Recombinant Expression and Purification of Dehydratase Domains from the Enzymatic Complex Responsible for the Biosynthesis of Omega-3 Fatty Acids in Marine Bacteria

Delise J. Oyola-Robles, Monica Rivera, Abel Baerga-Ortiz*

University of Puerto Rico, Medical Sciences Campus, Department of Biochemistry, University of Puerto Rico
Medical Sciences Campus, PO BOX 365067, San Juan, 00936, Puerto Rico
delise.oyola@upr.edu, abel.baerga@upr.edu

The enzyme complex responsible for the production of omega-3 fatty acids (ω-3 FA) in deep-sea bacteria contains two distinct dehydratase (DH) domains, DH1 and DH2. DH domains are the enzymes responsible for the introduction of cis or trans double bonds during the biosynthesis of ω-3 FA. In order to study the mechanism by which double bonds are introduced during the biosynthesis of ω-3 FA, as well as the substrate specificity of both DH domains in Photobacterium profundum, different constructs of both DH1 and DH2 domains were cloned and expressed with the goal of assaying them separately against synthetic surrogate substrates. The DNA fragments amplified by PCR correspond to two different constructs of DH1 and DH2 domains and a fragment comprised of both domains, DH1-DH2. The PCR-amplified DNA fragments were cloned into expression vectors pBADHis and pGEX4T-3 and expressed as fusion proteins in Escherichia coli. A scheme for the synthesis of soluble surrogate substrates will also be presented. This work will enable us to assay the enzymatic activity of DH domains against a variety of different substrates to elucidate the substrate specificity and to study the mechanism of cis and trans double bond formation during FA biosynthesis. Results will be discussed.

This work was funded by seeds funds from the office of the Dean of the UPR-School of Medicine and MBRS-RISE Program (R25GM061838) of the University of Puerto Rico, Medical Sciences Campus.
Isolation of Specific Toxin Component Recognition Peptides Using Phage Display

Ricardo D. Burgos-Muñiz¹, Selimar Ledesma-Maldonado¹ and Carlos Ríos-Velázquez¹

¹Department of Biology, University of Puerto Rico, Mayagüez Campus, P.O. BOX 9000, Mayagüez, Puerto Rico 00681-9000

PO Box 9012 Mayagüez PR 00681-9012, carlos.rios5@upr.edu

ABSTRACT. Phage Display technology has matured to the point where it is now a powerful tool in the post-genome era of Biology. Phage Display constructs of genetically tagged peptides and protein fractions allow converting pools of combinatorial nucleotides, mRNA’s or fragmented genomes into populations of viruses that contain the nucleotides coding for the elements that are displayed on their viral surfaces. The main focus of this project is to use T7 phage display technology to map and isolate possible interacting partners of the highly specific lethal factor, a component of the tripartite protein toxin secreted by Bacillus anthracis. The display of human cDNA fragments on the surface of T7 bacteriophages has successfully been used to identify candidates interacting proteins, from purified lethal factor. Phages clones displaying putative toxin specific peptides were isolated after several rounds of affinity selection and the cDNA amplified by PCR. The products from amplifications where sequence and analyzed In silico using available nucleic acids and protein databases. Preliminary data suggest consensus with synaptotodin, a dual actin/actinin binding protein, cholinergic receptors and myosin regulatory light chain.
The Evolutionary Role of Tandem ACP Domains in Polyketide Synthases

Uldaeliz Trujillo¹, María Rodríguez-Guilbe¹, Delise Qyola Robles¹, Jennifer Santos² and Abel Baerga-Ortiz¹.

¹Department of Biochemistry, School of Medicine, University of Puerto Rico, Medical Sciences Campus, PO BOX 365067 San Juan, P.R. 00936-5067, ²University of Puerto Rico, Rio Piedras Campus.

