Please cite this article in press as: Mass et al., Cloning and Characterization of Four Novel Coral Acid-Rich Proteins that Precipitate Carbonates In Vitro, Current Biology (2013), http://dx.doi.org/10.1016/j.cub.2013.05.007

Current Biology *23*, 1–6, June 17, 2013 ©2013 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2013.05.007

Report

# Cloning and Characterization of Four Novel Coral Acid-Rich Proteins that Precipitate Carbonates In Vitro

**Tali Mass,[1] Jeana L. Drake,[1] Liti Haramaty,[1] J. Dongun Kim,[1,3] Ehud Zelzion,[2] Debashish Bhattacharya,[2] and Paul G. Falkowski[1,3,4,*]**

[1]Environmental Biophysics and Molecular Ecology Program, Institute of Marine and Coastal Sciences

[2]Department of Ecology, Evolution, and Natural Resources Rutgers University, New Brunswick, NJ 08901, USA

[3]Department of Chemistry and Chemical Biology

[4]Department of Earth and Planetary Sciences Rutgers University, Piscataway, NJ 08854, USA

## Summary

**Biomineralization is a widely dispersed and highly regulated but poorly understood process by which organisms precipitate minerals from a wide variety of elements [1]. For many years, it has been hypothesized that the biological precipitation of carbonates is catalyzed by and organized on an extracellular organic matrix containing a suite of proteins, lipids, and polysaccharides [2, 3]. The structures of these molecules, their evolutionary history, and the biophysical mechanisms responsible for calcification remain enigmatic. Despite the recognition that mineralized tissues contain proteins that are unusually rich in aspartic and glutamic acids [4–6], the role of these proteins in biomineralization remains elusive [5, 6]. Here we report, for the first time, the identification, cloning, amino acid sequence, and characterization of four highly acidic proteins, derived from expression of genes obtained from the common stony coral, *Stylophora pistillata*. Each of these four proteins can spontaneously catalyze the precipitation of calcium carbonate in vitro. Our results demonstrate that coral acid-rich proteins (CARPs) not only bind $Ca^{2+}$ stoichiometrically but also precipitate aragonite in vitro in seawater at pH 8.2 and 7.6, via an electrostatic interaction with protons on bicarbonate anions. Phylogenetic analysis suggests that at least one of the CARPs arose from a gene fusion. Similar, highly acidic proteins appear to have evolved several times independently in metazoans through convergence. Based purely on thermodynamic grounds, the predicted change in surface ocean pH in the next decades would appear to have minimal effect on the capacity of these acid-rich proteins to precipitate carbonates.**

## Results and Discussion

Despite the broad interest in coral calcification and the potential for climate-driven adverse effects, the molecules and biophysical mechanism responsible for the precipitation of carbonates in corals are poorly understood. To date, we lack both a characterization of molecules involved in calcification and a mechanistic understanding of processes that lead to and control calcification. This lack of knowledge limits our ability to predict the response of corals to increasing atmospheric $CO_2$.

*Correspondence: falko@marine.rutgers.edu

To date, the best-characterized highly acidic proteins that catalyze the precipitation of carbonates are from mollusk shells and an echinoderm [4, 7–9]. However, in spite of the fact that stony corals are among the most abundant biomineralizing metazoans on Earth, surprisingly little is known about their skeletal organic matrix (SOM) proteins or how they regulate crystal formation. Furthermore, despite the fact that the amino acid composition of coral SOM proteins is characterized by a predominance of acidic amino acids [6, 10, 11], the only coral SOM protein fully characterized, i.e., galaxin, which is isolated from the stony coral *Galaxea fascicularis*, possesses neither acidic regions nor obvious $Ca^{2+}$-binding domains [12]. Recently, Drake et al. [13] identified 36 SOM proteins from a stony coral, of which two are highly acidic. They suggested, however, that their approach, based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) protein sequencing, might not be the most efficient method for identifying these molecules, due to highly redundant sequences. Although a number of N-terminal SOM protein sequences have been reported both in scleratinian and alcyonian corals, none of these share strong sequence similarity to other known proteins or to each other, and only one exhibits a highly acidic sequence [14–16]. A group of hypothetical, soluble acidic proteins (SAPs) has been identified in the *Acropora digitifera* genome, a transcriptome from *A. millepora*, and in expressed sequence tag (EST) libraries [17, 18]; however, the role of these hypothetical proteins in calcification was not examined, and the structure of the encoded genes was not validated using RT-PCR.

To better characterize the proteins responsible for coral calcification, we generated a draft genome assembly from the cnidarian host cells of the Indo-Pacific stony coral, *Stylophora pistillata* (see Supplemental Experimental Procedures available online). In combination with transcriptomic data from EST libraries and RT-PCR, we identified and characterized four coral acid-rich proteins (CARPs 1–4; GenBank accession numbers KC148537–KC148539 and KC493647) (Figure S1 and Table S1). Three of these, CARPs 1–3, contain a secretory signal peptide, suggesting that they are discharged from the cells. CARP1 also contains an EF hand $Ca^{2+}$-binding domain. CARPs 2 and 3 contain an isoleucine-proline-valine (IPV)-like motif following the signal peptide that has previously been suggested to assist in the trafficking of secreted, acidic, calcium-binding proteins out of the rough endoplasmic reticulum in metazoans [19] (Figure S1). An additional gene encodes a protein, CARP4, which lacks a targeting sequence but contains regions that have previously been identified in proteins extracted from the *S. pistillata* aragonite skeletal matrix [13, 14] and is therefore highly likely to be a component of the SOM. The calculated isoelectric points (pI) of CARPs 1–4 are 4.23, 4.78, 3.04, and 3.99, respectively (Table S1). Isolated SOM proteins from corals and other marine invertebrates are often posttranslationally modified [20, 21]; analysis of the amino acid sequence of the four CARPs reveals several potential sites of posttranslational modification, including phosphorylation and O- and N-glycosylation sites (Figure S1). Indeed, glycoproteins were recently reported to be present in the SOM of *S. pistillata* [13].

