Evolution of Biomineralization Proteins in Cnidaria:

Sequencing a Stony-Coral Skeletal Protein from the Soft-Coral, *Lobophytum crassum*

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Abstract— **Recently, the effects of ocean acidification on coral calcification have received significant interest. However, similar information is lacking for soft corals, which produce internal calcite sclerites. This poor understanding of the calcifying mechanisms makes it difficult to predict the response of soft corals to increased ocean acidification. In order to better understand the potential effects of increased atmospheric CO_2, it is crucial that we expand and enhance our knowledge of soft coral calcification biomolecules. Toward this end, we use molecular biology techniques to understand the evolutionary history of a Cnidarian highly acidic protein, previously thought to be limited to stony corals.**

Coral acid rich protein (CARP) 4, is a coral-specific protein exhibiting a high proportion (>20%) of the acidic amino acids (aspartic and glutamic acids). Previously thought to be stony coral-specific, the partial sequence of a soft coral sclerite protein appears similar to CARP4. An alignment of homologues of four stony corals and CARP4 proteins with an N-terminal peptide from *Lobophytum crassum* sclerites suggests that soft corals possess a member of the CARP4 subfamily. Through a series of molecular biology techniques, the presence of CARP4 in soft corals can be verified.

If *L. crassum* does indeed possess the CARP4 gene, phylogenetic comparison of soft versus stony coral CARP4 will be performed to trace the evolutionary track of this Cnidarian-specific biomineralization protein.

key words- Biomineralization; Cnidaria; Climate Change; Ocean Acidification; Proteins

I. INTRODUCTION

The start of the industrial revolution has led to an increase anthropogenic CO_2 emissions released into the atmosphere and are expected to decrease the pH of surface oceans by .2 to .5 pH units by the year 2100 [1]. A drop in pH will lead to nearly 50% decline in carbonate ion concentration [2]. This change could potentially result in a reduced ability to produce $CaCO_3$. Recently, the effects of ocean acidification on coral calcification have received significant interest [2]. Genetic and biochemical advances have allowed development of a mechanistic model for calcification by stony corals, which make an external aragonite skeleton [3]. However, similar information is lacking for soft corals, which produce internal calcite sclerite [2]. This poor understanding of the calcifying mechanisms makes it difficult to predict the response of soft corals to increased ocean acidification. In order to better understand the potential effects of increased atmospheric CO_2, it is crucial that we expand and enhance our knowledge of soft coral calcification biomolecules. Toward this end, we use molecular biology techniques to understand the evolutionary history of a Cnidarian highly acidic protein, previously thought to be limited to stony corals.

Both stony and soft corals are marine invertebrates in the phylum Cnidaria, class Anthozoa, and produce calcium carbonate either externally (stony) or internally (soft). In stony corals, it is thought that the aragonite exoskeleton provides an adherent substrate while, in soft corals, the calcitic endo-sclerites stiffen the tissue. The

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production of aragonite skeleton by stony corals takes place between the calicoblastic epithelium and the substrate [3]. In fact, known coral biomineralization proteins have been immunolocalized particularly to the calicoblastic epithelium [4, 5]. In contrast, soft corals possess calcifying cells throughout the tissue.

II. Coral Acid-Rich Proteins

Coral acid rich protein (CARP) 4, is a coral-specific protein exhibiting a high proportion (>20%) of the acidic amino acids (aspartic and glutamic acids). It precipitates CaCO3 in unamended seawater and has been detected by multiple means in the skeleton from several stony corals. Previously thought to be stony coral-specific, a partial sequence of a soft coral sclerite protein appears similar to CARP4 (Figure 1). An alignment of homologues of four stony corals and CARP4 proteins with an N-terminal peptide from *Lobophytum crassum* sclerites (Figure 1) suggests that soft corals possess a member of the CARP4 subfamily.