Medical Sciences Campus, PO BOX 365067, San Juan, 00936, Puerto Rico uldaeliz.trujillo@upr.edu

ABSTRACT. Acyl carrier protein (ACP) is a highly conserved protein domain that participates in the biosynthesis of fatty acids and polyketides. It functions as a support for covalently bound products and intermediates during biosynthesis. Most enzyme complexes, which require the presence of an ACP, contain either one or many of these domains arranged in a modular fashion. However, the polyketide synthase (PKS) complex responsible for the production of omega-3 fatty acids in deep-sea bacteria contains a total of five (5) ACP domains in tandem. This unprecedented arrangement of ACP domains is well conserved among the different species of marine bacteria, although it has been shown that the presence of a single ACP domain is sufficient for omega-3 fatty acid production at a much reduced yield. No satisfying explanation exists for why omega-3 fatty acid synthases have been selected with multiple ACP domains in tandem. One possible explanation is that different ACP domains have different binding specificities towards the enzyme domains, which “service” them. In order to investigate whether ACP domains have specificity toward other enzyme domains, we have expressed ACP domains individually in Escherichia coli and purified them using a combination of nickel chromatography and anion exchange chromatography. Each individual ACP will be assayed for binding to the different purified enzyme domains, such as the thioesterase, Orf6, and the dehydratase domains and well as other PKS domains. ACP domains have been expressed with and without the phosphopantetheine modification by co-expression with a phosphopantetheine transferase. The results from this work will facilitate the mechanistic understanding of marine biosynthetic machineries and will allow the harnessing of genes of marine origin for the production of new natural product with therapeutic potential.

This work was funded by the MBRS-RISE Program of the University of Puerto Rico Medical Sciences Campus, Grant R25GM061838.
Preliminary Structure of Orf6: Confirmation of a Thioesterase Hot-dog Fold

María M. Rodríguez-Guilbe¹, Uldaeliz Trujillo¹, Eric Schreiter¹² and Abel Baerga-Ortiz¹

¹ University of Puerto Rico, Medical Sciences Campus, Department of Biochemistry, Puerto Rico, 2 University of Puerto Rico, Rio Piedras Campus, Department of Chemistry, Puerto Rico

ABSTRACT. The polyketide synthases (PKS) are multifunctional enzymes responsible for the biosynthesis of omega-3 fatty acids in microbes and algae. They are organized into modules, each containing a set of distinct, non-iteratively acting activities responsible for the catalysis of one cycle of elongation in the polyketide chain. The PKS genes responsible for the biosynthesis of EPA in the deep-sea bacterium, Photobacterium profundum, have been cloned and sequenced. A total of five polyunsaturated fatty acid (pfa) genes (pfaA, pfaB, pfaC, pfaD and Orf6) have shown to be enough for eicosapentanoic acid (EPA) production. These domains presumably catalyze the reiterative steps in processing the growing acyl chain, where each condensation reaction is followed by complete or abbreviated reductive reaction cycles of keto-reduction, dehydration/isomerization (DH/I) and enoyl reduction (ER or pfaD). Orf6 that is the thioesterases (TE) domain, catalyzes the last step in the biosynthesis of fatty acid. Unlike most TE domains which have an α/β-hydrolase fold, orf6 has a novel hot-dog fold. The orf6 gene was amplified by PCR, cloned into pGEX vector, expressed in E coli and purified to homogeneity. Attempts to crystallize the protein in the presence of coenzyme A are under way. The structure of orf6 will be of great importance in the eventual engineering of TE domains of the hot-dog protein family. This type of enzymatic manipulation could be of great benefit in the design of lipid-based drugs or in the generation of biofuels.
Exploring Bacterial Diversity of Forests of Puerto Rico Using Metagenomics

Miosotis Acevedo-Esquín¹, Viviana Melendez-Muñiz¹ and Carlos Ríos-Velázquez²

¹University of Puerto Rico at Mayagüez, Industrial Biotechnology Program, ¹²University of Puerto Rico at Mayagüez, Department of Biology, Laboratory of Microbial Biotechnology and Bioprospects