Please cite this article in press as: Mass et al., Cloning and Characterization of Four Novel Coral Acid-Rich Proteins that Precipitate Carbonates In Vitro, Current Biology (2013), http://dx.doi.org/10.1016/j.cub.2013.05.007



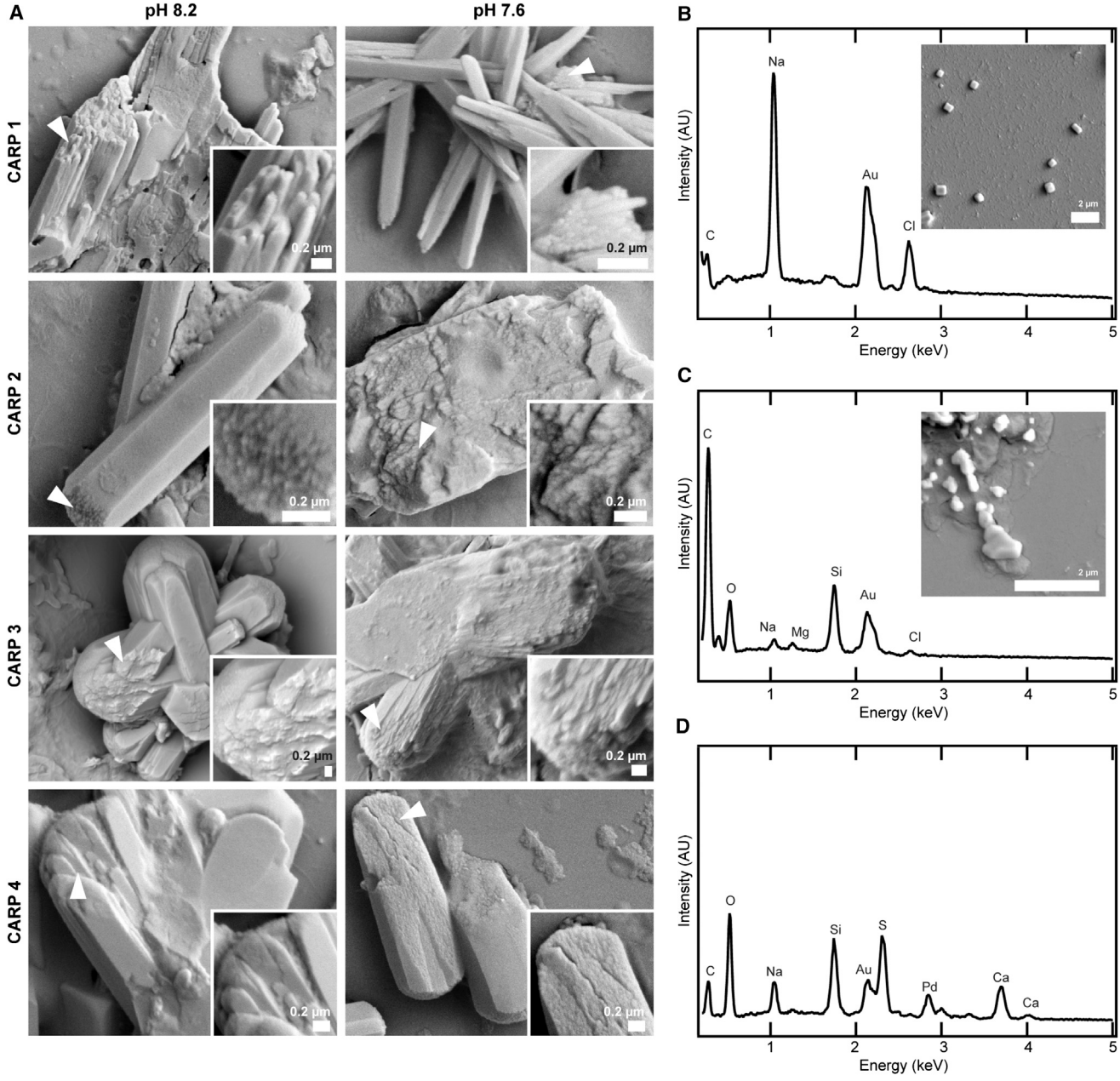


Figure 1. Scanning Electron Microscope Images and Elemental Composition of $CaCO_3$ Crystals Grown in Artificial Seawater

Scanning electron microscope (SEM) images and elemental composition of $CaCO_3$ crystals grown in artificial seawater containing 0.1 μM of CARPs 1–4 at pH 8.2 and 7.6 (A). In both pH treatments, a reticulate pattern is clearly visible (insets in A). EDS and SEM images (inset) of salts precipitated in protein-free artificial seawater (B) and in artificial seawater containing BSA (C) confirm that calcium carbonate does not precipitate in the absence of CARPs. Elemental composition of the crystals grown in artificial seawater containing CARPs 1–4 in both pH treatments reveal their calcium and carbonate composition (D). The Au and Pd peaks derive from the gold coating of the sample, and the Si peak derives from the silica wafer base.

