producers so that they could be grown in culture as a sustainable means of production. That goal was not accomplished, though this line of research continues to a limited degree.

The research team, led by Susan Sennett and Peter McCarthy, of the Harbor Branch Oceanographic Institution, took a two-pronged approach in its efforts. The first line of investigation was to determine if any of the over 100 microbial isolates the group had previously cultured from the sponge were the producers of the known important compounds. Each strain was fermented and extracts tested for bioactivity. During such experiments, if a microbe that produced one of the compounds of interest had indeed been isolated, then its bioactivity would have been similar to that of the compound itself.

The second approach involved the use of anti-discodermolide antibodies, produced by a commercial company that specializes in custom antibodies. This was accomplished by inoculating rabbits with the compound bound to a carrier molecule causing an immune response in the rabbit.

This antibody procedure involves a sort of product sandwich, the detection of which can lead to discovery of the location of production for a given compound. In theory, a compound-specific antibody attaches to the compound of interest in the sponge, at sites where the compound is being produced and, hence, is most concentrated. Next, a fluorescently labeled anti-rabbit antibody (commercially available) attaches to the compound-specific antibody. This secondary antibody can then be detected using fluorescence microscopy, allowing identification of production sites.

Other investigators have used this method to detect compounds such as Taxol® in blood, and it has also been used in one study to localize production of a marine natural product. However, the Harbor Branch team was not able to localize *Discodermia* metabolites using this method.

Neither approach identified a microbe that produces discodermolide or the other compounds with therapeutic potential produced by the sponge. Sennett says the group continues to pursue isolation of such microbes, as this would be helpful for basic research related to the compounds and provide more insight into marine invertebrate-associated microbial communities. However, she says, there is little need now for a sustainable microbe culture production method because Harbor Branch's pharmaceutical partner has developed a synthetic production method for discodermolide.

"We still suspect that [the compounds] are produced by microbes, but we don't have anything definite," she says, "We haven't been able to get to that point."

The work did have a potential spinoff. Discodermide, another bioactive compound isolated from *Discodermia*, is an antifungal agent, so the group studied fungi isolated from the sponge in hopes of identifying other novel antifungal compounds. An intern working with the group identified a microbial extract with antifungal activity. However, this activity could not be reproduced consistently and so work with this compound was pursued no further.

Students Involved in Project:

Schneider, W. Illinois Wesleyan University, Bloomington, IL

Walls, B. William Smith College, Geneva, NY

Harber, A. Lincoln Park Academy High School, Fort Pierce, FL

No Resulting Papers or Presentations

Methods to Increase Microbial Recovery From Deep-Water Sponges

Project Number: R/LR-MB-11
October 1, 1999 through September 30, 2001

Principal Investigator: Julie Olson, University of Alabama (Formerly at Harbor Branch Oceanographic Institution)
Co-Principal Investigator: Peter McCarthy, Harbor Branch Oceanographic Institution

Many microorganisms harbored within marine sponges are known to produce or are suspected of producing chemicals with therapeutic value for humans. But it is estimated that current methods only allow about 0.1 to 12.5% of the microbe species in a targeted microbial community to be cultured and studied. This project was motivated by the assumption that the as yet untapped majority of microbes must almost certainly include countless undiscovered beneficial natural products. Its main objective--to improve existing culturing methods to increase the diversity of microbes available for study--was achieved.

Though the methods developed through this project can be applied to work with various microbial communities, the focus was on those associated with deepwater sponges. Sponges have proven a good source of compounds with pharmaceutical value in part because they are stationary and therefore must have chemical or other defenses against predators. These are typically secondary metabolites, or compounds not biologically required to sustain life, and they are the main drug discovery target.

However, research has shown that microorganisms harbored within sponges may actually be the producers of many secondary metabolites of interest, so culturing microbes from sponge samples is a topic of great interest. This task has proven challenging because the variety of microbes present is vast. "Sponges tend to be filter feeders", says Olson, "so they're actually filtering bacteria and other particles from the water column to use as their food sources. By the same token, it's been shown that up to 60% of the biomass of some sponges is microbial."

To maximize the diversity of microbes cultured from sponge samples, the Olson group tested a variety of media additions chosen based on clues from previous research, such as catalase, sodium pyruvate, and sponge extract to learn which would lead to the isolation of the most microbial species. These experiments did in fact allow dramatic increases in the diversity of microbes cultured. Of the additions tested, 35% led to isolation of at least 50% more microbial species than control groups. Recoveries greater than 100% higher than controls were achieved with 21% of the media tested.

Sodium pyruvate was the most successful addition, stimulating a roughly threefold increase in recovery over controls. This success was likely due to the fact that sodium pyruvate protects microbes, many of which have little tolerance for oxygen, against harmful oxygen radicals, combined with its being long-lived under culturing conditions. Other additions, such as the enzyme catalase, offered the same protection, but only for a brief period because they quickly degrade.

A second priority of the research was to increase production of secondary metabolites by isolated microbes, because for cultured microbes to be of significant research value, they must produce enough secondary metabolite for use in bioassays. Also, when a metabolite with potentially beneficial activity is discovered, production is critical if a metabolite is to be produced economically for pharmaceutical use.

To maximize the expression and production of secondary metabolites, Olson and her team studied both the physical conditions, including temperature, oxygen concentration and salinity, as well as growth

medium, that would best support the targeted microbial communities. For physical parameters, the group strove to match conditions measured at sites where sponge samples were collected. Culture experiments were also conducted using a variety of growth media. Data produced suggested that SYZ, which can be produced using commercially available products, was the best fermentation medium.

As a rough measure of the genetic diversity of both initial sponge samples and the collections of microbes cultured from them, the team used Polymerase Chain Reaction (PCR) techniques to amplify segments of microbial ribosomal RNA from microbes present. These were then run on denaturing gradient gel and the resulting banding patterns examined. Sponge samples, which would include all microbes present in the sponges, had a significantly higher level of genetic diversity than that of the cultured organisms, indicating that a significant number of microbial species remained uncultured.

Also based on this gel work, it was apparent that the cultivation techniques used did not allow the growth of some groups of bacteria, particularly those with high content of the nucleic acids guanine and cytosine. This group has historically produced the most beneficial secondary metabolites as compared to microbes highest in adenine and thymine, which the techniques tended to favor.

Using funding from other sources, members of Dr. Olson's lab are currently working with additional media additions and other techniques to further improve the diversity of cultured organisms, with an emphasis on increasing isolation of organisms from the high guanine and cytosine group. Now, says Olson, "We're trying to get better recoverability of specific communities rather than of the entire community, for drug discovery purposes." The Sea Grant project work was focused more on the recoverability of the entire community because, she says, "When you're looking at environments very few people have looked at, it's hard to know where to start."

As a result of this project, 330 microorganisms were isolated, and with 252 of these the group was able to ferment and extract metabolites. Samples of these have been sent to Novartis, the group's industrial partner, for ongoing testing using the company's proprietary bioassays. The isolates are also maintained at Harbor Branch and are part of its Biotic Surveys and Inventories project, which is defining the biodiversity of the institution's culture collection of more than 16,000 isolates.

Resulting Publications:

Olson, J.B., C.C. Lord, and P.J. McCarthy. 2000. Improved recoverability of microbial colonies from marine sponge samples. Microbial Ecology. 40:139-147.

Olson, J.B. and P.J. McCarthy. Re-submitted-2003. Sponge-associated microbial communities reveal distinct differences when examined by cultivation and DGGE approaches. Aquatic Microbial Ecology.

Resulting Presentations:

Olson, J.B. and P.J. McCarthy. Methods to increase recoverability of sponge-associated marine microorganisms. Presented at the 7th European Symposium on Marine Microbiology, Sept 17-22, 2000.

Olson, J.B. and P.J. McCarthy. Methods to increase recoverability of sponge-associated marine microorganisms. Presented at the Florida Marine Biotechnology Summit II, Oct 16-17, 2000.

Olson, J.B. and P.J. McCarthy. Genetic diversity of deep-water sponge-associated microorganisms. Presented at the ASM General Meeting, May 20-24, 2001.

Students Involved In Project:

Walls, B. - Intern presentation: “The search for secondary metabolites produced by microbes isolated from the sponge *Scleritoderma cyanea*.” undergraduate during project, currently pursuing graduate degree.

Russo, R. - Intern presentation: “Isolation and antimicrobial activity of bacteria from deep sea sponges.” Graduate student during project and still enrolled in a Ph.D. program at the University of Florida.

Henderson, D. - Intern presentation: “Molecular analysis of the microbial communities of deep sea sponges.” Undergraduate during project, planning to pursue graduate degree.

Investigation of the Molecular Target of the Lasonolides, Potent Antitumor Agents Isolated from the Marine Sponge *Forcepia* Sp.

Project Number: R/LR-MB-17
February 1, 2002 through December 31, 2004 (Work in progress as of this summary)

Principal Investigator: Amy Wright, Harbor Branch Oceanographic Institution

Finding bioactive compounds produced by marine organisms that show pharmaceutical potential is only the first step in the very long process of commercializing a new drug. Before a pharmaceutical company will make the decision to take on that costly process, researchers must first determine how a bioactive compounds works. If, for instance, it kills cancer cells in laboratory tests, the mechanism of cell death must be understood so that the compound's effect on humans can be predicted.

