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The Skeletal Proteome of the Coral Acropora millepora: The Evolution of Calcification by Co-Option and Domain Shuffling

Paula Ramos-Silva,1,2 Jaap Kaandorp,*,2 Lotte Huisman,2,3 Benjamin Marie,4 Isabelle Zanella-Cléon,5 Nathalie Guichard,1 David J. Miller,3 and Frédéric Marin*1

1UMR 6282 CNRS, Biogéosciences, Université de Bourgogne, Dijon, France
2Computational Science Section, Informatics Institute, Universiteit van Amsterdam, Amsterdam, The Netherlands
3ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland, Australia
4UMR 7245 CNRS, MCAM, Muséum National d’Histoire Naturelle (MNHN), Paris, France
5IFR 128 BioSciences Gerland-Lyon Sud, UMR 5086 CNRS, Institut de Biologie et Chimie des Protéines, Université de Lyon, Lyon, France

*Corresponding author: E-mail: j.a.kaandorp@uva.nl; frederic.marin@u-bourgogne.fr.

Abstract

In corals, biocalcification is a major function that may be drastically affected by ocean acidification (OA). Scleractinian corals grow by building up aragonitic exoskeletons that provide support and protection for soft tissues. Although this process has been extensively studied, the molecular basis of biocalcification is poorly understood. Notably lacking is a comprehensive catalog of the skeleton-occluded proteins—the skeletal organic matrix proteins (SOMPs) that are thought to regulate the mineral deposition. Using a combination of proteomics and transcriptomics, we report the first survey of such proteins in the staghorn coral Acropora millepora. The organic matrix (OM) extracted from the coral skeleton was analyzed by mass spectrometry and bioinformatics, enabling the identification of 36 SOMPs. These results provide novel insights into the molecular basis of coral calcification and the macroevolution of metazoan calcifying systems, whereas establishing a platform for studying the impact of OA at molecular level. Besides secreted proteins, extracellular regions of transmembrane proteins are also present, suggesting a close control of aragonite deposition by the calicoblastic epithelium. In addition to the expected SOMPs (Asp/Glu-rich, galaxins), the skeletal repertoire included several proteins containing known extracellular matrix domains. From an evolutionary perspective, the number of coral-specific proteins is low, many SOMPs having counterparts in the noncalcifying cnidarians. Extending the comparison with the skeletal OM proteomes of other metazoans allowed the identification of a pool of functional domains shared between phyla. These data suggest that co-option and domain shuffling may be general mechanisms by which the trait of calcification has evolved.

Key words: biomineralization, calcium carbonate skeleton, scleractinian, proteomics, evolution.

Introduction

It is generally accepted that anthropogenic CO2 emissions cause deleterious effects, not only on the atmosphere (i.e., global warming) but also on seawater chemistry. Since pre-industrial times, the average pH of seawater has fallen from 8.2 to 8.1, and this ocean acidification (OA) is thought to have significantly affected marine organisms in a variety of ways (Caldeira and Wickett 2003; Kline et al. 2012), including impacts on important physiological functions such as calcification (Orr et al. 2005; Cohen et al. 2009; Kroeker et al. 2013). This process is essential for a wide range of marine metazoans and fulfills a diverse array of important physiological and ecological roles (Cusack and Freer 2008). In the neritic domain of tropical and subtropical regions, the most prominent calcium carbonate producers are cnidarians, in particular scleractinians (stony corals), where coral reefs have an annual net production ranging from 5 to 126 mol CaCO3 m−2 year−1 (Milliman and Droxler 1996; Gattuso et al. 1998). Recent studies have shown that OA and increasing sea surface temperatures decrease coral calcification rates (Cohen and Mcconnaughey 2003; Langdon and Atkinson 2005; Orr et al. 2005; De’ath et al. 2009; Kleypas and Yates 2009; Pandolfi et al. 2011; Chan and Connolly 2013). However, most of the data supporting these negative effects are obtained at the organism/colony level and only few studies have considered the underlying molecular and cellular mechanisms. Of particular significance is the fact that the molecular machinery of coral biomineralization (the “calcification toolkit”) is, as yet, poorly characterized. Thus, a better understanding of the process is required, in particular at the level of the mineralizing space, where CaCO3 crystals are formed and shaped (Allemand et al. 2011). A number of candidate genes...
for roles in coral calcification have been identified for being expressed in the calcicoblastic ectoderm (Zoccola et al. 2004, 2009; Moya et al. 2008). This number was recently increased with high-throughput analyses (Meyer et al. 2011; Shinzato et al. 2011; Moya et al. 2012), whereas microarray studies led to the identification of the SCRPs, a family of small cysteine-rich proteins showing molecular features suggestive of an involvement in calcification processes (Sunagawa et al. 2009). Amongst the most obvious candidates for roles in coral calcification are the genes that encode skeletal organic matrix proteins (SOMPs), that is, proteins secreted by the calcifying tissues—the calcicoblastic ectoderm in corals—which are occluded in the skeleton during its formation (Tambutté et al. 2008). These proteins are the main components of the skeletal organic matrix (OM), which in corals contains also polysaccharides (Goldberg 2001), glycoproteins (Dauphin and Cuif 1997), and lipids (Farre et al. 2010). The SOMPs have been characterized biochemically “in bulk” (i.e., without fractionation) in different coral species (Cuif, Dauphin, Gautret 1999; Cuif, Dauphin, Freiwald, et al. 1999; Ingalls et al. 2003; Puverel et al. 2005), the compositional analyses have shown that it is enriched in aspartic acid and, in lesser extent, in glutamic acid and glycine. This feature, together with the presence of saccharidic moieties, makes the OM unusually acidic. Moreover, its interaction with calcium carbonate was observed experimentally in the scleractinians Balanophyllia europaea (Goffredo et al. 2011), Acropora digitifera, Lophelia pertusa, Montipora calciculata (Falini et al. 2013) as well as by OM produced by coral cells of Stylphora pistillata (Helman et al. 2008), suggesting an important role of the OM in coral calcification.