To examine the effect of CARPs on $CaCO_3$ precipitation, we expressed and purified the acidic domain (excluding the putative signal peptide) from CARPs 1 and 2 and the entirety of CARPs 3 and 4. The recombinant His-tagged proteins were visualized, and their identities were verified by western blot analysis using polyclonal antibodies generated against each recombinant protein (Figure S2). In addition, the predicted amino acid composition of the pure proteins was verified by high performance liquid chromatography (HPLC). The molecular masses, estimated from SDS-PAGE for these proteins, are slightly higher than predicted from their sequences (14, 20, 18, and 37 kDa, respectively), probably because of the high levels of negatively charged amino acids [8].

To determine the stoichiometry of acidic residues involved in binding calcium ions, we developed a $^{45}Ca^{2+}$ assay, in which 20 μCi of the chloride salt of the radiotracer, diluted in 20 mM $^{40}CaCl_2$, was overlaid on a polyvinylidene fluoride membrane containing various dilutions of pure CARPs 1 and 3 (Supplemental Experimental Procedures). $Ca^{2+}$ binds to CARPs 1–4 (Figure S2). Based on the binding affinity, we calculated the molar ratio between acidic amino acid residues and $Ca^{2+}$ in

Please cite this article in press as: Mass et al., Cloning and Characterization of Four Novel Coral Acid-Rich Proteins that Precipitate Carbonates In Vitro, Current Biology (2013), http://dx.doi.org/10.1016/j.cub.2013.05.007



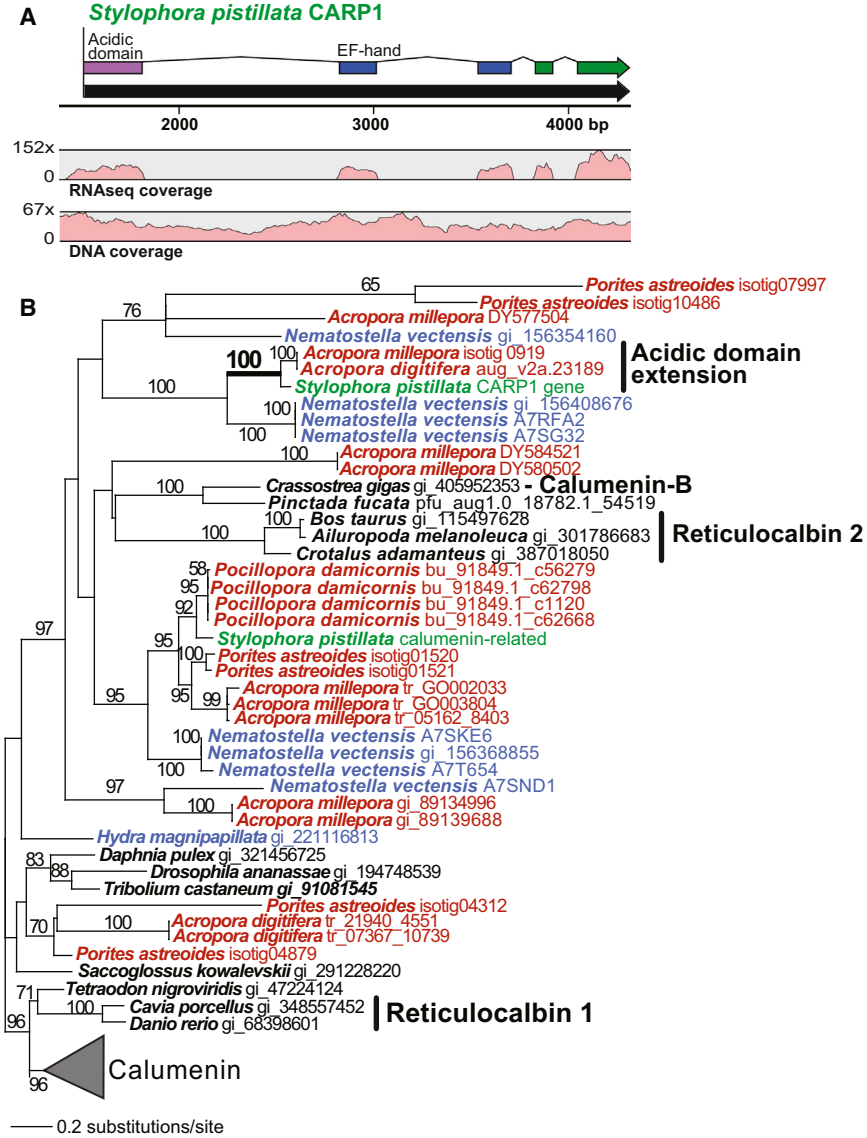


Figure 2. Evolutionary Analysis of the CARP1 Gene in *Stylophora pistillata*

(A) Genome region that encodes the CARP1 gene, showing the intron/exon structure, transcript coverage using mRNA-seq, and genome coverage in the draft assembly. Note the strong support for the N-terminal exon that encodes the acidic domain (shown with the purple box). The EF hand-encoding exons are shown with the blue boxes.

(B) Maximum likelihood (RAxML) tree showing the phylogenetic position of CARP1 among other calumenin-related homologs in corals and other taxa. The *S. pistillata* genes are in green text, other corals are in red, sea anemone is in blue, and other taxa are in black. RAxML bootstrap values, when greater than 50%, are shown at the nodes. The monophyletic clade of coral genes that contains the N-terminal extension is marked by the vertical bar.