This project, led by Dr. Amy Wright, of Harbor Branch Oceanographic Institution, is aimed at reaching that level of understanding for the lasonolides, a promising group of anti-cancer compounds produced by a deep-sea sponge. At this point only preliminary work has been done toward that goal.

The lasonolides are particularly intriguing for scientists because research to date has shown that the compound has a novel effect on cancer cells. The National Cancer Institute tested the compound against its 60 cancer cell line panels, and did not find a match with the patterns of activity for known cancer-fighting agents whose mechanisms are understood. Harbor Branch has done a number of experiments to determine if lasonlide has an effect on the cell cycle, as many cancer drugs do, but found no indications of such activity. "All we figured out was that it doesn't do any of the obvious things we would think it might do," says Wright.

So, with Sea Grant funding, the group is now turning to a new approach using a process called affinity chromatography. The method's first step calls for chemically attaching lasonolide to tiny micrometer-range beads that are used to fill a column and act as an "affinity matrix." Cancer cells are then broken down and all their proteins isolated together as a mixture, which is then added to the column in solution. In theory, those proteins that lasonolide has an effect on will bind to the beads to be later removed from the beads and isolated for study.

The idea is that determining which proteins are affected by lasonolide is a first step in determining how it affects cancer cells. Once the affected protein or proteins are identified, the team can then sequence the protein/s and compare them to the sequences of known proteins. If similarities with known proteins are found, researchers may be able to infer important information about how lasonolide works by considering biological interactions in which the known protein is involved. If similarities are not found, then researchers will have to embark on a much more lengthy process of close study of the sequence. This could include inferring the sequence of the gene that produced it based on the protein's sequence then seeking out that gene to determine if it can give any information about what might be involved in the lasonolide activity.

The work has proven challenging. Wright says the lasonolides are somewhat unstable and preparation of a good affinity matrix has been difficult. In addition the degree to which proteins that do not bind with the lasonolides have nonetheless been binding to the beads, called non-specific binding, has been greater than predicted. Work is ongoing to optimize factors such as the type of bead used in the affinity matrix as well as exploring other methods to link lasonolide to the matrix.

As another approach, the team also plans to use a different affinity technique that operates on the same principal as affinity matrix chromatography but avoids the use of beads and therefore problems with non-specific binding. This process involves tagging lasonolide molecules with an ultraviolet activated label. These tagged lasonolides are then reacted with the cancer cell protein mixture and separated using a process called gel electrophoresis. Those proteins that bind to the lasonolide can then be identified by the light emitted when they are illuminated with ultraviolet light. The target protein can then be cut from the gel, purified further and sequenced.

The group is also collaborating with Harbor Branch commercial partner, Novartis Institute of Biomedical Research in East Hanover, NJ. Through this collaboration, Novartis is applying modern genetic methods to help define the molecular target of the lasonolides.

No publications or presentations have yet resulted from this work.

Biosynthesis and Enzymology of the Pseudopterosins: Anti-inflammatory Agents from the Soft Coral *Pseudopterogorgia elisabethae*

Project Number: R/LR-MB-8
February 1, 2000 through May 3, 2002

Principal Investigator: Russell Kerr, Florida Atlantic University

and

Development of a Biotechnological Production Method of Elisabethadione -- A Potent Marine Anti-inflammatory Agent

Project Number: R/LR-MB-14
February 1, 2002 through May 3, 2004 (Work in progress as of this summary)

Principal Investigator: Russell Kerr, Florida Atlantic University
Co-Principal Investigator: Jose Lopez, Harbor Branch Oceanographic Institution

Pseudopterosins and seco-pseudopterosins, a group of compounds isolated from the soft coral *Pseudopterogorgia elisabethae*, are powerful anti-inflammatory and analgesic agents and are used in commercial production of skin cream products. Nonetheless, the only current source for pseudopterosins is collection from nature, meaning *P. elisabethae* is likely the marine invertebrate most heavily collected as a source of natural products. It is also likely that some researchers and pharmaceutical companies have been reluctant to take promising pseudopterosins into clinical trials because without a sustainable method of production, their commercial viability remains questionable.

The main purpose of these two projects, both led by Dr. Russell Kerr of Florida Atlantic University, was to take critical steps toward the development of a biotechnological production method for pseudopterosins. This goal was accomplished, due in part to the successful application of techniques developed and lessons learned through a previous Sea Grant project, "Development and Optimization of In Vitro Production Methods of Bioactive Marine Alkaloids." The Kerr group is now close to the development of a sustainable biosynthetic production method for pseudopterosins or anologues with similarly promising bioactivity, and is also working to culture the algae recently discovered to produce the pseudopterosins as an alternative sustainable production method.

The first step in the research was to produce crude cell-free extracts that included all the key chemical components produced by the source organism, *P. elisabethae*. This was accomplished using a protein precipitation technique known as acetone powder formation, which leads to the production of a catalyst powder containing all the proteins found in a sample organism. Those compounds in the *P. elisabethae* extract powder that were structurally related to pseudopterosins and seco-pseudopterosins were identified as possible biosynthetic intermediates in the production of these products.

Of these potential intermediates, those that looked most promising were generated in a radioactively labeled form and then added individually to extract solutions. When radioactive pseudopterosin products were produced it confirmed that the initial compound was in fact an intermediate in the biosynthesis of pseudopterosins.

Kerr's team believes that, using these methods, it has now identified many key intermediates in the biosynthetic pathway that produces the pseudopterosins and seco-pseudopterosins, though this will

require further verification. The importance of this accomplishment is emphasized by the fact that only two steps in the biosynthetic pathway of the important cancer-fighting drug Taxol have been confirmed.

The most critical intermediate in pseudopterosin production is a diterpene cyclase now known as elisabethatriene cyclase, which is responsible for production of much of the structure of the final product. By subjecting the crude protein extract powder to numerous repeated purification protocols, the group has been able to produce this enzyme in purified form.

With the elisabethatriene cyclase purified, the team was able to take a major step toward a sustainable means for producing pseudoterosins. They have sequenced the cyclase and converted this information to identify the corresponding DNA sequence information that codes for production of the cylcase. Using a technique called Reverse Transcriptase PCR, or RT-PCR, the group has been searching for the gene containing this DNA sequence identified and, hence, the gene responsible for the cyclase production.

Kerr says the group has identified DNA fragments that may contain the gene responsible for the cyclase, and is in the process of testing this assertion. To do that, the DNA fragments have been inserted in the genome of the common and relatively easy to work with bacteria E. coli, to see if it produces the enzyme, though this has not yet been completed. More efficient and commercially viable production of the enzyme, however, would require insertion of the gene in yeast, which naturally, and unlike E. coli, produces the precursors needed for the enzyme's activity.

Successful expression of the enzyme would not only lay the groundwork for a biotechnological production method for pseudoterosins, it could also enable production of an analogue that could be used in place of pseudopterosins. This analogue, called elisabethadione, is an advanced intermediate in the production of pseudopterosins identified during the project that itself shows promising bioactivity similar to that of the pseudopterosins. Producing it would be easier than full production of the pseudopterosins and therefore may prove the most direct route to harnessing the desired pharmaceutical benefits.

Another potential sustainable route for pseudopterosin production has also emerged during the group's work. Kerr and his team have discovered that pseudopterosins are not produced, as previously thought, by the coral *P. elisabethae,* but instead by the coral's symbiotic algae, *Symbiodinium* sp. A patent on this discovery has already been filed. "We now have two competing goals," says Kerr, because the algae could potentially be cultured in the lab to sustainably produce pseudopterosins, which could prove a more favorable source for these products than the other productions methods under investigation.

A sub-goal of this research was to produce novel pseudopterosins and seco-pseudopterosins by using commercially available glycosyl transferases and glycosidases to complete the glycosylation step in pseudopterosin biosynthesis. However, none of the roughly 20 products tested allowed successful glycosylation, which Kerr says is not surprising given that glycosyl transferases are typically very selective in the substrates they accept. This line of work has led to a new project funded by the National Science Foundation in which the group is working to purify the enzyme naturally responsible for the glycosylation. This would not only allow advancement in the goal of biosynthetic pseudopterosin production, but could also allow production of novel compounds.

Resulting Publications:

Ata, A., R.G. Kerr and R.S. Jacobs. Identification of anti-inflammatory diterpenes from *Pseudopterogorgia elisabethae*. Tetrahedron, in press.

Kohl, A. and R. Kerr. Purification and characterization of the diterpene synthase from Pseudopterogorgia elisabethae. Manuscript in preparation.

L.D. Mydlarz, R.S. Jacobs, J. Boehnlein and R.G. Kerr. Evidence that the origin of pseudopterosin biosynthesis resides in the dinoflagellates symbiont of *Pseudopterogorgia elisabethae*. Submitted to P. National Academy of Science.

R. Thornton and R. Kerr. 2002. Induction of pseudopterosin biosynthesis in the gorgonian *Pseudopterogorgia elisabethae*. Journal of Chemical Ecology, 28, 2083-2090.