Although several OM proteins have been extensively studied in other calcifying metazoans, such as molluscs (Marin et al. 2012) and sea urchins (Veis 2011), their characterization is still in its infancy for corals. Only recently an approach combining proteomics with genomics was applied unveiling a set of partial and complete sequences of proteins in the skeleton of the hexacoral S. pistillata (Drake et al. 2013). Prior to this study, only the full sequence of two SOMPs, galaxin from the scleractinian Galaxea fascicularis (Fukuda et al. 2003), and scelritin from the octocoral Corallium rubrum (Debreuil et al. 2012) were published and a number of other partial sequences from octocorals (Rahman and Isa 2005; Rahman et al. 2005; Rahman, Isa, Takemura, et al. 2006; Rahman, Isa, Uehara 2006) and scleractinians (Puverel et al. 2005).

One prerequisite for understanding the process of coral calcification is a comprehensive survey of the skeletal matrix proteins. In this article, we address this requirement, by describing 36 extracellular proteins that constitute the SOMP repertoire, or “skeletome,” of the scleractinian coral Acropora millepora. This is the first large-scale survey of the proteins present in the skeleton of a member of the acroporid family and the second in cnidarians. These proteins give new insights on the molecular tools required for controlling the deposition of the calcium carbonate and provide a platform for investigating the impact of environmental factors on calcification at the molecular level. In addition, they open novel perspectives on the mechanisms of evolution of SOMPs within the Cnidaria and, more broadly, within other calcifying metazoans.

Results and Discussion

Analysis of the Matrix on Gel

Coral skeleton was treated with bleach twice to remove contaminating tissue, symbionts, and bacteria, that can be entrapped in the highly porous skeleton (supplementary fig. S1, Supplementary Material online), the second treatment being conducted after the skeleton had been reduced to fine powder (granulometry < 200 μm). The residual powder was then extracted with 10% acetic acid, yielding acid-soluble matrix (ASM) and acid-insoluble matrix (AIM) fractions, assumed to be contaminant-free and closely associated with the aragonite skeleton. This extraction process yielded approximately 0.034 ± 0.01% of ASM and 0.23 ± 0.03% (wt/wt) of AIM relative to the skeleton weight. 1D-gel electrophoresis of the ASM extract (fig. 1A) showed three main discrete bands at around 120, 90, and 64 kDa embedded in a diffuse background of more dispersed “smearing” macromolecules, whereas the AIM was characterized by a smear lacking discrete bands. On the 2D gel (fig. 1B), the 90 and 64 kDa fractions, and, to a lesser extent, the 120 kDa fraction, in the ASM are characterized by large spots circa pl 3, suggesting that these macromolecules are very acidic.

Identification and Characterization of SOMPs

Raw data generated by LC-MS/MS on the ASM and AIM were analyzed with the software MASCOT to search against a pooled database consisting of transcripts and predicted proteins of A. millepora and the genus Symbiodinium.

This procedure was initially made on the AIM and ASM prepared after only a single (whole skeleton) bleach treatment (“first batch”), but was then repeated on OM fractions (“second batch”) after the second bleaching step (i.e., on skeleton reduced to fine powder) outlined earlier. This enabled to analyze the effect of extended bleaching on removal of contaminants (fig. 2). Although the proteomic analyses of the “first batch" revealed intracellular coral proteins and proteins of symbiont origin (Ramos-Silva et al. 2013), the second batch was free of such contaminants, enabling the detection of 43 unique A. millepora proteins likely to constitute the SOMP repertoire, that is, proteins that are strongly associated to the skeleton. Of these 43 proteins, 36 assignments could be made with high confidence (i.e., with more than one unique peptide matching the sequence, supplementary table S1, Supplementary Material online), whereas seven sequences each with only a single peptide match were dropped from the list (supplementary table S2, Supplementary Material online) despite their properties being generally consistent with those of the high-confidence data set. Figure 3 provides a schematic overview of the domain structure and general properties of the Acropora SOMPs that were classified based on the in silico sequence analyses. From the 36 SOMPs identified, 22 proteins were common to both ASM and AIM (supplementary table S1, Supplementary Material online).
Similar ASM and AIM protein repertoires have also been observed in the case of mollusc shell matrices (Marie et al. 2009). On the other hand, 12 SOMPs were exclusively associated with the insoluble organic fraction of the skeleton, and only 2 with the soluble matrix (supplementary table S1, Supplementary Material online). Possible causes for this apparent bias (fig. 2) between matrices include the over/under representation of certain peptides that is inherent to the proteomic approach, and the fact that some proteins from the acid-soluble fraction may bind acid-insoluble components.