CARPs 1 and 3 to be 1.6 and 2.5 with coefficients of determination of 0.98 and 0.91, respectively.

To assess whether the CARPs catalyze precipitation of $CaCO_3$, we conducted in vitro experiments in which the four purified proteins were incubated in artificial seawater adjusted to pH 8.2 and 7.6. Given the measured alkalinity of 2,221 and 2,114 μmol $kg^{-1}$, the calculated $\Omega_{arag}$ for these conditions was 4.15 and 1.27, respectively. These experiments were designed to simulate contemporary and predicted pH conditions in the upper ocean in the coming century [22]. In addition, in vitro aragonite precipitation has been previously reported in the presence of primary cell culture in enriched seawater [11]. Figure 1 shows Scanning electron microscope (SEM) images of $CaCO_3$ crystals grown in the presence of CARPs 1–4, at pH 8.2 and 7.6. In all cases, a typical reticulate crystallization pattern is visible at nanometer length scales (Figure 1, insets). The mineral phases of biominerals, regardless of their origin, always show a reticulate structure that is different from the typical crystalline organization. This structure occurs due to the presence of organic remains surrounding the crystallized units [23]. In control experiments, either with no addition of CARPs, with BSA, or with His-tagged glycosyl hydrolase 2 (*E. coli* M863), only NaCl crystals were observed (Figures 1B and 1C). The chemical composition of the crystals precipitated in the presence of CARPs, determined by energy-dispersive X-ray spectroscopy (EDS), confirmed that they are calcium carbonate (Figure 1D). A typical X-ray spectrum of the crystals from each CARP treatment reveals a similar elemental composition, as previously reported [11], with a sulfur peak in addition to the prominent calcium and carbon peaks (Figure 1D).

In order to identify the carbonate mineral, we used both Feigl's stain (Supplemental Experimental Procedures) and high-resolution inductively coupled plasma mass spectrometry (ICP-MS). The positive stain (Figure S2) and the Sr/Ca ratio (7.83 ± 1.53 mmol $mol^{-1}$), measured by ICP-MS, suggest that the crystals are indeed aragonite, similar to the results of Helman et al. [24]. Although the specific form of the carbonate mineral is not critical to the role of CARPs in the precipitation process, the thermodynamically favorable form is aragonite in modern seawater [25].

The wide distribution of calcification in protists and metazoans [26] leads to the question of the evolutionary history of this process. To address this question, we looked for putative homologs of CARPs 1–4 in our comprehensive local database derived from NCBI Reference Sequences (RefSeq) v51. This database also included 13 genomes or EST data sets from biomineralizing metazoans that are not in RefSeq (listed in Table S2). The sequence comparisons showed that CARP1 is closely related to two calumenin-related proteins widely distributed in metazoans (Figure 2).

Analysis of the (noncalcifying) sea anemone *Anemonia viridis* shows calumenin to be the most strongly upregulated protein when symbiotic dinoflagellates (i.e., *Symbiodinium* species) are present in animal cells [27]. In *S. pistillata*, CARP1 is comprised of five exons, with the N-terminal exon

Please cite this article in press as: Mass et al., Cloning and Characterization of Four Novel Coral Acid-Rich Proteins that Precipitate Carbonates In Vitro, Current Biology (2013), http://dx.doi.org/10.1016/j.cub.2013.05.007