PO Box 9012 Mayagüez PR 00681-9012, carlos.rios5@upr.edu

ABSTRACT. From helping animals to digest their food, participate symbiotically with plants and even survive in extreme conditions in ecological mutualism, bacteria are undoubtedly a fundamental part of life. With this knowledge and with the curiosity of identifying the secrets our land conceals, we decided to analyze with culture independent techniques the soil samples from two contrasting environment in Puerto Rico: Guanica dry forest and Yunque tropical forest. Two years ago, metagenomic libraries were generated from these soil samples. Functional analyses have been performed, but the microbial community represented is still unknown. In this research, we attempt to characterize the microbial communities present in the libraries by analyzing the rDNA genes. Two fosmid metagenomic libraries master pool of approximately 40kb insert size and grown in Luria Bertani Media, were extracted using mini preparation technique. The inserts of interest into the fosmids were amplified using 16S rDNA specific primer, and the amplifications were cloned using the TOPO-TA systems. After isolating potential clones and confirming the presence of the inserts of the expected size, the fragments were amplified, and sent to be sequenced to further in silico analysis. We have been able to amplify sequences of rDNA clones generated from the metagenomic libraries from both forests and we will be in the process of increasing the number and sequences of rDNA clones generated from the metagenomic libraries in order to unravel the diversity of the soil from both forests.

This work was funded by the USDA-CSREES-HSI, Grant 2007-02386-18042.
Screening for Antifungal Activity in Forest Soils in Puerto Rico Using Culture Independent and Functional Genomics Approaches

Manuel A. Ortega¹, Pedro J. Ondina¹, Rosivette Santiago¹, Jose M. Cruz¹ and Carlos Ríos Velázquez²

¹Industrial Biotechnology Program, University of Puerto Rico, Mayagüez, PR, ²Department of Biology, University of Puerto Rico, Mayagüez, PR

PO Box 9012 Mayagüez PR 00681-9012, carlos.rios5@upr.edu

ABSTRACT. Studies have demonstrated that standard cultivable methods fail to access a representative amount of the microbes available from a microbial environment population since they only obtain a 1% of the whole population. This leaves behind the other 99% of the microbes, which can represent organisms with possible phenotypes never described in scientific literature. To access this diversity we created two fosmid libraries of soil genomic DNA from El Yunque Rain Forest, and Guanica’s Dry Forest. The total library consisted of 795,830 clones from which 781,199 clones were from Guanica’s Dry Forest and 14,631 clones from El Yunque. Among the several novel biomolecules searched, novel antifungal molecules able to inhibit the growth of Sacharomyces cerevisiae are of main interest. In order to access this possible antifungal component, the library stock pools were grown for 2 hrs and serially diluted until obtaining 600-800 clones/plates. Once the dilution factor was obtained the library was cultured in LB agar supplemented with chloramphenicol for 3 days at 37°C. The precultured clones were overlaid with 5 mL of LB soft agar containing a S. cerevisiae suspension in Yeast Potato Dextrose broth adjusted to an optical density of 0.2-0.3 Abs. The plates were incubated overnight at 30°C and scored for activity looking for inhibition zones in the S. cerevisiae lawn. To date 95,600 clones from El Yunque Rain Forest have been screened and no antifungal activity has been detected under the current experimental conditions.

This work was funded by the USDA-CSREES-HSI, Grant 2007-02386-18042.
Intracellular Thermometry using *lacI* Temperature-Sensitive Vectors with LacZ and EGFP Reporters

Ivan Albino¹, Kevin McCabe², Mark Hernandez ³

¹Center for Hemisphere Cooperation, ²University of Puerto Rico- Mayaguez, ³University of Colorado-Boulder