Multiple Sequence alignment of Carp4 sub-family homologues

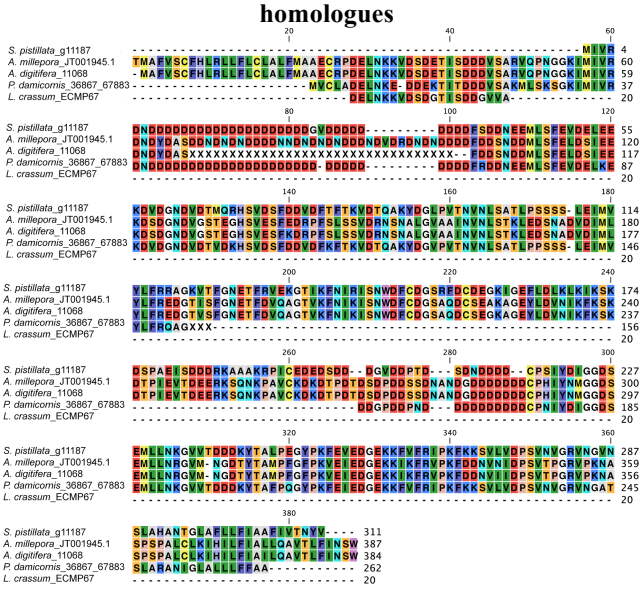

Fig. 1. Alignment of homologues from four stony-corals places a previously un-aligned soft coral N-terminal sequence into this family. The CARP4 subfamily is not found in sea anemones even though anemones are thought to have diverged from stony-corals after the soft-coral divergence.

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Through a series of molecular biology techniques, the presence of CARP4 in soft corals can be verified and used to aid in the understanding of the evolution of biomineralization proteins in Cnidaria. Understanding its evolutionary history will inform our hypotheses about the ability of soft and stony corals to persist during dramatic climate change such as ocean acidification [6].

III. Materials and Methods

A. Sequencing Alignment

The CARP4 gene from 10 stony coral species was aligned by BLASTing. The sequence alignment of CARP4 was then used to determine regions for which existing *Stylophora pistillata* (stony coral) CARP4 primers would be most likely to amplify *L. crassum* (soft coral) CARP4. (Figure 2).

B. DNA Extraction PCR Amplification and Sequencing

L. crassum RNA was extracted from colony fragments using TRI Reagent (Sigma) and manufacturer protocols. RNA was converted to cDNA using SuperScript III First Strand Synthesis System (Thermo Fisher) and then amplified using primers S. pistillata CARP4-specific (F: AGACCCATTTGTGAAGAC; R: CGGTAACCACTCCTTTATT). Amplicons were gel-extracted and cloned using TOPO TA Cloning Kit (Thermo) for sequencing by Genewiz.

C. Rapid Amplification of cDNA Ends (RACE) Protocol

Fresh RNA was extracted from L. crassum cuttings, as described above. RNA quality was confirmed on a Bioanalyzer (Agilent). 5' and 3' RACE were performed using the SMARTer RACE 5'3' Kit (Clontech) with L. crassum CARP4-specific primers (5' RACE: CGGGGACGAGTTTCCTGTTCTTGGAGATTACGCCAAGCTT; 3' RACE: GATTACGCCAAGCTTTTGCCGGAGAACGAAATGAGCCACT) following manufacturer instructions. sequencing.Amplicons of correct sizes were gel-extracted and cloned in pUC19 Vector into

Stellar Competent Cells (Clontech) by manufacturer protocol.

Partial Sequence Alignment of Stony Corals

Fig. 2. Partial segment of a sequence alignment of stony-coral. One of the several regions of gene conservation across species

IV. Results and Discussion

Stony coral CARP4-like genes contain several regions of conservation across species (Figure 2). *S. pistillata* CARP4-specific primers against the highlighted region were chosen to amplify a portion of the gene in the soft coral *L. crassum*. A ~150 nt region was amplified from *L. crassum* cDNA which is similar to the 134 nt anticipated size from *S. pistillata* (Figure 3). After sequencing, the translated amino acid sequence contains the FRIPKF which is conserved across stony corals (Figure 2), providing additional support for the presence of a CARP4 gene in soft corals.