Resulting Presentations:

Coleman, A. and R.G. Kerr. "Biosynthetic studies of pseudopterosin in the soft coral *Pseudopterogorgia elisabethae*." Presented at the Annual meeting of the American Society of Pharmacognosy, Seattle, Washington; July 2000.

Coleman, A., R. Thornton, A. Ata, T. Ferns and R. Kerr. "Development of biotechnological production methods for pseudopterosins and seco-pseudotperosins, marine-derived anti-inflammatory agents." Presented at the BIO+Florida annual meeting, Tampa 2000.

Kerr, R. "The pseudopterosins -- elucidation of the biosynthetic origin, discovery of anti-inflammatory intermediates and proposal of a biotechnological production method." Invited presentation at the Gordon Research Conference on Marine Natural Products, Ventura, California, February 2002.

Kerr, R. "Biosynthesis of Marine Diterpenes." Colloquium on Marine Natural Products, Bremerhaven, Germany; 2002.

Kerr, R. "On the Biosynthesis of the Pseudopterosins." Euro Conference on Marine Natural Products, Elmau, Germany; 2002.

Kerr, R. "Pseudopterosin Biosynthesis: Pathway Elucidation and Enzymology." The Society for Industrial Microbiology, Hawaii; 2002.

Kerr, R. "Biosynthesis of Marine-Derived Anti-inflammatory Agents." The University of Kentucky; 2003.

Kohl, A. and R. Kerr. "Purification of the elisabethatriene cyclase from the marine soft coral, *seudopterogorgia elisabethae*." Presented at the Gordon Research Conference on Marine Natural Products, Ventura, California, February 2002.

Students Involved with Project:

Boehnlein, J. (PhD), biosynthetic studies, cell biology.

Coleman, A. (PhD), enzyme purification studies, biosynthetic studies.

Ferns, T. (PhD), enzymatic glycosylation reactions.

Frenz, J. (PhD), biosynthetic studies.

Thornton, R. 2000. Aquaculture of Pseudoterogorgia elisabethae and induction of pseudoterosins. Master's thesis. Florida Atlantic University.

Bipyridyl Marine Natural Products as Anti-Fouling Agents
Project Number: R/LR-MB-9
February 1, 2000 through January 31, 2002

and

Nemertine and Sponge Pyridyl Marine Natural Products as Anti-Fouling Agents
Project Number: R/LR-MB-16
February 1, 2002 through June 30, 2004 (Work in progress as of this summary)

Principal Investigator: William Kem, University of Florida
Co-Principal Investigator: F. Soti

Development of new marine paints to protect boats and ships from encrustation by barnacles and other organisms is an important goal not only because of economic significance, but also because of environmental concerns. The world market for marine paints is worth billions, and currently most boats and ships are covered with paints containing heavy metals that are a threat to marine organisms, especially when vessels are in port and the additives in bottom paints have time to leach out. The goal of improving on existing marine bottom paints is now especially urgent because paints containing common additives are now being outlawed.

The main goal of these two projects, both led by Dr. William Kem of the University of Florida, was to isolate and test compounds from marine organisms that can prevent settlement of barnacles and other organisms but that are not toxic to marine organisms. The team was successful in this effort and has recently filed a patent for the most promising compound so far identified.

The Kem team's main focus was the nemertine worm *Amphiporus angulatus*, known commonly as the Chevron nemertine, which produces a wide range of pyridine alkaloids. Many compounds from this group have medicinal or poisonous properties, and they have especially strong odors. For reasons that are not yet completely clear, some of the many pyridine alkaloids the worms produce are toxic to marine organisms. "My hypothesis is that these flatworm-like marine worms use the pyridine alkaloids as a means of paralyzing their prey and also to defend themselves against animals that might want to make a meal of them," says Dr. Kem.

The group was able to isolate about 20 different pyridyl alkaloids and then study their structures using a variety of methods including High-pressure Liquid Chromatography and sequential Gas Chromatography-Mass Spectrometry-Infrared analysis in collaboration with a National Institutes of Health laboratory.

Laboratory tests of barnacle settlement on surfaces treated with isolated alkaloids identified the Aa alkaloid 2,3'-bipyridyl as having excellent ability to inhibit barnacle settlement, as well as high toxicity for crustaceans, a potential problem for bottom paints. However, there had been earlier work by Dr. Kem and others on mammals and crustaceans suggesting that animals can detect some pyridine alkaloids with nicotine receptors, rather than simply respond to any harmful effects they may inflict. So, the team set out to separate the settlement prevention ability of the compound from its toxicity.

To accomplish this separation, the team began by synthesizing a variety of compounds with structures similar to the promising 2,3'-bipyridyl. Some of these compounds did in fact block settlement with dramatically reduced lethality.

One of the most promising of these synthetic compounds was a substituted bipyridyl, which showed no significant toxicity in tests with crustaceans. A patent has now been filed on this and related compounds because of their potential for use in bottom paints.

The group plans to run its first field tests with some of the compounds that have been identified and synthesized. Panels covered in paint containing individual compounds will be placed in the water and exposed to ocean currents and conditions in Beaufort, North Carolina, to test settlement on the panels. Growth on these panels will then be compared to control panels covered with paint that does not contain any of the compounds being studied.

The second project has also included experiments to study the specific effect of some of the alkaloid compounds on spiny lobster chemoreceptors and whether the compounds also bind to nicotine receptors in the brains of crustaceans, given that some nemertine alkaloids are known to stimulate mammalian nicotine receptors.

The scope of the second project has expanded to include testing of the anti-settlement and crustacean toxicity properties of a group of sponge toxins called collectively halitoxin. The work with these compounds has not progressed to the point where settlement prevention and toxicity can been separated, so their commercial promise is not yet clear. However, regardless of commercial potential, the compounds are scientifically interesting because they have certain structural similarities but are much longer than the nemertine compounds. For this reason Dr. Kem believes it is likely their anti-settlement properties and toxicity involve distinct mechanisms, which could allow for interesting comparisons that may increase understanding of all the compounds.

In addition to exploring potential marine paint applications of the compounds, the Kem group is also examining their possible use as pesticides. Several of the compounds have been subjected to insecticide assays where they are injected into roaches. Convulsions or death in the roaches indicates pesticide potential. Only a few compounds have so far been tested, and some have shown promise. As evidence of the importance of this line of research, Japanese and German companies are exploring use of derivatives of another nemertine alkaloid originally discovered by Kem through past research as a pesticide.

Dr. Kem also suggests that some of the compounds his group is studying could potentially be used as "lead" compounds in the design of muscle relaxants because nicotine receptors are very important in the excitation of mammalian skeletal muscles. Blocking this activity could prevent a muscle from responding to a nerve during surgery.

Resulting Publications:

Kem, W.R., and F. Soti. 2001. *Amphiporus* alkaloid multiplicity implies functional diversity: initial studies on crustacean pyridyl receptors. Hydrobiologia 456: 221-231.

Kem, W.R., D. Rittschof, and F. Soti. Synthesis and chemical-biological characterization of all eight possible methyl-2, 3'-Bipyridyls. (re-submitted to Journal of Natural Products; 10/03.)

Kem, W.R., F. Soti, and D. Rittschof. 2003. Inhibition of barnacle larval settlement and crustacean toxicity of some hoplonemertine pyridyl alkaloids. Biomolecular Engineering 20:355-361.

Soti, F., J. Rocca, and W.R. Kem. Isolation and structure elucidation of two new alkaloids from the marine work, Amphiporus angulatus. (re-submitted to Journal of Natural Products; 12/03.)

Resulting Presentations:

Kem, W.R., and F. Soti. Pyridyl alkaloids of the hoplonemertine *Amphiporus angulatus*. 13th Congress, International Society of Toxinology, Paris, Sept. 2000.

Kem, W.R., F. Soti, B.M. Ache, and D. Rittschof. Pyridyl alkaloids of a marine hoplonemertine worm: neurotoxic, feeding repellant and anti-fouling activities. Florida Marine Biotechnology Summit II, Tampa, Oct. 2000.

Kem, W.R. GTS-21, a synthetic derivative of anabaseine (*paranemertes*) toxin, is an alpha7 nicotinic agonist which may be useful in the treatment of Alzheimer's disease. 5th international Nemertean Conference, Alcala, Spain, June 2000.

Kem, W.R. Nemertine Natural Products. Florida Marine Biotechnology Summit III Meeting at Harbor Branch Oceanographic Institution in October, 2002.

Kem, W.R., Michalski, Wildeboer, Soti. Hoplonemertine pyridyl alkaloids. Florida Marine Biotechnology Summit III Meeting at Harbor Branch Oceanographic Institution in October, 2002.

Students Involved in Projects:

Wildeboer, K. 2005. “Effects of nemertine alkaloids on high affinity nicotine receptors." Doctoral Dissertation.

Michalski, S. 2005. "Anabaseine derivatives affecting alpha7-type nicotine receptors." Doctoral Dissertation.

Patent:

Kem, W. R., F. Soti, and D. Rittschof. 2003. Materials and methods for inhibiting fouling of surfaces exposed to aquatic environments (UF#-11120, submitted Feb, 2002).