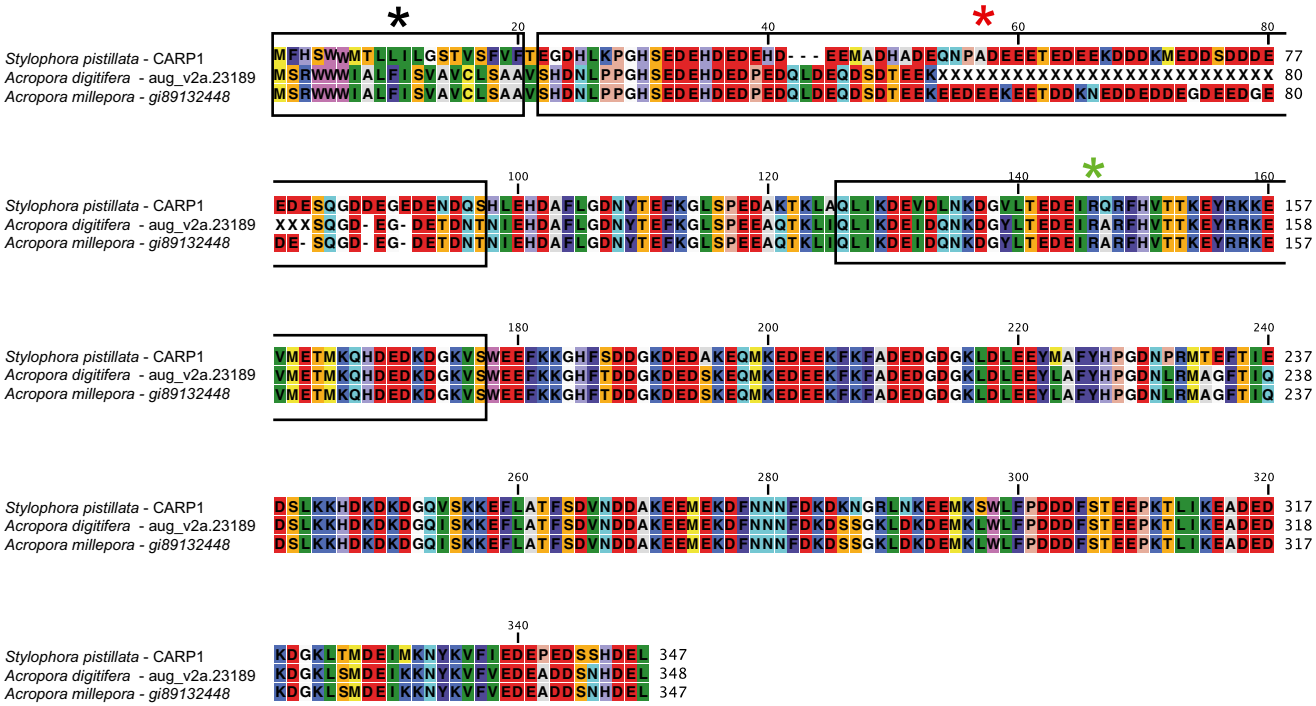


Figure 3. Alignment of CARP1 and Coral Homologs Containing the Acidic Domain

Note the three blocks denoted by the black, red, and green asterisks, which represent the secretion signal, acidic domain, and EF hand domain, respectively.

containing the acid-rich domain described above that is absent in other calumenin-related proteins. The second and third exons encode the EF hand $Ca^{2+}$-binding domain (Figure 2A) and are similar to calumenin-related proteins. The exon-intron structure of CARP1 is strongly supported by mRNA-seq data, and independent assembly using transcriptome data recovered the intact cDNA that includes the five exons shown in Figure 2A. Phylogenetic analysis of CARP1 (Figure 2B) reveals that the N-terminal acid-rich domain is coral specific (Figures 2A and 3). The observation that neither calumenin nor other related nonacidic $Ca^{2+}$-binding proteins are known to take part in the calcifying process, together with the fact that CARP1 contains an N-terminal acid-rich domain capable of precipitating $CaCO_3$, is consistent with the idea that CARP1 results from a gene fusion. Fusion of EF hand domains to heterologous genes, leading to novel functions, has been previously observed (e.g., the NEFA and nucleobindin genes [28]) and, in general, allows the translation of a regulatory signal (i.e., $Ca^{2+}$-binding) to different functional responses [29]. These results demonstrate that CARP1, while widely dispersed among metazoans, evolved independently [26] but converged in function to other metazoan calcifying proteins. The search also revealed a high similarity of CARP2 (Figure 4A) and CARP4 (see Figure S3 in Drake et al. [13]) only to other acidic scleractinian proteins. The fact that CARP2 and CARP4 exhibit a high similarity only to other acidic scleractinian proteins supports the argument suggested by Drake et al. [13], that CARP4 and potentially CARP2 belong to highly acidic subfamilies of proteins that are well conserved across order Scleractinia. Interestingly, CARP3 revealed high similarity both to acidic scleractinian proteins and to the prismatic shell matrix protein family of the bivalve *Atrina rigida*, Asprich [4] (Figure 4B).

The convergent sequence evolution of highly acidic proteins in all calcium carbonate-precipitating organisms identified to date suggests a common underlying mechanism for biomineralization. Calcium is an s block element that forms a metal-ligand complex via coordinate bonds, typically with oxygen atoms from carboxylates in either a bidentate or syn/anti monodentate mode [30]. We propose that the acid-rich regions in CARPs effectively localize calcium ions, thereby increasing the ionic strength in the microenvironment and leading to an increased local dielectric constant, which, in turn, decreases the pKa of the microenvironment [31] (Figure S3A). The coordination bond strength of calcium with carboxylates is not as strong as for transition metals, such as zinc; the carboxylates are readily replaced by a stronger Lewis base [32]. The net negative charge on the oxygen atoms in the carboxyl groups leads to electrostatic displacement of protons from bicarbonate anions, thereby allowing the precipitation of inorganic carbonates on an organic scaffold [33] (Figure S3A).

A $\beta$ sheet conformation has been shown to accelerate calcification [34], but acidic protein domains of CARPs 1–4 form neither $\alpha$ helices nor $\beta$ sheets (Figure S3B). Indeed, aspartic acid favors neither $\alpha$ helices nor $\beta$ sheets but has a relatively higher propensity to form $\alpha_L$ conformational states compared to other amino acid residues [35]. The electrostatic interactions between calcium and acidic residues may confer an $\alpha_L$ conformation in a number of residues of the experimentally determined acidic domains. For example, in a catgrip $Ca^{2+}$-binding motif, the backbone residues have alternating $\alpha_L$ and $\alpha_R$ conformations, which is a characteristic of an $\alpha$ sheet structure [36]. We propose that calcium carbonate precipitation and its higher-order assembly might be a consequence of $\alpha$ sheet-mediated protein aggregation. This reaction is almost certainly controlled by other, nonmineralizing proteins, such as collagens [13], which help guide the arrangement of the crystals to achieve the

Please cite this article in press as: Mass et al., Cloning and Characterization of Four Novel Coral Acid-Rich Proteins that Precipitate Carbonates In Vitro, Current Biology (2013), http://dx.doi.org/10.1016/j.cub.2013.05.007