**Acidic Proteins**

In both ASM and AIM, the most abundant proteins (high emPAI values; Ishihama et al. 2005; supplementary table S1, Supplementary Material online) were acidic (fig. 1B), that is, with pI < 4.5 (Marin and Luquet 2007) and rich in negatively charged residues (Asp, Glu). Asp and Glu-rich proteins are supposed to interact directly with calcium carbonate crystals promoting crystal nucleation, determining the growth axes and inhibiting the crystal growth (Wheeler et al. 1981; Addadi et al. 1987). Because of their polyanionic character, Asp and Glu-rich proteins usually have high-capacity, but low-affinity, calcium-binding properties (Maurer et al. 1996). Among the *A. millepora* SOMPs, six acidic proteins were identified (fig. 3), including two similar (48.8% identity; supplementary fig. S2, Supplementary Material online) proteins each containing approximately 20% of Asp residues and named as SAARP1 (B3EWY6) and 2 (B3EWY8) (secreted acidic Asp-rich protein). These proteins are likely to belong to a family of highly acidic proteins conserved across scleractinians. Indeed two Asp-rich proteins, named coral acid-rich proteins (CARPs), were recently identified in *S. pistillata* skeleton (Drake et al. 2013). Similarly to CARPs, SAARPs contain two Asp-rich regions intercalated by two nonacidic regions (fig. 3). In particular, CARP 4 (M1SN56) exhibits the highest identity percentage with the two SAARPs, 60.6% and 45.2%, for SAARP 1 and 2, respectively. However, the predicted protein sequences of CARP4 and CARP5 (M1RYM2) are shorter than SAARPs and lack a putative peptide signal required for targeting these proteins to the secretory pathway. Interestingly, the four acidic proteins also show significant identity, in the nonacidic regions, with the protein fragment P27 (M1TA76).
**Fig. 3.** List of SOMPs identified by proteomics in the acid soluble and insoluble matrices extracted from *Acropora millepora* skeleton. Proteins were named according to the characterization of their primary structure.
Identified in the skeleton of S. pistillata (Drake et al. 2013) (supplementary fig. S3, Supplementary Material online); however, this similarity is not understood from a functional viewpoint. The two SAARPs together with a third protein, acidic SOMP (B3EWY7), corresponded to the identifications with the highest emPAI scores (supplementary table S1, Supplementary Material online). The acidic SOMP has lower Asp content (9.9%) and its sequence does not contain two Asp-rich regions as in SAARPs (fig. 3). Still, it shows significant similarities in the nonacidic region with both SAARPs (supplementary fig. S4, Supplementary Material online).

The three other skeletal proteins included the secreted acidic proteins Amil-SAP1 (B3EWZ0, B3EWZ1), Amil-SAP2 (B3EWZ4) (19% and 21.6% of Asp and Glu, respectively), and one Glu-rich protein (B7W112). Amil-SAP1 and Amil-SAP2 are the counterparts of Adi-SAP1 (supplementary fig. S5, Supplementary Material online) and Adi-SAP2, respectively, which have been hypothesized to have roles in calcification along with seven other proteins, Adi-SAP3 to -SAP9, in Acropora digitifera, on the basis of their high Asp plus Glu content (Shinzato et al. 2011). Of these acidic proteins, only SAP1 and two exhibit a predicted signal peptide, and are confirmed here to be true SOMPs. BLASTP searches with the six acidic SOMPs retrieved significant matches (E value < 10−4) with proteins in the UniprotKB database. Interestingly, among the best matches for Amil-SAP1 and the SAARPs were shell matrix proteins (aspeins) from Pinctada fucata, P. maxima, and Isognomon perna. However, in these cases, similarity scores might be largely misleading due to the relatively low complexity of the sequences, dominated by acidic residues; note also that none of these SOMPs had significant similarity to annotated domains from the InterPro database. Moreover, these data confirm the direct involvement of acidic proteins in skeletal formation in A. millepora.

**Unique Uncharacterized Proteins**

High-throughput approaches such as proteomics and transcriptomics have often revealed completely novel proteins that lack conserved domains or significant database matches that would allow to classify them into families and to hypothesize about their functions (Ramos-Silva et al. 2012). Galaxin exemplifies the case of such “orphans” because its function remains unknown (Reyes-Bermudez et al. 2009). In A. millepora skeleton, we identified two distinct galaxins along with further eight uncharacterized “orphan” SOMPs (fig. 3) referred here as USOMPs. These proteins do not constitute a single group and, with the exception of three proteins, exhibit no significant similarities. Two of these exceptions are galaxin proteins, and the third is provided by USOMP-4 (B8UU74), with a region near the C-terminus that has similarity to three of the acidic proteins discussed earlier (supplementary fig. S4, Supplementary Material online). The presence of multiple galaxin-related genes has previously been reported (Reyes-Bermudez et al. 2009; Shinzato et al. 2011); one of the galaxins identified here corresponds to GenBank ADI50283.1, a previously known gene from A. millepora (Reyes-Bermudez et al. 2009). The second sequence (galaxin-2, B8UU51) is orthologous with galaxin 2 from A. digitifera (Shinzato et al. 2011). The two A. millepora galaxins have 31.5% identity overall, the di-Cys repeat region being most similar. Note that the di-Cys pattern might indicate a role in the assembly and support of the macromolecular framework (Fukuda et al. 2003). Indeed, a multiple alignment with five galaxins and similar sequences from Nemastostella vectensis (Gls: 156374951 and 156377965) and Hydra magnipapillata (Gln: 221103149) shows the conservation of at least nine double Cys motifs (supplementary fig. S6, Supplementary Material online). Similarly in S. pistillata skeleton, three proteins were identified, P12 (M1GFJ4), P16 (M1SN43), and P22 (M1T243) (Drake et al. 2013), which can fit in our description of uncharacterized proteins. From these, P16 and P22 are completely unique, whereas P12 shows similarities with USOMP8 (supplementary table S6, Supplementary Material online).