Isolation and Characterization of Novel Pharmacological Agents from Atlantic and Panamic Cone Snails

Project number R/LR-MB-18
February 1, 2002 through January 31, 2004 (Work in progress as of this summary)

Principal Investigator: Frank Mari, Florida Atlantic University
Co-principal Investigator: Gregg B. Fields, Florida Atlantic University

Cone snails are found in reef areas throughout the world. They are a family of mollusks that includes more than 1,000 known species, some of which produce venom that can be fatal to humans. The venom of a single cone snail can contain as many as 300 different chemical components. Such phenomenally complex mixes are important targets for marine biotechnology research because individual cone snail venom components, or conopeptides, can have powerful neurological and other effects. For instance, Prialt^{tm} (formerly known as Ziconotide), a synthetic form of a cone snail-derived peptide, is the most powerful painkiller known and has already received FDA approval. Several related cone snail drugs are in clinical trials and could eventually be used to treat everything from Alzheimer's to epilepsy to Parkinson's.

The goal of this project was to isolate and sequence new components from the venom of several cone snail species in hopes of identifying compounds with pharmaceutical potential. This main goal was achieved, and the research team is in the process of applying for a patent on a new superfamily of compounds identified that has already shown promising biological activity. Other important components were also isolated.

Separating individual components is a complex task that required development of several novel laboratory techniques by the Mari team. The group's research focused on six cone snail species collected off Central America, the Bahamas and Florida. Venom was extracted from sample snails and their various components separated according to molecular weight using Size-Exclusion High Performance Liquid Chromatography (SE-HPLC). In terms of pharmaceutical importance, compounds with smaller molecules are of greatest interest because they are simpler and easier to study and eventually produce.

Once the team isolated venom components, each was put through a series of bioassays to determine its activity. The pharmaceutical importance of conopeptides comes from their ability to regulate the traffic of specific ions within and between cells, which is the basis of neurological functioning, among other biological activities.

The main bioassay was to expose neuronal cells injected with fluorescent dyes to individual isolated components. The dyes, which bind selectively to specific ions within the neuronal cells, allowed ion activity to be monitored using fluorescent probes. Those components with the most promising effects on ion activity were then characterized at atomic resolution level using Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) to determine the sequence and the three-dimensional structure of each component's molecules.

This sequencing is a critical aspect of drug discovery work as it allows the researchers to compare components they isolate to those that other researchers have already studied, to ensure focus on novel compounds. The Mari group compiled all the sequences from this work into an Atlantic-Panamic conopeptide library that has now grown 50-fold as a result of this Sea Grant project.
The group was able to isolate and sequence a number of promising compounds. These included a completely new superfamily of compounds dubbed conophans, for which a patent application, the first

of several planned as a result of this work, will soon be filed. Mari and his team also found an unusually small cone snail venom component they named nux-1. If the pharmaceutical activity of nux-1 proves to be commercially important, it would likely be relatively cheap to produce because its molecules are smaller and simpler than those of the conopeptides now being produced as drugs.

Testing of these novel compounds is ongoing and all data currently available are preliminary, but Mari says they have already shown great promise. The conophans, he says, appear to target sodium channels, which are involved in numerous aspects of brain functioning from mood to pain. "We believe that these new compounds have neuronal targets that are associated with numerous neurological conditions; therefore, we can envision numerous therapeutic applications out of this discovery," says Mari, "This is extremely exciting."

Though initial focus has been on activity related to conditions of the central nervous system, Mari points out that the conopeptides can have important effects through ion regulation on non-neuronal cells. He suggests, for instance, that conopeptides could be used as cancer therapies to kill cells by controlling the flow of ions in or out of them.

Mari and his colleagues have had discussions with several potential pharmaceutical partners evaluating the novel compounds and they are exploring the possibility of establishing a company of their own to commercialize the results of this and related work. As follow-up research the team plans to work to identify and isolate more compounds within the new conophans family and to work to better understand the conophans' biological activity.

Resulting Publications:

Mora, D., F. Pflueger, G.B. Fields and F. Mari, First example of γ-Hydroxyvaline (ghb) within a polypeptide chain: A novel conopeptide from *Conus gladiator* contains ghv. Submitted to *Journal of American Chemical Society*.

Franco, A., C. Moller, D. Mora, H. Cano and F. Mari. Size exclusion HPLC separation of complex mixtures of marine neuropeptides. *Submitted to Journal of Chromatography*.

Resulting Presentations at the 3rd Florida Marine Biotechnology Summit, Fort Pierce, October 2002:

Mari*, F., G.B. Fields, F. Pflueger, A. Franco, H. Cano, H. Abbasi, E. Matei, C. Moller, J. Lauer-Fields, D. Mora, R. Ramlahkan, O. Williams, K. Pisarewicz and M. Swanson. “Drugs from the Sea: Atlantic and Panamic Cone Snail Species are a Rich Source for Novel Neuropharmacological Agents”.

Mora, D. and Frank Mari*. “First example of γ-Hydroxyvaline (ghv) within a polypeptide chain: A novel conopeptide from *Conus gladiator* contains ghv.”

Ramlakhan, R. and Frank Mari*. “Isolation of a Novel μ-Conotoxin from the Venom of *Conus nux”*.

Pflueger, F. and Frank Mari*. “*Conus brunneus*: Isolation and Characterization of Novel Conopeptides from an Eastern Pacific Worm-hunting *Conus.”*

Franco, A., M. Swanson and F. Mari*. "*Conus regius*: Isolation and Characterization of Novel Conopeptides from a Western Atlantic Worm-hunting *Conus.*"

Cano, H. and F. Mari*. "*Conus purpurascens*: Isolation and Characterization of Novel Conopeptides from the Only Panamic Fish-hunting *Conus.*"

Abbasi, H., R. Ramlakhan and F. Mari*. "*Conus dalli*: Isolation and Characterization of Novel Conopeptides from a Panamic Mollusk-hunting *Conus*."

Matei, E., F. Pflueger and F. Mari*. "Structural Analysis of Nanomolar Quantities of Conopeptides Using 2D-NMR Methods."

Moller, C., K. Pisarewicz, J. Lauer-Fields, G.B. Fields and F. Mari*. "Evaluation of the Neuromodulatory Effects of Conopeptides of Atlantic and Panamic Origin."

Students Supported Through Project:

Franco, A. "Novel conopeptides from the venom of *C. regius* and *C. spurious."* Doctoral Dissertation. Department of Chemistry and Biochemistry, Florida Atlantic University. Expected date of completion: May 2004.

Pflueger, F.C. "Novel conopeptides from the venom of *C. brunneus.*" Doctoral Dissertation. Department of Chemistry and Biochemistry, Florida Atlantic University. Expected date of completion: May 2004.

Cano, H. "Novel conopeptides from the venom of *C. purpurascens* and *C. ermineus.*" Doctoral Dissertation. Department of Chemistry and Biochemistry, Florida Atlantic University. Expected date of completion: May 2005.

Abbasi, H. "*Conus dalli*: Isolation and Characterization of Novel onopeptides from a Panamic Mollusk-hunting *Conus*." Doctoral Dissertation. Department of Chemistry and Biochemistry, Florida Atlantic University. Expected date of completion: May 2005.

Matei, E. "3D structure and Dynamics of Conopeptides in Solution using Nuclear Magnetic Resonance." Doctoral Dissertation. Department of Physics, Florida Atlantic University. Expected date of completion: December 2005.

Moller, C. "Novel conopeptides from the venom of *C. floridanus and C. villepini."* Doctoral Dissertation. Department of Biology, Universidad Simon Bolivar, Caracas, Venezuela. Expected date of completion: December 2005.

Ramlakhan, R.E. "Novel conopeptides from the venom of *C. nux."* Master's thesis. Department of Chemistry and Biochemistry, Florida Atlantic University. Date of completion: December 2002.

Mora, D. "Novel conopeptides from the venom of *C. gladiator."* Doctoral Dissertation. Department of Chemistry and Biochemistry, Florida Atlantic University. Expected date of completion: December 2003. Currently employed at Ivax Pharmaceuticals, Miami Gardens, Fl.

Williams, O. “Novel conopeptides from the venom of *C. jaspedius.”* Master’s thesis. Department of Chemistry and Biochemistry, Florida Atlantic University. Expected date of completion: May 2004.

Pisarewicz, K. “Novel conopeptides from the venom of *C. mus.”* Master’s thesis. Department of Chemistry and Biochemistry, Florida Atlantic University. Expected date of completion: May 2004.

Swanson, M. Undergraduate student – Scientific diver: assisted with sample collection and the chromatographic separation of the venom of *C. regius*. Currently employed at a local environmental analysis company.

Egelhoff, T. Undergraduate student – Scientific diver: assisted sample collection and the chromatographic separation of the venom of *C. brunneus*

Giraldo, C. Undergraduate student – Assisted with chromatographic separation of the venom of *C. jaspedius.*

Anthony, J. Undergraduate student – Assisted with bioassays development and in charge of maintaining the aquaria.