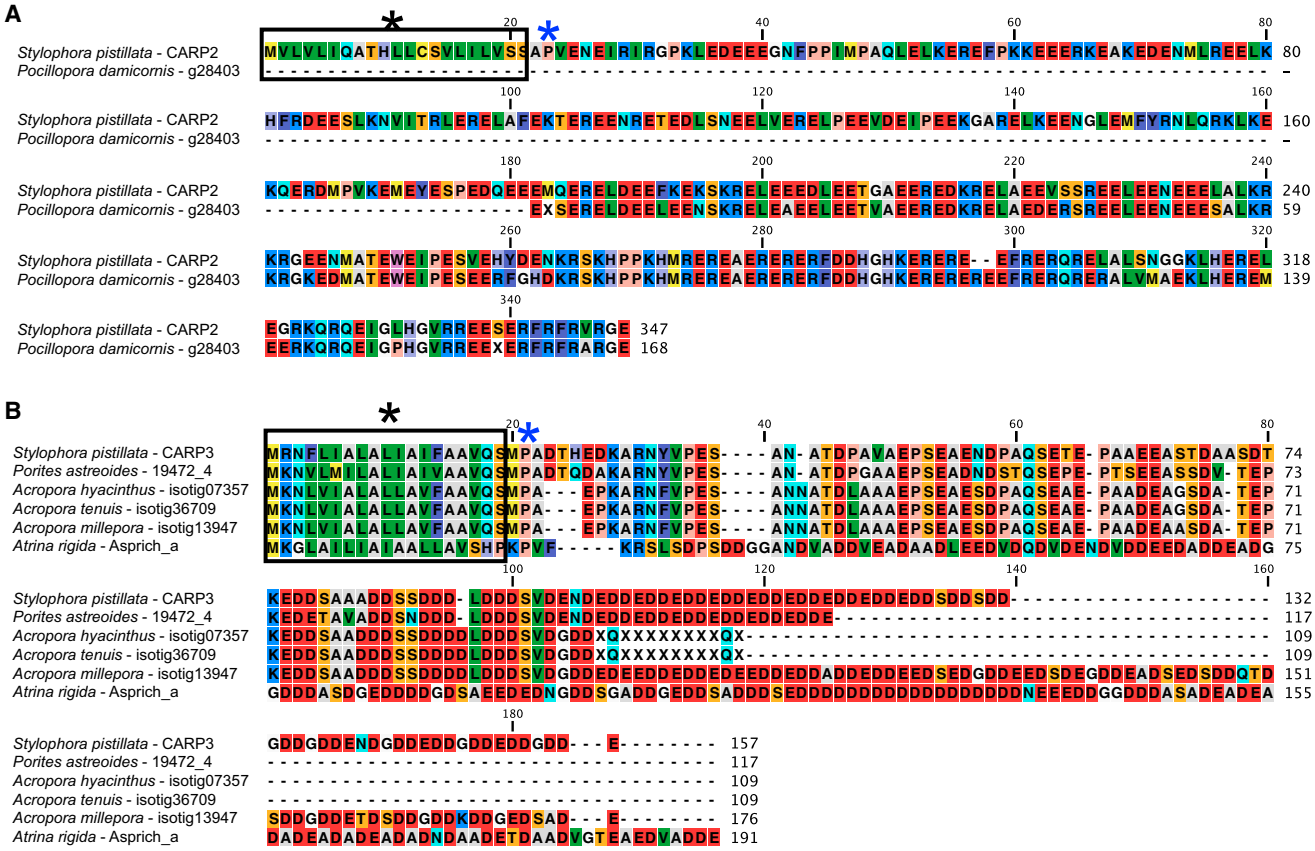


Figure 4. Alignment of CARP2 with Coral Homologs and CARP3 with Coral and Bivalve Homologs

(A) Alignment of CARP2 with coral homologs.

(B) Alignment of CARP3 with coral and bivalve homologs.

Note the block denoted by the black and blue asterisks, which represent the secretion signal and the IPV-like motif, respectively.

macroscopic architecture characteristic of each species of coral.

## Conclusions

Our research has identified and characterized four unique, acidic proteins in corals that catalyze the precipitation of $CaCO_3$ in vitro and opens an approach not only for understanding how the process is controlled in vivo but also for in vitro applications in novel biomaterials. The precipitation reaction is likely to be driven by an electrostatic interaction of $Ca^{2+}$ ions with the carboxylate groups on the proteins, followed by dehydration and precipitation of carbonate. Whereas calcification is widely dispersed across metazoan phyla [26], cnidarians (corals in particular) appear to be the most anciently diverged extant phylum possessing this trait [26]. To the extent that the animal can buffer the reaction from the external pH of the bulk fluid [37], the calculated pI values strongly suggest that these proteins will continue to catalyze calcification reactions at ocean pH values projected in the coming century, assuming that the animals can physiologically acclimate and/or genetically adapt to these changing conditions.

## Experimental Procedures

### DNA and RNA Purification and Complementary DNA Synthesis

Total genomic DNA that was free of cells from the dinoflagellate symbiont *Symbiodinium* species was extracted from harvested cells using a blood and cell culture DNA Mini Kit (QIAGEN) with small modifications. Total RNA was extracted using TRIzol Reagent (Life Technologies) following the manufacturer's protocol with small modifications. Further details are described in Supplemental Experimental Procedures.

### *Stylophora pistillata* Draft Genome and Gene Model

Genomic DNA that was free of cells from the dinoflagellate symbiont *Symbiodinium* species and total RNA were used to produce a draft genome as well as transcriptome data using single-read and paired-end protocols on an Illumina Genome Analyzer IIx. Further details are described in Supplemental Experimental Procedures.