**Extracellular Matrix-Like Proteins**

Other SOMPs, although in some cases lacking clear overall matches, contain conserved regions or domains. The largest group comprises those with one or more domains usually associated with extracellular matrix (ECM) or cell-adhesion proteins from other metazoans. Amongst the 15 ECM-related proteins identified in the skeletal OM of A. millepora most contained multiple domains (fig. 3). Several of these ECM-related SOMPs resembled human cell-adhesion proteins such as mucin-4 (mucin-like SOMP, B3EWY9) and hemicentin-1 (coadhesin, B3EWZ3) (supplementary table S1, Supplementary Material online), which are heavily glycosylated and rich in cysteine residues, thereby forming disulfide bridges involved in modulating their adhesive properties. SOMPs that exhibit mucin-like or co-adhesin signatures have previously been reported in the mineralizing matrices of molluscs (Marin et al. 2000; Suzuki et al. 2009; Joubert et al. 2010), such as thrombospondin type 1 repeats, von Willebrand factor type A and epidermal growth factor-like domains, which are typical of ECM proteins involved in cell–cell and cell–substrate adhesion and in the binding of other macromolecules (Engel 1991; Mosher and Adams 2012). Moreover, many ECM domains present in Acropora’s skeletal proteome have been detected in previous high-throughput proteomic studies on the OM of molluscs, sea urchin, chicken eggshell, and coral (supplementary table S5, Supplementary Material online) but only the polycystic kidney disease 1 (PKD1)-related protein (B8UU59) contains domains (lipoygenase, egg-jelly receptor, and PKD/chitinase) not previously found in proteomic analyses of other skeletal organic matrices. On the other hand some of the data sets used in these comparisons consist of broader lists of proteins, including some of intracellular origin that may be derived from cell debris and are most likely not involved in biomineralization (Mann et al. 2010; Zhang et al. 2012). Thus, comparisons were interpreted carefully and taking into account other evidences such as the expression in skeleton secreting-tissues (Jonchère et al. 2010; Marie et al. 2012; Zhang et al. 2012), the direct interaction with CaCO₃ or the detection in the calcifying...
media (Hincke et al. 2000) (supplementary table S5, Supplementary Material online). From the 15 ECM-related SOMP s, 8 have homology domains with proteins from the S. pistillata skeletal proteome (supplementary tables S5 and S6, Supplementary Material online): mucin-like, coadhesin, eectin (B3EWZ8), epidermal growth factor (EGF) and laminin G domain-containing protein (B8UU78), Meprin, A-5 protein, and receptor protein-tyrosine phosphatase Mu (MAM) and LDL-receptor domain-containing protein 1 (B3EWZ5) and 2 (B3EWZ6), zona pellucida domain-containing protein (G8HTB6), and proteocaherin-like (B8V7Q1). Still, apart from protocadherin, we could not confirm candidate orthologous for these SOMP s in S. pistillata because most sequences correspond to fragments. Four ECM-related SOMP s were exclusively detected in the AIM, two of them having homologs usually found in the central nervous system in metazoans (Bieber et al. 1989; Frank and Kemler 2002)—protocadherin-like and neuroglant-like protein (B8VIV9), also the EGF and laminin G domain-containing protein and finally collagen (B8V7R6). The latter is homologous to the human alpha-2 type I collagen (P08123), which is found in bone (Glimerch 1959; Veis and Sabsay 1987). Fibrils of collagen are generally in close contact with the mineral phase and remain as an insoluble component of the organic fraction throughout the extraction process. Although collagen has been detected in the spicules of gorgonian (Goldberg 1974; Kingsley et al. 1990) and scleractinian corals (Drake et al. 2013), this is the first report of the occurrence of collagen type I in the OM of a coral skeleton.

**Enzymes**

Four of the SOMP s identified correspond to three types of enzymes (fig. 3). The first enzyme corresponds to a 148 amino acid (aa) fragment (B8V7P3) of an alpha-type carbonic anhydrase (CA), a zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide. CA activity has been detected in the skeletal OM of both zooxanthellate (Acropora hebes; Isa and Yamazato 1984) and azooxanthellate (Tubastrea aurea; Tambutté et al. 2006) scleractinians.

In the case of the CA identified here, its best BLAST hit in UniprotKB is the α-CA from S. pistillata, STPCA-2 (C0IX24) (supplementary table S1, Supplementary Material online). This enzyme was previously shown to be highly active compared with other CAs and was localized in the oral endoderm and aboral tissue (Bertucci et al. 2011). Despite its high similarity with the cytosolic human carbonic anhydrase II, STPCA-2 has unusual features such as a putative signal peptide and an insert of 30 amino acids between positions 212 and 243. Moreover, the same protein was also identified in the skeletal OM of both zooxanthellate (Acropora hebes; Isa and Yamazato 1984) and azooxanthellate (Tubastrea aurea; Tambutté et al. 2006) scleractinians.

The second SOMP (B3EWZ9), hephaestin-like, is a secreted member of the copper oxidase family with 1,114 aa length, containing a copper-binding site and a cupredoxin domain. Hephastin proteins function as copper-dependent ferrooxidases, mainly involved in iron and copper metabolism at membranes (Vulpe et al. 1999; Lang et al. 2012). Although to date there is no experimental evidence of activity of this type in mineralizing matrices, it is reasonable to propose that the hephaestin-like protein catalyzes the oxidation of iron (from Fe$^{2+}$ to Fe$^{3+}$) in the compartment where aragonite precipitation occurs. It was previously suggested that corals may entrap iron in the skeleton as a detoxification mechanism when high concentrations of the metal are present in the reef (Brown et al. 1991). Alternatively, the incorporation of iron in the skeleton of A. millepora may be a normal process, regulated by hephaestin. Interestingly, cupredoxin domains have also been identified in the shell of the mollusc Crassostrea gigas (in the L-ascorbate oxidase, K1P1Z9; Laccase-15, K1QAQ2 and Laccase-18, K1PMS4) and in the tooth and spicules of the echinoderm S. purpuratus (in a protein similar to hephaestin, H3JMP5) (supplementary table S5, Supplementary Material online). Finally, the third enzymatic function assigned is observed in two SOMPs (B8V7S0 and B8VIV4), exclusive to the AIM. It corresponds to proteases containing peptidase S1/S6, chymotrypsin/Hap and C1r/C1s, Uegf, Bone morphogenetic protein 1 (CUB) domains.