Development of a Marine Prophage Induction Assay for Detection of Mutagens in Seawater Samples

Project Number: R/LR-MB-3
February 1, 1998 through July 31, 2000

and

Molecular Detection of Enteroviruses in Florida's Coastal Waters

Project Number: R/LR-MB-12
June 1, 2000 through December 31, 2001

Principal Investigator: John Paul, University of South Florida

Florida has the eighth highest tonnage of toxic waste buried within its borders, according to the Environmental Protection Agency, and effluent and seepage from this waste containing cancer-causing agents, or carcinogens, invariably makes its way into state waters, posing a potentially serious health threat. Minimizing the impacts of these carcinogens requires rapid detection of those that are genotoxic and, hence, can cause mutations in flora and fauna. Such carcinogens pose the greatest risk to humans, nonetheless, current mutation assays designed to detect genotoxic agents do not work with seawater samples. The first project was designed to correct this deficiency. The overall goal, which was achieved, was to develop an assay that would detect carcinogens in saltwater samples through the use of a marine phage-bacterial host system.

Such assays take advantage of the fact that bacterial cells infected by phages (bacterial viruses), when exposed to certain carcinogens, shift into what is known as the lytic cycle. In this cycle a bacteria cell replicates the phage's DNA independent of its own DNA. This allows production of new phage particles that fill and eventually burst, or lyse, the bacteria cell. These phage particles can be detected, signaling a carcinogen's presence. In the absence of carcinogens, cells remain in a lysogenic cycle where the phage DNA is incorporated into the bacteria cell's own DNA. The genetic information needed to produce the phage is therefore preserved, but the bacteria cell is not harmed and does not produce phage particles unless it shifts into the lytic cycle..

To accomplish the project's goals, the group identified and isolated 19 marine prophage-host systems. These were screened to determine which mutated in response to exposure to the known carcinogen mitomycin C, leading to the production and release of free viral particles through cell lysis. The team used a variety of standard techniques to detect the free viral particles, including transmission electron microscopy, plaque agar overlays, and epifluoresence microscopy using Sybr Green to stain the viral particles. The staining technique was chosen as the best method because it was the simplest and most cost efficient of the methods tested.

Two bacterial strains, *Halomonas aquamarina* and *Psueomonasa aeruginosa*, produced large numbers of viral particles upon treatment with the mitomycin C. However, those produced by *H. aquamarina* were small and difficult to detect by epifluorescence microscopy, so *P. aeruginosa*, which produced larger, more easily recognizable viral particles, became the focus of the work. On further testing, the team also found that several other mutagens, including phenanthrene, aldrin , and naphthalene also induced lysis, confirming this phage-host system as suitable for use in assays to test for carcinogens in salt water. Using funding from the National Oceanic and Atmospheric Administration, a Ph.D. student in Paul's lab, Lauren McDaniel, has successfully used this marine prophage induction assay to examine the distribution of mutagens in marine sediments at various locations in Rookery Bay in Naples, Florida.

In the process of testing one of the isolated phage-host systems, the group also stumbled onto an interesting phenomenon that while rendering the system inappropriate for use as a standard assay, is proving quite valuable scientifically. The system turned out to exhibit what is known as pseudolysogeny, and it is one of only a handful of such systems that have ever been isolated. "The interesting thing about pseudolysogeny," says Paul, "is that the organism grows *and* it spits out viruses." Normally bacterial cells produce viral particles only in response to a mutation, and the process kills them. But pseudolysogens produce viral particles even when no mutagen is present. Paul's lab, in collaboration with a group in California, has received a nearly $2 million National Science Foundation grant to study this phenomenon as part of a larger study entitled “Marine Viromics: The Interaction of Viral Genomes with the Environment."

A second project led by Paul, titled "Molecular Detection of Enteroviruses in Florida's Coastal Waters," focused on identification and quantification of a different type of potential water contaminant: enteroviruses. Enteroviruses are viruses that replicate in the human intestinal track. Some are harmless, but others can cause diseases such as meningitis. Despite their potentially harmful effects on the environment and humans, prior to this work there was no method for detecting the amount of enteroviruses found in natural waters. The only test that was available gave no quantitative information about the viruses, simply a positive or negative indicator of their presence, even though health risk increases as enterovirus concentrations increase.

The Paul group was able to develop new methods to allow quantitative detection of enteroviruses affordably and relatively quickly. They began by searching portions of various enterovirus genomes so that they could identify specific RNA sequences unique to the viruses. These sequences, or primers, were then used as the basis for assays using PCR (Polymerase Chain Reaction) techniques. PCR amplifies the genetic material in a sample that matches that of a primer, and the amount of amplification gives a quantitative measurement of how much enterovirus is present in a given sample.

To fulfill the goals of this Sea Grant project, the Paul team then tested water samples from the Florida Keys to determine the amount of enteroviruses they contained. This was the first time quantitative information about enteroviruses had ever been collected in the region. "We found enteroviruses in Key West coastal waters and also in sponge tissues in the Dry Tortugas," says Paul. Species identified in the Keys included Coxsackie viruses and Poliovirus. This was the first time that human wastewater indicators had been detected in benthic reef dwelling invertebrates in the Florida Keys reef tract, and Paul says the work lends further credence to the hypothesis that wastewater is in part responsible for the deterioration of Florida's reefs.

This project also supported work that later formed the basis for a successful NSF grant. The grant is providing funding for the group to develop methods and equipment for accomplishing enterovirus detection *in situ* as they have already done laboratory techniques. The team is now working to create *in situ* samplers that use NASBA (Nucleic Acid Sequence Based Amplification) rather than PCR and that could be deployed for long-term monitoring.

Resulting Publications:

Donaldson, K.A., D.W. Griffin, and J.H. Paul. 2002. Detection, quantitation, and identification of enteroviruses from surface waters and sponge tissue from the Florida Keys using Real Time RT-PCR. Water Res. 36:2505-2514.

McDaniel, L, D.W. Griffin, J. Crespo-Gomez, M.R. McLaughlin and J.H. Paul. 2001. Development of a marine prophage induction assay. Marine Biotechnology 3:528-535.

Paul,J.H., M.R. McLaughlin, D.W. Griffin, E.K. Lipp, R. Stokes , and J.B. Rose. 2000. Rapid movement of wastewater from on-site disposal systems into surface waters in the lower Florida Keys. Estuaries 23(5):662-668.

Williamson, S.J., M.R. McLaughlin, and J.H. Paul. 2001. Interaction of the φHSIC virus with its host: Lysogeny or pseudolysogeny? Applied and Environmental Microbiology. 67(4):1682-1688.

Resulting Presentation:

Paul, J.H., D.W. Griffin, J. Crespo-Gomez, L. McDaniel, and M.R. Mclaughlin. 2000. Evaluation of marine bacterial lysogens for use in a mutagen detection (Prophage induction) assay. American Society for Microbiology Meeting, Los Angeles, CA, May 21-25, Abs. N-58

Students Involved in Project:

McDaniel, L. a Ph.D. student in Dr. Paul's lab.

Gray, M. a master's student in Dr. Paul's lab.

Quantitative Real-time PCR Probes for Pathogenic *Vibrio* Species

Project Number: R/LR-MB-15
February 1, 2003 through July 31, 2004 (Work in progress as of this summary)

Principal Investigator: Anita Wright, University of Florida
Co-Principal Investigator: G. Rodrick, University of Florida
Associate Investigator: K. Schneider, University of Florida

The genus *Vibrio* includes a number of bacterial species found commonly in coastal waters and, hence, in a variety of animals consumed as seafood. When seafood is eaten raw, as is especially common with oysters, large concentrations of certain *Vibrio* species can accumulate and lead to serious human infections. *Vibrio vulnificus* poses the most serious threat. Though only about 50 infection cases per year are reported in the U.S., about half of those prove to be fatal. Most persons are generally not susceptible to this disease, but life-threatening infections can occur in people with serious underlying liver disease or defects in iron metabolism such as hemochromatosis. Another species, which poses a threat to healthy humans, is *Vibrio parahaemolyticus*. Though almost never fatal, this bacterium has caused on the order of about 1000 cases of illness in past years.

Preventing *Vibrio* illnesses has been a priority of the Food and Drug Administration, but bacterial testing in foods such as oysters is not currently economical or quick enough to make regular assessment feasible. Existing methods tend to require about a week in order to determine if a given lot of oysters is contaminated.

The main goal for this project, led by Dr. Anita Wright of the University of Florida, was to develop more rapid and accurate testing methods for both *V. vulnificus* and *V. parahaemolyticus*. A method for *V. vulnificus* has in fact already been developed and is currently being improved through this research, and work on a method for *V. parahaemolyticus* is now underway.

The group's work revolved around the increasingly common genetic technique known as Real-time Polymerase Chain Reaction (PCR). This technique allows the rapid matching of a specific segment of an organism’s genetic sequence, or primer, with genetic material in a given sample, positively identifying the organism if it is present in the sample. Unlike other PCR assays, it will also measure the number of bacteria in a contaminated sample based upon real-time assessment of the amount of PCR product formed.

In previous work Dr. Wright's lab had already identified a gene unique to *V. vulnificus* that codes for the production of cytolysin. Using a segment of this coding region as a primer in real-time PCR tests, the group was able to detect low levels of the bacterium in samples.