2032 Colinas de Alturas de Mayaguez Mayaguez, Puerto Rico 00682 - ivan.albino@upr.edu

**ABSTRACT.** Knowing bacterial intracellular temperature is critical for the structure and function of the cell. Lac-operon derived plasmids have been adapted to be used as intracellular thermometers, exploiting the *lacI*-ts system. These “bacterial thermometers” are capable of measuring a range of temperatures, from 25- 45°C, using the chemical catalysis of the *lacZ* gene product, β-galactosidase. Temperatures were quantitated using a spectrophotometric assay (OD630) measuring the catalysis of X-gal to insoluble blue; the higher the temperature, the greater the absorbance observed. Most recently, there has been an interest in inserting the Enhanced Green Fluorescent Protein Gene in *lacZ's* locus, since it would not require from expensive reporter reagents like X-gal, to measure intracellular temperature. The EGFP would also permit to measure the temperature and analysis real-time. *E. coli* DH5 alpha colonies with their respective lac-derived plasmids were grown, their DNA was purified, and sequenced to find the convenient restriction endonuclease sites (BamHI), which we used to exchange the EGFP for the *lacZ gene* using DNA ligase and bacterial transformation. It is important to mention that a crucial biomedical-involved aspect of these temperature-sensitive bacterial vectors, is the utilization of them as massive protein/drug production systems. Instead of EGFP or *lacZ* gene, another gene of interest, Human Insulin for example, could be inserted and by just increasing temperature, large amounts of protein would be produced. This way, we would identify intracellular temperature and make protein/drug production for a cheaper and more efficient way.
Unraveling Activities from Metagenomic Libraries Generated from Dry and Rain Forest Soils in Puerto Rico Using Functional Approaches

José M. Cruz-García¹, Pedro J. Ondina¹, Manuel Ortega¹, Rosivette Santiago², Amaris Torres¹, and Carlos Ríos-Velázquez³

¹ Industrial Biotechnology Program at University of Puerto Rico – Mayagüez, ² Microbiology Program at University of Wisconsin – Madison, ³ Biology Department at University of Puerto Rico – Mayagüez

P.O. Box 9012, Mayagüez, PR, 00681-9012; carlos.rios5@upr.edu

ABSTRACT. Molecular studies have demonstrated that only 1% of the microorganisms occurring in nature can be cultivated, leaving 99% of the soil microbiota uncultivated and an invaluable source of unstudied microorganisms with potentially novel metabolic activities. We propose to study the functional genomics of the microbiota of Puerto Rico’s forest soils to access the diversity and complexity of its uncultivable majority. The generation and screening of metagenomic libraries has become a powerful culture-independent technology that has facilitated the unraveling of the roles of microbes in many environments. Our goals are the generation of metagenomic libraries from dry and rain forest soils in Puerto Rico, and searching for activities using functional and sequence-based approaches. A total of eight soil metagenomic libraries were generated [3 small (4-10 kbp) insert (all from the rain forest), and 5 large (25-40kbp) insert size (2 from the rain forest and 3 from the dry forest)]. The total number of clones in the libraries is approximately 850,000 clones. Selections, overlay assays and colorimetric tests were used to detect presence of antibiotic resistance, production of antimicrobial agents, and DNase activities respectively. While the presence of PKS amplicons in all the samples indicated the presence of antimicrobial genes in the soil libraries, functional evidence of the production of such molecules has not been detected yet. Clones showing resistance to tetracycline (10µg/mL), ampicillin (50µg/mL) and kanamycin (25µg/mL) were isolated. Transposon mutagenesis is being performed to the positive clones in order to determine the gene(s) associated with the activities found.

This work was funded by the USDA-CSREES-HSI, Grant 2007-02386-18042.
β-lactam Resistance Genes and Proteins from the Human Gut Microflora

José M. Cruz García¹, Diego Hernández Aranda¹, Gautam Dantas², Morten Sommer³, George Church³

¹University of Puerto Rico at Mayagüez, Industrial Biotechnology Program, ²Washington University in St. Louis, Department of Pathology and Immunology, ³Harvard Medical School, Genetics Department

P.O. Box 9012, Mayagüez, PR, 00681-9012; jose.cruz30@upr.edu; dantas@path.wustl.edu