Even though they are present at relatively lower abundance (supplementary table S1, Supplementary Material online), the enzymes detected here may play crucial roles in the supply of bicarbonate ions at the site of calcification (CA), in the regulation of iron and copper metabolism (hephaestin), and in the assembly/cleavage of the OM (proteases).

**Toxin-Like**

One SOMP (B7W114) corresponds to a secreted protein that has high similarity (50%) with the N-terminus of a toxin (of 1,052 aa) from the cephalopod Sepia esculenta (SE-cephalotoxin) (B2DCR8; BLASTP E value = 8$^{-48}$), whereas a number of toxins have been characterized from cubozoans (box jellyfish) (Brinkman et al. 2012) and anthozoans (sea anemones) (Frazão et al. 2012). However, very little is known about toxins in scleractinian corals, though studies have demonstrated antibacterial activity of the mucus (Gochfeld and Aeby 2008; Geffen et al. 2009).

The level of identity between B7W114 and the cephalotoxin (supplementary fig. S7, Supplementary Material online) is suggestive of conserved function. If the toxic character of the toxin-like SOMP is later confirmed, we may hypothesize that this protein acts in the coral skeleton in a similar way to the lysozyme in the chicken eggshell (Hincke et al. 2000), which due to its well-known anti-microbial properties was suggested to have a protective function in the eggshell in addition to its ability to interact with calcium carbonate.

**Proteins with Transmembrane Domains**

The occurrence of transmembrane (TM) proteins associated to calcium carbonate biominerals has been explained as contamination by soft tissues (Zhang et al. 2012). However, with the double-bleaching procedure used in the present case, this
Hypothesis is very unlikely and we suggest another explanation. Eleven SOMPs identified among the different groups described above contain putative TM domains. Ten of these SOMPs have a single TM domain located at their C-terminus (fig. 3), the exception being the PKD1-related protein, which is predicted to contain 11 TM helices, 4 of which are part of the polycystin cation channel domain. SOMPs with TM domains represent about one half of the ECM group, one protein (hephaestin-like) in the enzyme group, one protein (uncharacterized SOMP-3) of the “orphan” group and two in the acidic group (SAARP 2 and SAP-1). We propose that the TM proteins detected here are cleaved in their extracellular region, becoming subsequently occluded in the forming biomineral. Three lines of evidence support this hypothesis. First, without exception, the 75 peptides detected by LC-MS/MS that correspond to the 11 TM domain-containing SOMPs belong to the extracellular region located at the N-terminal side of the proteins (supplementary table S1, Supplementary Material online). Second, two proteases were detected amongst the SOMPs having chymotrypsin/Hap domains that may constitute the required tool for cleaving the extracellular region of the TM domain-containing SOMPs. Third, the 11 TM-containing SOMPs possess predicted chymotrypsin cleavage sites in their extracellular regions (supplementary table S1, Supplementary Material online). Specific cleavage sites for chymotrypsin and TM domains are illustrated in figure 4 for two SOMPs: SAARP2 and zona pellucida domain-containing protein. These lines of evidence are congruent with the recent data on the spicule matrix proteins of the sea urchin S. purpuratus (Mann et al. 2010), so the mechanism proposed here may apply more generally.

If this hypothesis is later confirmed by other approaches, this may call for a complete change of paradigm in the biomineralization field, whatever the biological model studied. Until now, the “molecular tools” controlling the mineralization process were all supposed to be secreted outside the calcifying epithelial cells to interact with the inorganic precursors of mineralization. The data presented here suggest that membrane-bound proteins may also contribute to the process via their extracellular domains, which are subsequently incorporated in the biomineral after being cleaved by peptidases. Furthermore, it suggests that there is a true connection between the epithelium and the mineral front, and that the mineral deposition is accomplished under the direct guidance of the cell surfaces, rather than remotely, as “classical” views tend to show, in particular in mollusc shell models (Wilbur and Saleuddin 1983).

SOMPs in Early Stages of Calcification Affected by High CO2

The availability of transcriptome data derived from A. millepora primary polyps (Moya et al. 2012) permitted investigation of the impact of elevated CO2 on expression of the SOMPs identified here. Comparisons with the data sets submitted with the NCBI Gene Expression Omnibus database (GEO) under the accession number GSE33016 confirmed that genes corresponding to all of the SOMPs are expressed during the early stages of calcification and at least 26 of these were up regulated, whereas 4 were down regulated in polyps exposed to higher concentrations of CO2 (supplementary table S4, Supplementary Material online). SOMPs are therefore appropriate molecular markers for the investigation of the impacts of elevated CO2 on Acropora calcification.