"We were able to detect between 100 and 1,000 bacteria per gram of oyster," says Wright. "That's basically as sensitive as most of the other assays out there," she says, "but we can do it in a couple of hours instead of days or a week." Wright says she believes that with further development the technique could eventually be applied for testing specific lots of commercially harvested oysters for contamination. Or, if effective methods for ridding oysters of the bacteria before they are sold are developed, the PCR techniques could be used to ensure that decontamination methods are working effectively.

The work could also have other applications because in some areas, especially Asia, a variety of marine animals are commonly consumed raw and could be tested for safety. Also, when seafood processing

methods are changed, the PCR technique could be used to ensure that the changes do not allow significantly increased bacterial contamination of processed seafood.
Work with *V. parahaemolyticus* has only just begun, but Wright says that the group will be working with PCR primers based on previous work done by the FDA.

As a result of Dr. Wright's Sea Grant project, the U.S. Department of Agriculture has provided her lab with funding for related research. Wright's team is working to better understand the products or characteristics of *V. vulnificus*, otherwise known as the virulence determinants, that cause its harmful effects on humans. If a specific virulence marker can be identified, it could have two important benefits. First, researchers suspect that not all strains of *V. vulnificus* are virulent, so identification of a virulence determinant could allow development of a PCR method that would use a primer from a gene related to the determinant. That would prevent foods containing no dangerous bacteria from being wrongly labeled as contaminated. A second possibility is that discovery of one or more virulence determinants could allow development of a treatment method capable of rendering the virulent bacteria harmless.

Resulting Publications:

Campbell, M.S. and A.C. Wright, Real-time PCR analysis of *Vibrio vulnificus* in oysters. Accepted by Applied and Environmental Microbiology

Wright, A.C. and M.S. Campbell, Rapid detection of *Vibrio vulnificus* by real-time PCR . In: "Molluscan Shellfish Safety" In Press.

Resulting Presentations:

Campbell, M., A.C. Wright. Real-time PCR assay for detection and enumeration of *Vibrio vulnificus*. Abstracts of the American Society for Microbiology, 102nd General Meeting. May 2002, Salt Lake City, Utah.

Wright, A.C. and M. Campbell. Rapid detection of *Vibrio vulnificus* in oysters. June, 2002. Fourth International Conference on Molluscan Shellfish Safety. Santiago de Compestella, Spain.

Wright, A.C. To probe or not to probe: molecular methods for detection of *vibrios* in oyster meats. October, 2002. Seafood Science and Technology, Orlando, FL. (Invited Speaker).

Wright, A.C. To probe or Not to Probe: Molecular Detection of Pathogens in Molluscan Shellfish. Nov. 2002, Southeastern American Society of Microbiology, Gainesville, FL. (Session Moderator)

Campbell, M.S. and A.C. Wright. Real-time PCR Assay for Detection and Enumeration of *Vibrio vulnificus* in oysters. November, 2002. Southeastern American Society of Microbiology, Gainesville, FL.

Students Involved in Project:

Campbell, M. "Development of Real-Time PCR Assay for Detection and Enumeration of *Vibrio vulnificus* in Oysters." Master's thesis.

Arzaga, R. undergraduate

Use of Bacteriophage for the Decontamination of Oysters Infected with *Vibrio vulnificus*

Project Number: R/LR-Q-20
October 1, 1999 through March 31, 2002

and

Strategies for the Decontamination of Oysters Infected with *Vibrio vulnificus*

Project Number:R/LR-Q-24
March 1, 2002 through August 31, 2004 (Work in progress as of this summary)

Principal Investigator: Donna Duckworth, University of Florida
Co-Principal Investigator: Paul Gulig, University of Florida

Human infection from the common marine bacteria *Vibrio vulnificus*, often found in oysters, is a constant and lingering threat for the numerous consumers who enjoy raw oysters. Though such infection is relatively rare compared to other diseases, afflicting perhaps 50 people per year, it is fatal in about 50% of reported cases. "The fact that it is almost always lethal is what makes it so scary," says Donna Duckworth, from the University of Florida, who led these two projects.

The main goal of the projects was to isolate bacterial viruses, or phage, capable of killing *Vibrio vulnificus* in oysters, with the ultimate goal of creating a commercially viable method for ridding oysters of the bacteria before they are sold. Duckworth's team has isolated phage for many different strains of *Vibrio vulnificus* and has shown that the phage are very effective in treating mice infected with the bacteria. Though met with some unexpected problems, the group is now trying to use the phage to kill the bacteria in oysters and has had some success.

The initial step in the work was to isolate a variety of phage that could potentially be used against the bacteria. Because the phage were most likely to be found where the bacteria are found, the group collected mud samples from oyster beds and incubated them with *V. vulnificus* on agar plates. Clear spots on the plate after incubation where bacteria had been eliminated indicated the presence of a phage, which was then grown with the bacteria as a host, for later use in experiments. This process yielded about 20 different phage.

In the process of growing the various phage the group found that maintaining maximum growth and toxicity required that sea salts be added to the growth medium. They also found that of the dozens of strains of *V. vulnificus* used during the project, those that did not produce protease, an enzyme involved in the breakdown of proteins, allowed more stable phage growth.

In order to test the toxicity of the various phage against the bacteria in oysters, the group first needed to develop a method to reliably infect oysters with high concentrations of the bacteria so that the phage's effect could be accurately measured. This goal was not accomplished until work began with the second project.

Besides working with oysters, another major goal was to study phage effects on mice injected with the bacteria, a more easily accomplished task given that researchers have been experimentally infecting mice with various bacteria for over a century, while intentional bacterial infection of oysters had never been attempted. Oysters could not simply be injected with the bacteria, as mice could, because this

would involve drilling a hole in the oysters, or slipping a needle in when they open. In either case, researchers would not be able to tell where specifically the bacteria were injected.

The mouse work was intended mainly as a means to explore the general concept of using phage to treat diseases in humans, an idea that has been around for many years but which garnered less attention after the advent of antibiotics. According to Duckworth, the team had remarkable success in eliminating bacterial infections in mice using phage. "I can honestly say I think it worked like a miracle," she says, "the mice that hadn't received phage were sick and dying, and the mice that got the phage were just running around and happy."

Duckworth says that the group's work stood apart from related past research because it was more quantitative, measuring the number of bacteria killed by a given phage to give an accurate view of its affect. The group has applied for funding from the National Institute of Health that would allow further research on phage activity in the mice.

During the second project, the group was able to develop a technique to allow reliable bacterial infection of the oysters. They discovered that experimental infection of the oysters was hindered when oysters were already infected to varying degrees with *V. vulnificus* bacteria, as is the case with all wild oysters. By applying an antibiotic to kill bacteria in the collected oysters, the team found that oysters would repeatedly take up the bacteria under controlled conditions as intended. This was, of course, a critical step in allowing them to perform the phage experiments.

Duckworth says experiments so far have shown that the phage, which are introduced to the filter-feeding oysters simply by addition to the water of their holding tanks, can reduce the number of bacteria in the oysters. However, their toxicity has been comparable to that of irradiating oysters with ultraviolet light and neither treatment is as effective as would be desired for reliable commercial treatment of oysters.

To increase effectiveness of phage treatment, the Duckworth group will be experimenting with different manipulations of their basic techniques. For instance, they are planning to test higher phage concentrations and treat the oysters with heat prior to phage introduction in hopes of boosting initial growth of the bacteria. Duckworth feels that one problem in earlier experiments has been that though the bacteria have been infecting the oysters, they have not been growing well, which could prevent complete phage infection. Bacteria found naturally in wild oysters likely have healthier populations than those on the experimental oysters.

Should the phage work prove successful enough for commercial application, phage could be introduced to harvested oysters in much the same manner as the ongoing experiments, with oysters being placed in a holding tank for a period of time before being marketed. Duckworth says techniques the group is developing could potentially also be applied elsewhere, such as in the treatment of fish at aquaculture facilities infected by different bacteria.

Throughout the phage experiments, the group has also been working to identify the virulence determinant that allows *V. vulnificus* to harm humans. This determinant is as yet unknown but could be anything from a toxin produced, to a chemical on the bacterial cells that allows them to attach to human cells. If phage toxicity were dependent on this virulence determinant, for instance if a phage attached to the same chemical that allows bacterial infection in humans, a strain of bacteria resistant to the phage and, hence, non-pathogenic to humans, might be isolated. Manipulation of this affect might offer an alternative route to treatment of commercial oysters, and could also have implication for treatment of

various bacterial infections. The group is applying for a second NIH grant to support this line of research.

Resulting Publications:

Duckworth, D.H. and P.A. Gulig. 2002. Bacteriophages: Potential treatment for bacterial infections. Biodrugs 16 (1): 57-62.

Cerveny, K.E., A. DePaola, D.H. Duckworth, and P.A. Gulig. 2002. Phage therapy of local and systemic disease caused by *Vibrio vulnificus* in iron-dextran-treated mice. Infection and Immunity. In revision.

Resulting Presentations:

Four presentations have been made on our use of phage as therapy against *V. vulnificus* disease in mice.