ABSTRACT. Though much is known about antibiotic resistance in clinical settings, it remains uncertain where antibiotic resistance determinants originated and how they are transferred to human pathogens. Most of the knowledge regarding β-lactam antibiotic resistance genes found in pathogens has been derived using traditional techniques, such as polymerase chain reaction (PCR) amplification and cloning from cultivable bacteria. PCR detection of resistance determinants relies on amplification using primers from previously described genes, and cloning from cultivable bacteria can only sample the <1% of readily cultivable microbes. These methods may not provide access to the >99% of bacteria that are not cultivable under normal laboratory conditions. To study β-lactam resistance determinants in a human context and potentially sample uncultivable pathogenic strains, DNA from microbes inhabiting two healthy individuals was isolated. Two small-insert (2-3 kbp) fragment metagenomic libraries were generated; one sampling genomic DNA from cultivable isolates and the other sampling metagenomic DNA, directly from human fecal samples. The metagenomic libraries were selected for resistance to β-lactam antibiotics, and inserts conferring resistance phenotypes were sequenced. Open reading frames (ORFs) within each of the inserts were analyzed and putative β-lactam resistance determinants were then restriction cloned to isolate functional genes and their gene products. Resistance phenotypes for each of the cloned genes were assessed by determination of minimum inhibitory concentration (MIC) values. β-lactam resistance gene over-expression was induced, and β-lactamases were purified by affinity chromatography. We describe recognized and novel β-lactamase genes and proteins isolated from microbes inhabiting the human gut.
Generation and Screening of Large Insert Metagenomic Libraries from Young and Mature Tropical Hypersaline Microbial Mats at Different Seasons

Irimar Torres¹, Adel González¹, Christian Castro¹, ²Lynn Williamson, ²Heather Allen, ³Lilliam Casillas, and Carlos Ríos Velazquez¹

¹University of Puerto Rico at Mayagüez, Mayagüez, Puerto Rico, ²University of Wisconsin, Madison, Wisconsin, ³University of Puerto Rico at Humacao, Humacao Puerto Rico

P.O. Box 9012, Mayagüez, PR, 00681-9012; carlos.rios5@upr.edu

ABSTRACT. The generation of metagenomic libraries to access microorganisms’ genetic traits has led to the discovery of gene products with potential biomedical applications. Some extreme environments, such as microbial mats, harbor high biological diversity for which they are valuable sources of novel activities. This research is focused on generating large-insert metagenomic libraries from a mature (Candelaria) and a young (Fraternidad) tropical hypersaline microbial mat on the dry and rainy seasons and their screening for antibiotic resistance. Finding novel antibiotic resistance genes is an important contribution for the development of new chemotherapeutics. Our approach consisted on cloning large fragments of DNA into a fosmid and transferring recombinant fosmids to the appropriate host strain by in vitro packaging. Four metagenomic libraries were generated with 32,000 (Candelaria) and 30,000 (Fraternidad) clones from the dry season, and 1,200 (Candelaria) and 1,400 (Fraternidad) clones from the rainy season. The libraries (with inserts from 25-50 Kbp) were screened for resistance to ampicillin 50 μg/ml and 100 μg/ml, tetracycline 10 μg/ml, spectinomycin 100 μg/ml and kanamycin 20 μg/ml. There were kanamycin resistant clones in the four libraries. Five clones from each library were characterized by the detection of a recombinant fosmid and the verification of the phenotype being encoded by the insert. All the monitored clones had a fosmid with a 30 Kbp insert. The retransformation of the host strain with this recombinant fosmid determined the phenotype is encoded by its insert. In the future, the kanamycin resistant insert will be sequenced and other antibiotics will be tested.

This work was funded by the NSF-RUI: Microbial Observatories
Screening for Urease Activity in Metagenomic Libraries from Tropical and Dry Forest in Puerto Rico

Jean C. Cruz Hernández1 and Carlos Ríos Velázquez1

1University of Puerto Rico at Mayagüez

P.O. Box 9012, Mayagüez, PR, 00681-9012; carlos.rios5@upr.edu, Jean.cruz1@upr.edu

ABSTRACT. Emerging disciplines and technologies, especially involving molecular biology, have allowed the study of microbes that are not able to grow using conventional culture media. The use of metagenomic libraries has allowed unraveling the presence of novel microbial groups and new enzymatic activities with application in biomedical sciences and biotechnology by using culture independent approaches. Urea is a product when proteins are metabolized, that can be used as fertilizer and also can become in some Industries a waste product. The main focus in this research is to detect the presence of urease activity in metagenomic libraries generated by direct DNA extraction method from El Yunque Tropical Rain Forest (two sites) and Guánica Dry Forest (one site). A total of approximately 18,000 clones were screened from both forest from Puerto Rico. The screened was performance by adding to urea broth media, washed clones with physiological saline from 16 sub-pools from Dry Forest and 4 sub-pools from El Yunque. The inoculated samples were incubated at 37°C from 24-48 hrs, and the presence of a change in color from yellow to pink was scored. There was only one sub-pool, from Tropical Forest positive for the presence of urease activity. The positive sub-pool has around 6,000 clones. Serial dilutions in ELISA plates as well as solid media are being performed in order to isolate the clone or clones with the proposed activity.