### $^{45}Ca^{2+}$ Overlay Assay

SDS-polyacrylamide gel was transferred to polyvinylidene fluoride (PVDF) membranes. CARPs 1–4 and control proteins (glycosyl hydrolase 2 [*E. coli* M863] and BSA) blotted on PVDF membrane were labeled with $^{45}Ca$, as described previously [38]. Further details are described in Supplemental Experimental Procedures.

### In Vitro $CaCO_3$ Precipitation

Calcium carbonate precipitation experiments were carried out by adding 0.1 μM of CARPs 1–4 to 1 ml of artificial seawater (Instant Ocean sea salt, Aquarium Systems; salinity = 34). Further details are described in Supplemental Experimental Procedures.

## Supplemental Information

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.05.007.

**Acknowledgments**

This research was supported by National Science Foundation Grant EF1041143 to P.G.F. We are grateful to J. Yaiullo of the Long Island Aquarium and F. Natale of the Institute of Marine and Coastal Sciences for providing and maintaining the corals used in this study. We thank members of the Genome Cooperative at Rutgers University for aid in generating and analyzing the draft genome data. We thank L. Fisher, K. Thamatrakoln, L. Mintz, K. Wyman, J. Kalansky, S. Murali, and V. Yamazaki for advice and technical support.

Received: March 19, 2013
Revised: April 17, 2013
Accepted: May 7, 2013
Published: June 6, 2013