American Society for Microbiology annual meeting, Chicago, 1999.
Millennial Phage Biology Meeting, Montreal, Canada, 2000.
Evergreen Phage Biology Meeting, Seattle, Washington, 2001.
Johnson and Johnson Focused Giving Symposium, New Brunswick, NJ, 2001.

Students Supported by the Projects:

Cerveny, K. 2001. “Use of bacteriophage as therapy for disease caused by *Vibrio vulnificus* in a mouse model and for decontamination of experimentally infected oysters.” Mster’s thesis. Department of Molecular Genetics and Microbiology, University of Florida College of Medicine. Karen is now working for the USDA in Ft. Pierce, FL.

Martin, J. It is anticipated that Julio will receive his M.S. from the University of Florida College of Medicine in 2003. His project is typing strains of *V. vulnificus* with phage.

Wilkenning, E. B.A. anticipated in May of 2003. Eric isolates and purifies the phage and is working on developing the experimental method for infecting oysters with *V. vulnificus*.

Genetic Characterization of Atlantic and Gulf Coast *Uniola paniculata* (Sea Oats) Populations: Implications for Varietal Selection Using Micropropagation Technology

Project Number: R/C-S-36
February 1, 1996 through December 31, 2001

and

Enhanced Commercial Selection and Micropropagation of Sea Oats for Dune Stabilization

Project Number: R/C-S-41
February 1, 2002 through January 31, 2005 (Work in progress as of this summary)

Principal Investigator: Michael Kane, University of Florida

The practice of planting nursery-propagated sea oats is widely used in Florida and throughout the Southeast because it is an effective means of stabilizing dunes and controlling erosion. However, in recent years scientists, state park managers, and others, have begun to question whether planting locally adapted sea oats found in one location at a different location. where a different set of environmental factors prevail, might have detrimental effects on both the ecological stability and long-term survival of plants. In addition, various factors such as the loss of significant numbers of wild seed-producing plants due to frequent storms have led to decreased availability of sea oats seed for commercial nursery production. The main goals of these two projects, led by Drs. Michael Kane and Sandy Wilson at the University of Florida, were to assess the genetic variation within and between sea oats populations from different locations, and the ecological importance of this variation, as well as to develop improved methods for the selection and efficient propagation of sea oats genotypes. The research is still underway, but the group has made substantial progress toward those ends.

The initial focus of the work was to examine genetic variability within and between sea oats populations using DNA-based molecular assays. The specific method used was a technique known as Random Amplified Polymorphic DNA (RAPD) analysis. This technique involves extracting the DNA of various plants and then cutting this genetic material into fragments using enzyme digestions. Many copies of these fragments are then made (amplified) using the Polymerase Chain Reaction (PCR). The banding patterns of the fragments are determined using gel electrophoresis. Plants of the same genotype produce identical PCR banding patterns, while those of different genotypes produce divergent patterns that can be analyzed to give a measure of how different they are. In later work the team turned from RAPD, to an improved technique called Amplified Fragment Length Polymorphism (AFLP), which relies on a single enzyme for digestion and separates DNA fragments more reliably.

Plants were collected from two sites on Florida's Atlantic coast at Anastasia State Recreation Area and Sebastian Inlet State Recreation Area, and two on the Gulf coast at St. George Island State Park and Egmont Key National Wildlife Refuge. Results of the RAPD analyses indicated distinct genetic differences between populations on opposite coasts. Greater genetic differences were observed between the two populations on the Gulf coast than between those on the Atlantic coast. About seven different genotypes were selected from each of the four populations. These genotypes were cloned in the lab using micropropagation, or plant tissue culture, procedures, then acclimated to greenhouse conditions, and finally planted on each coast at the St George Island and Anastasia sites.

"We're seeing tremendous differences in terms of flowering and morphology," says Kane of the various genotypes grown at the experimental dune plots. Several genotypes flowered after the first year's growth while others did not. The differences they see in productivity and growth success between genotypes appears to be roughly the same for plants growing at the site where they are normally found and on the opposite coast. The group is now working to determine whether the differences they see are ecologically important.

To help answer that question, the researchers are working to understand the physiological basis for why different genotypes grow differently. For instance, the group is now more closely evaluating growth differences of the same genotypes in culture and in the greenhouse. Understanding whether the same differences in performance are found in the field as in the lab and greenhouse will give an indication of whether these early differences in growth can be used to predict growth performance in the field. There are not enough data available yet to determine this, but if the same differences are found in the greenhouse and in the field, it will suggest that performance of a given genotype is not dependent on its location. This could mean that it is ecologically safe to restore dunes in one area with a sea oat variety from another area, something that is currently avoided. The group cannot currently match specific aspects of a genotype's performance to its genetics, though that is a long-term goal.

The group is also working with plants in the lab and greenhouse to optimize growth protocols to enhance research and commercial sea oat production. Differences in survivability and growth of the various genotypes are allowing the researchers to identify those that are easiest to raise successfully and that are most likely to survive and prosper once transplanted under different environmental conditions.

Another aspect of the growth optimization work has been an examination of the effects of micropropagation techniques on the ultimate growth and acclimatization success of various sea oats genotypes. Benzyladenine (BA) is an effective growth regulator that induces steps critical to micropropagation such as production and outgrowth of new shoots, but it is also known in some cases to have a detrimental carryover effect on some plants that can decrease acclimatization success. "It's essential, unfortunately, to produce the plants in culture," says Kane of BA.

The group is testing an analogue of BA called meta-topolin that may have a smaller carryover effect and serve as a potential replacement. The group has found that a genotype's later survival rate is in some cases tied to the amount of time plants are exposed to BA during the shoot multiplication stage and the length of the rooting stage. Of the two genotypes intensively studied so far, one was not affected by the exposure time, but another's survival increased when exposed for 8 weeks compared to four weeks, and decreased when exposed for 12 weeks compared to 8 weeks. Based on this work the team is recommending 8 weeks as optimal for commercial sea oat propagation.

The researchers are also examining the anatomical and physiological basis for genotypic variations in acclimatization from culture to the greenhouse. This research is being conducted by a doctoral student, Carmen Valero-Aracama. Microscopy is being used to compare anatomical differences of leaves of one genotype that has difficulty acclimatizing and one that does not. The difficult-to-acclimatize genotype appears to have a relatively large number of stomata that are closed which could be an indication of poor stomatal function and limited gas exchange. Leaves of poor-to-acclimatized genotype produced in culture also display limited cuticle development and disorganized anatomy of leaf tissues. Under the direction of Dr. Wilson at the Indian River Research and Education Center in Ft. Pierce, Florida, the team is also in the process of gearing up to grow micropropagated sea oats in growth chambers with and without enriched carbon dioxide levels to examine differences in photosynthetic capacity and other parameters for various genotypes.

Kane says a major goal for the team is to make all of its work available to commercial sea oat growers. Already the team has begun working with a commercial partner to provide information on protocols developed as well as micropropagated plants of various genotypes, which the company is currently evaluating for potential use.

Resulting Publications:

Kane, M.E. and N.L. Philman. 2001. *In vit*ro culture for habitat revegetation: issues and opportunities. In Vitro 37:7-A. (published abstract)

Kane, M.E. and N.L. Philman. 2001. Influence of Stage II cytokinin selection on rooting and acclimatization of native coastal and wetland plants. In Vitro 37:11-A. (published abstract)

Ranamukhaarachchi, D., M.E. Kane, D.W. Crewz, C.L. Guy and N.P. Philman. 2003. Molecular analysis of genetic diversity in four Florida Sea-oats (*Uniola paniculata* L. Poaceae) populations: Implications for restoration of dune communities. Ecological Restoration (submitted)

Ranamukhaarachchi, D., M.E. Kane, and T.H.M. Mes. 2003. Character incompatibility: an approach to determine reproduction mode of sea oats (*Uniola paniculata* L.) populations. (to be submitted).

Ranamukhaarachchi, D., M.E. Kane, C.L. Guy and Q.B. Li. 2000. Modified AFLP technique for rapid genetic characterization in plants. BioTechniques 29:858-866.

Valero-Aracama, C., M. E. Kane, S. B. Wilson, and N. L. Philman. 2002. Genotypic differences of *in vitro* propagated sea oats genotypes (*Uniola paniculata*). Proc. SNA Research Conference 47:357-360.

Resulting Presentations:

Kane, M. "Plant Production for Habitat Restoration." Technical paper for the Restoration Genetics Workshop, St. Petersburg, FL, December 4, 1997.

Kane, M. "Micropropagation Techniques for Native Wetland and Terrestrial Plant Conservation in the Southeast United States." Congress on In Vitro Biology - 1997 Meeting of the Society for In Vitro Biology, Washington, DC, June 16, 1997. (Invited speaker)

Ranammukha arachchi, D.G., M.E. Kane, C. L. Guy, N.L. Philman and D. W. Crewz. "Molecular Analysis of Genetic Diversity and Population Structure Between Atlantic and Gulf Coast Sea Oats Populations in Florida." Natural Resources Forum '98: Linkages in ecosystem Science, Management and Restoration. Gainesville, FL, June 11 – 12, 1998. (poster presentation)

Kane, M. "In Vitro Culture for Habitat Revegetation: Issues and Opportunities." 2001 Meeting of the Society for In Vitro Biology, St. Louis, MO June 18, 2001 (symposium organizer/ convener).