This work was funded by the USDA-CSREES-HSI, Grant 2007-02386-18042.
Utilization of Natural Genomic Variation to Enhance Nutritional and Health Values of Beef: Investigation of Potassium and Magnesium

Jean C. Cruz1, Richard G. Tait, Jr.2, Qing Duan2, Mary S. Mayes2; Kadir Kizilkaya2,3, and James M. Reecy2

1Industrial Biotechnology, University of Puerto Rico at Mayagüez; 2Department of Animal Science, Iowa State University; 3Department of Animal Science, Adnan Menderes University, Turkey

jean.cruz1@upr.edu

ABSTRACT. Minerals are a requirement for life; specifically humans need minerals to complete more than 300 biochemical reactions. The main focus of this research project is to characterize the genetic control, or heritability, of potassium and magnesium concentration in beef, using traditional pedigree based analysis and new high density SNP panel based analysis. A total of approximately 1,078 cattle were screened for both mineral concentrations. The cattle population was managed in 19 different carcass contemporary groups and all cattle were harvested before 18 months of age. The minerals were extracted for each of the samples and the mineral concentrations were then analyzed with a five generation pedigree using MTDF-REML to determine the heritability of potassium and magnesium concentration. The MTDF-REML estimated heritability for potassium was 0.09 and magnesium was 0.11. We extended these results to a Bayesian analysis with the GenSel program to evaluate the effect of 54,001 SNP markers on mineral concentration and determined the proportion of variance attributed to the markers. GenSel estimated the proportion of variance attributed to markers to be 0.09 for potassium and 0.19 for magnesium. The concentration of potassium and magnesium are heritable and should respond to selection. Magnesium presents a slightly stronger heritability than potassium, so implementation of a breeding program for mineral concentration may be more effective for magnesium concentration in the beef than potassium concentration. In conclusion, potassium and magnesium are heritable; selection on these traits may lead to beef being a better nutritional source for potassium and magnesium in our diet.
Generating of Small-Size Metagenomic Libraries from Dry and Rain Forest Soils in Puerto Rico

Amaris Torres¹, Francisco Sanchez² and Carlos Ríos-Velázquez²

University of Puerto Rico at Mayagüez, ¹Industrial Biotechnology Program, and ²Biology Department, Mayagüez, Puerto Rico

P.O. Box 9012, Mayagüez, PR, 00681-9012; carlos.rios5@upr.edu

ABSTRACT. Metagenomics involves the analysis of microbial genomes from communities in environmental samples, using culture independent approaches. El Yunque (Caribbean National Rain Forest) and the Guanica Dry Forest; first and second International Biosphere Reserves in Puerto Rico, receive an average of 200 and 25-40 inches of rain per year, and a temperature from 17ºC - 28ºC and 24ºC – 39ºC respectively. To date there are no publications reporting metagenomic studies on these forests. The main objective of this project is to generate small-size DNA metagenomic libraries to soil samples from both forests. Direct DNA extractions were performed using a mechanical method. The correct endonuclease units to obtain the appropriate DNA size (3,000 – 10,000 bp) were determined by using the “rocket assay”. The resulting fragments were purified and cloned into the vector pCF430 and classified as small size (4,000 – 6,000 bp) and larger size (>6000 – 10,000 bp). After transforming the cloned DNA into Escherichia coli by electroporation, the positive clones were confirmed for the presence of inserts by restriction analysis. The data suggest the presence of high amounts of humic acids and nucleases activities which interfered and affected the effective generation of both libraries. Two libraries were generated from the samples, with a reduced number of clones. The metagenomic libraries contain clones with a DNA insert size that range from approximately 2,500 - 5,000 bp. There is ongoing research to optimize the process of generating this type of libraries and to analyze the cloned fragment at the level of function and sequence.

This work was funded by the USDA-CSREES-HSI, Grant 2007-02386-18042.