Polymerase Chain Reaction

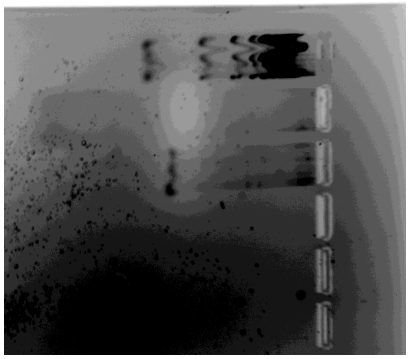

Fig. 3. Top lane: Molecular Weight ladder; Third lane: Polymerase Chain Reaction pruduct.

RACE will likely provide the complete *L. crassum* CARP4 gene sequence. RACE-specific primers designed using the PCR-amplified and sequenced internal region produced two DNA fragments of appropriate size as compared with the *S. pistillata* CARP4 gene (Figure 4).

RACE-PCR Product

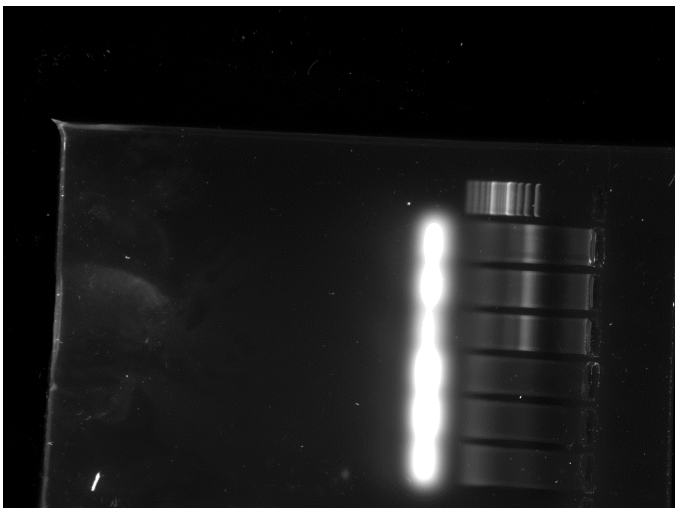

Fig. 4. Top lane: Molecular Weight Ladder; Second-fourth lane: 5' RACE-PCR product; Fifth-seventh lane: 3' RACE-PCR product

The 5' fragment was expected to be at least 800 nt. In *S. pistillata*, the N-terminal region information is lacking; hence, the 1200 nt RACE-PCR product is likely the correct band. Similarly, the *S. pistillata* C-terminus is anticipated to be ~400 nt, which is similar to the 300-400 nt 3' RACE-PCR product. Like the internal region described above, these RACE products provide further evidence that soft corals do indeed contain a CARP4-like gene. This is interesting because soft corals precipitate calcite whereas stony corals produce aragonite; while both are $CaCO_3$, they are two mineral polymorphs exhibiting different crystal structures, solubilities, and functions. Although CARP4 induces the precipitation of $CaCO_3$ in vitro (Mass et al. 2014 Current Biology), our results suggest that CARP4 may play other roles in biomineralization that are not crystallographically-specific. These include Ca concentration and crystal growth inhibition.

V. Future Implications

Through a series of molecular biology techniques, the presence of CARP4 in soft corals is extremely likely. This information will be used to aid in the understanding of the evolution of biomineralization proteins in Cnidaria. If *L. crassum* does indeed possess the CARP4 gene, phylogenetic comparison of soft versus stony coral CARP4 will be performed to trace the evolutionary track of this Cnidarian-specific biomineralization protein. Understanding its evolutionary history will inform our hypotheses about the ability of soft and stony corals to persist during dramatic climate change, such as ocean acidification.

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