Kane, M. "Influence of Stage II cytokinin selection on rooting and acclimatization of native coastal and wetland plants." 2001 Meeting of the Society for In Vitro Biology, St. Louis, MO June 19, 2001. (invited symposium speaker)

"Genotypic differences of *in vitro* propagated sea oats genotypes (*Uniola paniculata*)." SNA Conference, August 2002, Atlanta, GA (Valero-Aracama, C.).

Students Involved in Projects:

DeBatt, A. *Uniola paniculata* - sea oats genotype culture maintenance; nursery and field studies

Cerqueira, K. *Uniola paniculata* - sea oats genotype culture maintenance and comparative growth of sea oats genotypes.

Ranamukaarachchi, D. 2000– in charge of the genetic analysis of sea oats population completed "*Molecular analysis of genetic diversity and population structure of Atlantic and Gulf of Mexico coast populations of Uniola paniculata (Sea Oats)*. Doctoral Dissertation. Initial employment as a Research Associate, Centers for Disease Control, Atlanta, GA. He is now employed as a Research Scientist, US Food and Drug Administration, Rockville, MD.

Valero-Aracama, C. Physiological and anatomical studies. *Physiological and Anatomical Basis For Differences in In vitro and Ex Vitro Growth Performance of Sea Oats Genotypes.* Doctoral Dissertation.

High-throughput Molecular Genetic Identification of Shark Body Parts for Forensic Applications in Conservation, Fisheries Management and Trade Monitoring

Project Number: R/LR-B-54

February 1, 2002 through January 31, 2004 (Work in progress as of this summary)

Principal Investigator: Mahmood Shivji, Nova Southeastern University

Populations of numerous shark species are declining in the U.S. and worldwide due to overfishing, and in many cases the need for improved management and conservation efforts on a species-specific basis is urgent. However, such work has been hindered because there have been no efficient methods available to identify the species of a given shark carcass or body part so that shark catches can be monitored according to species and so that those catching or trading in protected species can be prosecuted.

"Nobody knows which shark species are being caught and in what numbers," says project leader Dr. Mahmood Shivji, of Nova Southeastern University's Guy Harvey Research Institute. The main goal of this project was to help correct that problem by developing a rapid and reliable method for shark body part identification, including fins, so that law enforcement agents could identify and prosecute fishers illegally catching protected species. Such methods are also intended to allow shark catches in the U.S. and globally to be sufficiently characterized to gauge the pressure being placed by fishing on specific shark species. The group was successful in this endeavor, and in fact has already applied its methods in collaboration with NOAA law enforcement agents to identify shark catches and has begun analyzing samples from Asian fin markets to allow study of the global shark fin trade.

The basis for this work was previous research on shark evolution by the group in which it analyzed a shark gene with a non-coding region of nuclear ribosomal DNA called ITS2. This region is common to all sharks and Shivji's research group has found that it is the same for sharks within a given species but different enough between shark species to allow development of genetic markers for reliable species identification.

To develop a test to identify an individual species, the group sequenced the ITS2 region from shark samples of that species from around the world to find a region that was conserved, which was then used to make a species-diagnostic primer, or targeted sequence using Polymerase Chain Reaction (PCR) techniques. To identify body parts of a shark from that species, they use PCR to determine whether a given sample contains the genetic sequence of the primer. If there is a match, then the sample is from that species.

So far the group has established diagnostic primers for about 18 common shark species such as bull (*Carcharhinus leucas*), blacktip (*Carcharhinus limbatus*), silky (*Carcharhinus falciformis*), and great white (*Carcharodon carcharias*) sharks. To avoid having to run a sample through dozens of assays to determine its identity , the group developed a technique dubbed high-density multiplex PCR. Each of the primers the group targeted for use identifying a species was carefully chosen so that it was from a distinct section of ITS2 that does not overlap the section used for other species. This has made it possible to test a sample against primers for eight species with a single reaction.

Shivji's team also developed an innovative method for avoiding a problem common with PCR analyses. Using traditional PCR methods, when results are negative for the presence of a primer sequence, it can mean that that sequence is not found in the genome of the sample, but it can also mean that there was a problem with the analysis. This is a reasonably common result with PCR, and it is difficult to decipher which explanation is accurate. To avoid this problem, the team identified two primers universal to all

shark species, and they use these in all analyses. If a sample tests negative for all the diagnostic primers in an analysis, it should still test positive for the universal primers. If it does not, it clearly indicates there is a problem, preventing “false negative" test results.

Already the shark identification techniques have been used to begin studying the global trade in sharks, especially the shark fins prized in Asia and elsewhere for use in soup. Because shark fins and other body parts are so difficult to identify, little is known about which shark species are under the greatest fishing pressure, and hence management and conservation efforts are difficult.

One of the group's successful efforts in this area has been to identify the main shark species sold in China. Fins there are sold under approximately 100 different trade names, but no information has been available about what species corresponded to what name. By analyzing samples from markets in Hong Kong, the group has created a concordance linking species to trade name. With that information Shivji and his research collaborator Shelley Clarke of the Wildlife Conservation Society were able to analyze Hong Kong market records to determine the quantity of various species being caught to support the fin trade. Further identification work of this type in other countries will yield vital information about global shark catches to aid resource managers and others in establishing better practices for shark conservation.

Ultimately Shivji and Clarke intend to carry global analysis of shark fishing a step further by modeling what impact the fishing pressure placed on various species is having on their populations. This work is now underway with funding from the Wildlife Conservation Society, the Packard Foundation, the Guy Harvey Research Institute, and other sources.

The team published a paper in the journal Conservation Biology on its work developing and using the species forensic markers that received significant coverage from the international press in such publications as Science, Nature, The New York Times, National Geographic Today, New Scientist, Science News and others. That exposure led the National Oceanic and Atmospheric Administration's Office of Law Enforcement to contact Shivji to enlist his help in identifying illegally harvested shark fins confiscated from U.S. fishing vessels. Already the team has identified the species involved for four cases, and so far they have been able to analyze samples in just two days per case. In all cases, fins from prohibited shark species were found.

Practical application of the work will not be limited to work with sharks. "One of the things we are quite pleased about is the fact that the basic methods developed can be used for any wildlife," says Shivji. They are already working to identify grouper body parts, and their methods could eventually be applied to other fish such as tuna, as well as land animals.

Resulting Publications:

Chapman, D., D. Abercrombie, C. Douady, E. Pikitch, M. Stanhope and M. Shivji. 2003. A streamlined, bi-organelle, multiplex PCR approach to species identification: application to global conservation and trade monitoring of the great white shark, *Carcharodon carcharias*. Conservation Genetics. (In Press)

Douady, C.J., M. Dosay, M.S. Shivji, and M.J. Stanhope. 2003. Molecular phylogenetic evidence refuting the hypothesis of Batoidea (rays and skates) as derived sharks. Molecular Phylogenetics and Evolution 26: (215-221)

Shivji, M.S., S. Clarke, M. Pank, L. Natanson, N. Kohler and M. Stanhope. 2002. Genetic identification of pelagic shark body parts for conservation and trade monitoring. Conservation Biology 16(4): 1036-1047.

Resulting Presentations:

Abercrombie, D., D. Chapman, E. Pikitch and M. Shivji. 2002. A novel, bi-organelle PCR approach to forensic identification of shark body parts: Application to great white shark conservation and trade monitoring on a global scale. Florida Marine Biotechnology Summit III. Ft. Pierce, FL. USA. October 2002.

Nielsen, J.T. and M. Shivji. 2002. Development of a high-density multiplex PCR assay for rapid identification of non-ridgeback shark species. Florida marine Biotechnology Summit III. Ft. Pierce, FL. USA. October 2002.

Hoenig, J. and M. Shivji. 2002. Estimating species composition of shark catches from DNA assays: group testing reduces the number of laboratory tests. Elasmobranch Symposium. North Atlantic Fisheries Organization. Spain. Sept. 2002.

Abercrombie, D., D. Champan, E. Pikitch and M. Shivji. 2002. Rapid detection of great white shark tissues for international conservation and trade monitoring using a streamlined, nuclear (ITS2) and mitochondrial (cyt *b*) bilocus, multiplex PCR approach. American Elasmobranch SOciety 18th Annual Meeting, July, 2002. Kansas City.

Students Involved in Project:

Jennifer Magnussen
Debra Abercrombie
Janne Nielsen
Marcy Henning
Vince Richards
Veronica Akle (undergraduate)

About the Author

Mark Schrope is presently a science writer for Harbor Branch Oceanographic Institution in Ft. Pierce, Florida. Before joining the Harbor Branch staff, he was a successful freelance writer and editor with frequent contributions to New Scientist, Discovery, Popular Science, National Geographic Online, Nature, Discovery Channel Online, and Outside magazines. His expertise ranges from topics in biology and medicine to oceans and atmosphere. He is president of Open Water Media, Inc., Melbourne, Florida, and holds a certificate in science writing from the University of California Santa Cruz; a M.S. degree in chemical oceanography from Florida State University, and a B.S. degree in biology from Wake Forest University.