Homology Comparison between Acropora, N. vectensis, and H. magnipapillata

In an attempt to unravel the origins of the A. millepora “skeletome”, homologs of the 36 biomineralization proteins
were searched amongst the predicted proteins (using BLASTP) and corresponding transcripts (BLASTN and TBLASTX) from the three cnidarians for which whole genome sequence data are presently available—A. digitifera, a scleractinian coral which diverged from A. millepora in the Mio-Pliocene (Van Oppen et al. 2001), the sea anemone N. vectensis, and H. magnipapillata. Sea anemones are classified as a distinct Order (Actinaria) from corals (Scleractinia) but in the same subclass (Zoantharia = Hexacorallia) within Class Anthozoa. Although sea anemones are considered to be “close,” but skeleton-less, relatives of corals, Hydra is a phylogenetically remote cnidarian belonging to Class Hydrozoa (Collins 2002; Ball et al. 2004; Collins et al. 2006). This approach enabled proteins that are involved in Acropora biomineralization but have ancient cnidian origins (i.e., are present in Hydra as well as Acropora) to be resolved from those that may be anthozoan-specific but not restricted to calcifying anthozoans (i.e., present in Nematostella as well as Acropora) and those that are so far unique to stony corals. Clear orthologs of each of the 36 A. millepora SOMPs could be identified in A. digitifera (supplementary tables S1 and S3, Supplementary Material online), confirming that all the sequences assigned to A. millepora are of coral origin and are not derived from zooxanthellae.

BLAST comparisons against the predicted proteins of N. vectensis and H. magnipapillata, indicate that eight of the SOMPs do not have counterparts in the two noncalcifying cnidarians: five “orphan” proteins (USOMPs), SAP-1, SAP-2, and the SOMP similar to cephalexotoxin (outer circle, fig. 5). Indeed, SAPs were previously suggested to be restricted to members of the genus Acropora (Shinzato et al. 2011).

To date, a BLAST search against the cnidian sequence data on NCBI with these proteins only retrieved orthologous sequences in other Acropora species (supplementary table S3, Supplementary Material online) and low sequence similarities (E value < 10⁻⁵⁰) in Porites astreoides (USOMP-2), Montastrea faveolata (USOMP-2, USOMP-4), Aiptasia pallida (USOMP-1, USOMP-3), Anemonia viridis (USOMP-3), and Clytia hemisphaerica (similar to cephalexotoxin). In particular, the apparent absence of homologs of the cephalexotoxin-like sequence in other cnidarians is surprising given the similarity between the Acropora and cuttlefish (Sepia) proteins. Note that the SE-cephalexotoxin (B2DCR8) identified in the salivary glands of the cuttlefish Sepia esculenta (Cephalopoda, Mollusca) does not exhibit any significant similarity to known proteins (Ueda et al. 2008). In turn, the striking similarity (50%) between the Acropora and Sepia sequences (supplementary fig. S6, Supplementary Material online) suggests that the ancestor of these proteins predate the divergence of molluscs and corals (i.e., 550–600 Ma or more) but could have been lost in most of the other eumetazoan lineages. Such a genome restructuring, that is, massive loss, has been recently documented via large-scale comparisons of distantly related genomes: for example, the gastropod Lottia and Nematostella share exclusively 89 gene families that are not retrieved in other phyla investigated (Ptitsyn and Moroz 2012).

Amongst the other SOMPs, we have distinguished similar (white circle area, fig. 5) proteins taking into account the statistical significance of BLAST searches, the percentage of identity (cutoff 30%) in the pairwise global alignment, the common domain architecture and the fact that many of the SOMPs are mosaic proteins, that is, proteins with multiple domains (Bork 1992; Doolittle 1992) (supplementary table S3, Supplementary Material online). In particular for those proteins from the ECM-like group and enzymes, it is difficult to infer homology merely on the basis of BLAST searches (generally highly significant within a certain domain) together with identity percentages and alignment coverage (generally low). To overcome this difficulty, the Neighborhood Correlation (NC) method was applied to the 3 genomes. This method has been developed and used to accurately identify homologs in complex multidomain families (Song et al. 2008; Joseph and Durand 2009), enabling the prediction of similar proteins (due to domain insertion) from homologous proteins (due to common ancestor).

This combined analysis implied that at least three SOMPs have homologs in the anthozoan but not in H. magnipapillata (middle circle, fig. 5): USOMP-7, neuroglian-like and zona pellucida domain-containing protein, whereas other 9 SOMPs, all from the ECM-like group, have homologs in the three genomes (inner circle, fig. 5). Several of these conserved ECM-like SOMPs are also found in vertebrates, such as the promegaculin-like, collagen, and PKD1-related proteins (supplementary table S1, Supplementary Material online). Whether these proteins function in similar ways in the skeletal matrix and in the ECM remains to be clarified. However, these results strongly corroborate the idea of a modern skeletal matrix derived from the recruitment of noncalcifying ECM-components.

Conversely, homologs for 16 of the SOMPs could not be confidently identified in the noncalcifying cnidarians due to specific factors: incompleteness of at least one of the sequences in study (putative carbonic-anhydrase, peptidase, Glu-rich, and USOMP-5), low identity percentages (cutoff 30%) (hephaestin-like, galaxins, SAARPs, acidic SOMP, and USOMP-8), different combination of domain architectures and neighborhood coefficients above the threshold (mucin-like, coadhesin, threonine-rich, and CUB domain-containing protein, peptidase) (supplementary table S3, Supplementary Material online). In consequence, these SOMPs were considered as “similar to” and not confirmed as homologous or nonhomologous (white circle area, fig. 5). Interestingly, homologs of hephaestin are present in both chordates and the symbiotic sea anemone Anemonia viridis whereas only low similarity matches were identified in N. vectensis and H. magnipapillata (supplementary fig. S8, Supplementary Material online). Also the SOMP mucin-like has similar domain architecture in the C-terminus side (extracellular nidogen, adhesion associated domain in mucin-4 and other proteins, von Willebrand factor D, and EGF) to the human mucin-4 found in chordates; however, the same domain combination was not identified in the genomes of Nematostella and Hydra (supplementary table S3, Supplementary Material online).
Conclusions

The work described here represents the first comprehensive survey of the skeletal molecular toolkit of the scleractinian coral *A. millepora*. In total, 36 proteins were identified from the acid soluble and insoluble skeletal organic matrices. The proteins of these two fractions overlapped significantly, only 2 proteins being exclusively associated with the soluble fraction and 12 with the insoluble fraction. It is unclear how complete this survey is; trypsin-resistant skeletal proteins, in particular those that do not possess suitable trypsin cleavage sites, may not have been detected. It is also possible that the calicoblastic cells secrete proteins that guide the calcification process but are not occluded in the skeleton and would therefore not be detectable by the approach used here.