**References**

1. Lowenstam, H.A. (1981). Minerals formed by organisms. Science *211*, 1126–1131.
2. Mann, S. (2001). Biomineralization: Principles and Concepts in Bioinorganic Materials Chemistry (New York: Oxford University Press).
3. Watanabe, T., Fukuda, I., China, K., and Isa, Y. (2003). Molecular analyses of protein components of the organic matrix in the exoskeleton of two scleractinian coral species. Comp. Biochem. Physiol. B Biochem. Mol. Biol. *136*, 767–774.
4. Gotliv, B.A., Kessler, N., Sumerel, J.L., Morse, D.E., Tuross, N., Addadi, L., and Weiner, S. (2005). Asprich: A novel aspartic acid-rich protein family from the prismatic shell matrix of the bivalve *Atrina rigida*. ChemBioChem *6*, 304–314.
5. Weiner, S. (1979). Aspartic acid-rich proteins: major components of the soluble organic matrix of mollusk shells. Calcif. Tissue Int. *29*, 163–167.
6. Mitterer, R.M. (1978). Amino acid composition and metal binding capability of the skeletal protein of corals. Bull. Mar. Sci. *28*, 173–180.
7. Suzuki, M., Saruwatari, K., Kogure, T., Yamamoto, Y., Nishimura, T., Kato, T., and Nagasawa, H. (2009). An acidic matrix protein, Pif, is a key macromolecule for nacre formation. Science *325*, 1388–1390.
8. Takeuchi, T., Sarashina, I., Iijima, M., and Endo, K. (2008). In vitro regulation of $CaCO_3$ crystal polymorphism by the highly acidic molluscan shell protein Aspein. FEBS Lett. *582*, 591–596.
9. Costa, C., Karakostis, K., Zito, F., and Matranga, V. (2012). Phylogenetic analysis and expression patterns of p16 and p19 in *Paracentrotus lividus* embryos. Dev. Genes Evol. *222*, 245–251.
10. Constantz, B., and Weiner, S. (1988). Acidic macromolecules associated with the mineral phase of scleractinian coral skeletons. J. Exp. Zool. *248*, 253–258.
11. Mass, T., Drake, J.L., Haramaty, L., Rosenthal, Y., Schofield, O.M.E., Sherrell, R.M., and Falkowski, P.G. (2012). Aragonite precipitation by "proto-polyps" in coral cell cultures. PLoS ONE *7*, e35049.
12. Fukuda, I., Ooki, S., Fujita, T., Murayama, E., Nagasawa, H., Isa, Y., and Watanabe, T. (2003). Molecular cloning of a cDNA encoding a soluble protein in the coral exoskeleton. Biochem. Biophys. Res. Commun. *304*, 11–17.
13. Drake, J.L., Mass, T., Haramaty, L., Zelzion, E., Bhattacharya, D., and Falkowski, P.G. (2013). Proteomic analysis of skeletal organic matrix from the stony coral *Stylophora pistillata*. Proc. Natl. Acad. Sci. USA *110*, 3788–3793.
14. Puverel, S., Tambutté, E., Pereira-Mouriès, L., Zoccola, D., Allemand, D., and Tambutté, S. (2005). Soluble organic matrix of two Scleractinian corals: partial and comparative analysis. Comp. Biochem. Physiol. B Biochem. Mol. Biol. *141*, 480–487.
15. Rahman, M.A., Isa, Y., and Uehara, T. (2005). Proteins of calcified endoskeleton: II partial amino acid sequences of endoskeletal proteins and the characterization of proteinaceous organic matrix of spicules from the alcyonarian, *Synularia polydactyla*. Proteomics *5*, 885–893.
16. Rahman, M.A., Oomori, T., and Wörheide, G. (2011). Calcite formation in soft coral sclerites is determined by a single reactive extracellular protein. J. Biol. Chem. *286*, 31638–31649.
17. Moya, A., Huisman, L., Ball, E.E., Hayward, D.C., Grasso, L.C., Chua, C.M., Woo, H.N., Gattuso, J.P., Forêt, S., and Miller, D.J. (2012). Whole transcriptome analysis of the coral *Acropora millepora* reveals complex responses to $CO_2$-driven acidification during the initiation of calcification. Mol. Ecol. *21*, 2440–2454.
18. Shinzato, C., Shoguchi, E., Kawashima, T., Hamada, M., Hisata, K., Tanaka, M., Fujie, M., Fujiwara, M., Koyanagi, R., Ikuta, T., et al. (2011). Using the *Acropora digitifera* genome to understand coral responses to environmental change. Nature *476*, 320–323.
19. von Marschall, Z., Mok, S., Phillips, M.D., McKnight, D.A., and Fisher, L.W. (2012). Rough endoplasmic reticulum trafficking errors by different classes of mutant dentin sialophosphoprotein (DSPP) cause dominant negative effects in both dentinogenesis imperfecta and dentin dysplasia by entrapping normal DSPP. J. Bone Miner. Res. *27*, 1309–1321.
20. Hecker, A., Testenière, O., Marin, F., and Luquet, G. (2003). Phosphorylation of serine residues is fundamental for the calcium-binding ability of Orchestin, a soluble matrix protein from crustacean calcium storage structures. FEBS Lett. *535*, 49–54.
21. Mann, K., Poustka, A.J., and Mann, M. (2010). Phosphoproteomes of *Strongylocentrotus purpuratus* shell and tooth matrix: identification of a major acidic sea urchin tooth phosphoprotein, phosphodontin. Proteome Sci. *8*, 6.
22. Hughes, T.P., Baird, A.H., Bellwood, D.R., Card, M., Connolly, S.R., Folke, C., Grosberg, R., Hoegh-Guldberg, O., Jackson, J.B.C., Kleypas, J., et al. (2003). Climate change, human impacts, and the resilience of coral reefs. Science *301*, 929–933.
23. Cuif, J.-P., Dauphin, Y., Nehrke, G., Nouet, J., and Perez-Huerta, A. (2012). Layered growth and crystallization in calcareous biominerals: Impact of structural and chemical evidence on two major concepts in invertebrate biomineralization studies. Minerals *2*, 11–39.
24. Helman, Y., Natale, F., Sherrell, R.M., Lavigne, M., Starovoytov, V., Gorbunov, M.Y., and Falkowski, P.G. (2008). Extracellular matrix production and calcium carbonate precipitation by coral cells in vitro. Proc. Natl. Acad. Sci. USA *105*, 54–58.
25. Flügel, E., and Senowbari-Daryan, B. (2001). Triassic reefs of the Tethys. In The History and Sedimentology of Ancient Reef Systems, G.D. Stanley, Jr., ed. (New York: Kluwer Academic Publishing/Plenum), pp. 217–249.
26. Knoll, A.H. (2003). Biomineralization and evolutionary history. Rev. Mineral. Geochem. *54*, 329–356.
27. Ganot, P., Moya, A., Magnone, V., Allemand, D., Furla, P., and Sabourault, C. (2011). Adaptations to endosymbiosis in a cnidarian-dinoflagellate association: differential gene expression and specific gene duplications. PLoS Genet. *7*, e1002187.
28. Karabinos, A., Bhattacharya, D., Morys-Wortmann, C., Kroll, K., Hirschfeld, G., Kratzin, H.D., Barnikol-Watanabe, S., and Hilschmann, N. (1996). The divergent domains of the NEFA and nucleobindin proteins are derived from an EF-hand ancestor. Mol. Biol. Evol. *13*, 990–998.
29. Frenkel-Morgenstern, M., and Valencia, A. (2012). Novel domain combinations in proteins encoded by chimeric transcripts. Bioinformatics *28*, i67–i74.
30. Holm, R.H., Kennepohl, P., and Solomon, E.I. (1996). Structural and functional aspects of metal sites in biology. Chem. Rev. *96*, 2239–2314.
31. Bashford, D., and Karplus, M. (1990). pKa's of ionizable groups in proteins: atomic detail from a continuum electrostatic model. Biochemistry *29*, 10219–10225.
32. Da Silva, J.F., and Williams, R.J.P. (2001). The Biological Chemistry of the Elements: The Inorganic Chemistry of Life (New York: Oxford University Press), p. 271.
33. Greenfield, E.D., Wilson, D.C., and Crenshaw, M.A. (1984). Ionotropic nucleation of calcium carbonate by molluscan matrix. Amer. Zool. *24*, 925–932.
34. Addadi, L., Moradian, J., Shay, E., Maroudas, N.G., and Weiner, S. (1987). A chemical model for the cooperation of sulfates and carboxylates in calcite crystal nucleation: Relevance to biomineralization. Proc. Natl. Acad. Sci. USA *84*, 2732–2736.
35. Annavarapu, S., and Nanda, V. (2009). Mirrors in the PDB: left-handed alpha-turns guide design with D-amino acids. BMC Struct. Biol. *9*, 61.
36. Watson, J.D., and Milner-White, E.J. (2002). The conformations of polypeptide chains where the main-chain parts of successive residues are enantiomeric. Their occurrence in cation and anion-binding regions of proteins. J. Mol. Biol. *315*, 183–191.
37. McCulloch, M., Falter, J., Trotter, J., and Montagna, P. (2012). Coral resilience to ocean acidification and global warming through pH up-regulation. Nat. Clim. Chang. *2*, 623–627.
38. Maruyama, K., Mikawa, T., and Ebashi, S. (1984). Detection of calcium binding proteins by 45Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis. J. Biochem. *95*, 511–519.