One tenet of our analyses is that the classical view of a coral skeletal matrix composed primarily or exclusively of acidic proteins (Mitterer 1978; Constantz and Weiner 1988) is eclipsed by the idea of a more complex matrix consisting of a diverse range of proteins with a larger spectrum of different potential functions. Whilst acidic proteins remain quantitatively key players in calcification (as aptly illustrated by the 2D gel), other SOMPs, some present in lower amount, are also likely to fulfill important roles in the deposition of the skeletal organic matrix.

**Fig. 5.** Resume of the results of similarity searches with BLAST and homology detection (by global alignment, domain architecture comparison and the NC method) using the 36 SOMPs (and corresponding transcripts) from *Acropora millepora* and the genomes of *A. digitifera, Nematostella vectensis*, and *Hydra magnipapillata*. Proteins in the outer circle (red) do not have similarities (and homologs) in the predicted proteins of *Nematostella* or *Hydra*. Proteins in the middle circle (green) have homologs in *Acropora* and *Nematostella*. Proteins from the inner circle (blue) have homologs in *Acropora, Nematostella*, and *Hydra*. SOMPs in the white region of the circle show considerable similarity with proteins from *Nematostella* and *Hydra* but their homology is not certain. The phylogenetic tree on the upright side represents the relationships previously purposed between Cnidaria (Collins et al. 2006); dcp, domain containing protein.
skeleton. The second tenet of our analysis is that the SOMPs are not exclusively secreted. Some SOMPs are membrane-bound proteins, the extracellular domains of which are subsequently cleaved in the mineralizing space and occluded in the growing biomineral.

What kind of information on the macroevolution of calcifying matrices can be inferred from this analysis? The evolutionary picture that emerges from our analysis is multifaceted. Whilst all of the SOMPs identified in *A. millepora* have homologs encoded in the genome of *Acropora digitifera*, surprising few of these are actually unique to the scleractinians, that is, present in the two acroporids but absent from noncalcifying cnidarians (*N. vectensis* and *H. magnipapillata*). These coral-restricted proteins may be “novelties” in the calcification repertoire. The timing of this innovation might have occurred deep in the Paleozoic when scleractinian corals emerged (~450 Ma) (Stolarski et al. 2011). In contrast, a considerable proportion of the ECM-like SOMPs identified here have homologous proteins in *N. vectensis* and *H. magnipapillata*, suggesting that their origins predate the divergence of the classes Anthozoa and Hydrozoa (Ball et al. 2004), which occurred in the Cambrian or earlier. Each of these proteins, and a number of others subsequently lost from *N. vectensis* and *H. magnipapillata* (typified by the proteins similar to cephalotoxin, hephaestin-like, and mucin-like), was presumably co-opted to function in calcification when this trait arose in the scleractinian lineage. Broadening the comparison with include other OM proteomes from metazoans revealed that most of the domains represented in the *A. millepora* skeletal proteome also occur in other OM, pleading for the concept of a pool of “shared domains” potentially involved in biomineralization. However, only few of the proteins containing these common domains are predicted to be homologous, suggesting that they have been independently recruited to roles in calcification at different times across the various taxa with this trait. Beyond macroevolutionary considerations, the data set presented here constitutes an important tool for understanding the molecular bases of calcification in stony corals, and for quantifying the impact of environmental changes (such as OA), on this process.

**Materials and Methods**

**Skeletal Collection and SEM Observations**

*Acropora millepora* colonies were collected at the Great Barrier Reef in Australia (Pioneer Bay, Orpheus Island) in November 2010, prior to the annual spawning event. Mother colonies that died after spawning were used to collect the skeletal material; animal tissue, symbionts, and other micro-organisms were removed by immersion in NaOCl solution (10 times dilution, 0.26% active chlorine) for 5 h and washed with milli-Q water several times until no trace of NaOCl was left. This treatment allows removing organic exogenous or endogenous contaminants that can be entrapped in the highly porous skeleton (supplementary fig. S1, Supplementary Material online), while keeping intact the most tightly bound skeletal matrix components (Gaffey and Bronnimann 1993). The extraction was performed according to a published procedure (Marin et al. 2001). In brief, the dried powder put in suspension in cold water was decalcified overnight in acetic acid (10% vol/vol) at 4 °C with an electronic burette (Titronic Universal, Schott, Mainz, Germany). The solution was centrifuged: the organic pellet (AIM) was rinsed several times with Milli-Q water and freeze-dried. The supernatant (ASM) was treated on an ultrafiltration cell (Amicon 400 mL, 10 kDa cutoff membrane) for volume reduction, then once concentrated, dialyzed several days against water at 4 °C, and freeze-dried. The extraction was performed in duplicate (2 × 30 g of skeletal powder under the same NaOCl treatment) to check the reproducibility of the results.

**ASM/AIM Analysis on 1D and 2D Gel Electrophoresis**

The skeletal matrix—both ASM and AIM fractions—was analyzed on 1D electrophoresis and stained with silver nitrate (Morrissey 1981). The ASM was directly denatured with Laemmli buffer (Laemmli 1970) according to standard conditions while the AIM was only partly solubilized by Laemmli buffer. The solubilized fraction is defined as the Laemmli-soluble/acetic acid insoluble fraction (LS/AIM). Electrophoresis was performed on discontinuous 12% acrylamide mini-gels at 100 V for 15 min and 150 V for 1 h. For the 2D electrophoresis, immobilized pH gradient (IPG) strips (ReadyStrip, BioRad) were loaded with 180 μg of ASM dissolved in 180 μL of rehydration buffer (6 M urea, 2 M thiourea, 4% [wt/vol] Chaps, 20 mM dithiothreitol, 0.1% ampholytes, 0.001% bromphenol blue) and rehydrated overnight at 50 V (25 °C) in a PROTEAN IEF cell (BioRad). Focusing was carried out at 250 V for 15 min, followed by 4,000 V for 2 h and 4,000 V until 10,000 Vh. Subsequently the IPG strips were equilibrated by transfer for 10 min into 1 mL of equilibration buffer I (6 M urea, 2% sodium dodecyl sulphate [SDS], 375 mM Tris/HCl pH 8.8, 20% glycerol) with 2% (wt/vol) dithiothreitol, followed by 10 min in equilibration buffer II, with 2.5% (wt/vol) iodoacetamide instead. Strips were rinsed in 25 mM Tris, 192 mM glycine and 0.1% SDS (TGS) and placed on the top of precast NuPAGE(R) BisTris Novex SDS-polyacrylamide gels (4–10%) (Invitrogen, Carlsbad, CA) with an overlay solution of 0.5% agarose/TGS.
Protocols for the separation of peptides were performed using a C18 PepMap micro-precolumn (5 μm; 0.3 mm × 5 mm) for a 3 min desalting and a C18 PepMap nano-column (3 μm; 100 Å; 75 μm × 150 mm) with a gradient elution at a flow rate of 300 nL/min. Eluent A was a mixture of 95% H2O, 5% CH3CN, and 0.1% formic acid (vol/vol). Eluent B was a mixture of 20% H2O, 80% CH3CN, and 0.1% formic acid (vol/vol). The gradient program was from 0% B to 50% B over 120 min and 100% B for 10 min.

LC was performed on an Ultimate 3000 nano-LC system (Dionex, Voisins Le Bretonneux, France). Chromatographic separation of peptides was obtained with a C18 PepMap micro-precolumn (5 μm; 0.3 mm × 5 mm) for a 3 min desalting and a C18 PepMap nano-column (3 μm; 100 Å; 75 μm × 150 mm) with a gradient elution at a flow rate of 300 nL/min. Eluent A was a mixture of 95% H2O, 5% CH3CN, and 0.1% formic acid (vol/vol). Eluent B was a mixture of 20% H2O, 80% CH3CN, and 0.1% formic acid (vol/vol). The gradient program was from 0% B to 50% B over 120 min and 100% B for 10 min.

In Silico Analysis of the SOMP

The data obtained with the LTQ-velos system were used for protein identification with the Mascot search engine (version 2.1, Matrix Science, London, UK). MS/MS raw data were searched with a carbamidemethylation as fixed modification, and methionine oxidation, asparagine, and glutamine de-amidation as variable modifications. The tolerance of the precursor and fragment masses was set to 0.2 Da. Peptides identified with at least two distinct peptides were considered valid assignments. The corresponding peptide sequences were validated by manually inspection of the MS/MS spectra. The subject of Mascot searches was a pooled protein dataset obtained from the three cnidian genomes (BLASTP), with default parameters; 2) the coding genes from N. vectensis and H. magnipapillata (TBLASTX), with default parameters; 3) the predicted proteins of the three cnidian genomes (BLASTP), with and without SEG (i.e., low complexity filter on query sequence) (Wootton and Federhen 1993). The best hit for each SOMP (E value threshold <10^-4) was selected and the sequences globally aligned using Needleman–Wunsch algorithm for pairwise alignment (cutoff: 30% identity, expect for mosaic proteins) (Supplementary Table S3, Supplementary Material online). The best matches were also manually compared for their domain architecture. Second, the NC method (http://www.neighborhoodcorrelation.org/, last accessed December 12, 2012) (Sung et al. 2008) was implemented for homology identification between the three genomes (A. digitifera vs. N. vectensis and A. digitifera vs. H. magnipapillata). Multidomain proteins with high confidence NC coefficients (>0.8) were considered strictly homologs (Joseph and Durand 2009). Orthologs of SOMP in A. digitifera genome were selected and subsequently analyzed for the presence of the homologs in N. vectensis and H. magnipapillata. The SOMP and corresponding best matches in the three genomes that were not complete sequences but showed significant blast scores (E value <10^-4), were not assessed for their homology and just considered similar.

High-throughput proteomic data sets obtained from OMs occluded in CaCO3 structures were collected from the literature and their primary sequences analyzed with InterProScan: Strongylocentrotus purpuratus (tooth: Mann et al. 2008a; spicules: Mann et al. 2010; test and spine: Mann et al. 2008b), Gallus gallus (eggshell: Mann et al. 2006; Miksik et al. 2010), Lottia gigantea (shell: Marie et al. 2013), Crassostrea gigas (shell: Zhang et al. 2012), and Pinctada (shell: Mann et al. 2006b).
Marie et al. 2012). The complete set of signatures obtained in these proteomes (InterPro entries - domains and repeats) was compared with those present in the SOMPs.

**Supplementary Material**

Supplementary tables S1–S6 and figures S1–S8 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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**References**


to CO(2)-driven acidification during the initiation of calcification. 
Mol Ecol. 21:2440–2454.


