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Archimer
<http://archimer.ifremer.fr>The original publication is available at <http://www.springerlink.com>**Characterization of MRNP34, a novel methionine-rich nacre protein from the pearl oysters**Benjamin Marie^{a, b, 1, *}, Caroline Joubert^a, Corinne Belliard^a, Alexandre Tayale^a, Isabelle Zanella-Cléon^c, Frédéric Marin^b, Yannick Gueguen^{a, *} and Caroline Montagnani^{a, 2}^a Ifremer, LBQP, Centre Océanologique du Pacifique, Taravao, French Polynesia^b UMR 5561, CNRS, Biogéosciences, Université de Bourgogne, Dijon, France^c UMR 5086, CNRS, IFR 128 BioSciences, IBCP, Université de Lyon 1, Lyon, France¹ Present address : UMR 7245, CNRS, MCAM, Muséum National d'Histoire Naturelle, Paris, France² Present address : UMR 5119, CNRS, Ifremer, IRD, Ecologie des Systèmes Marins Côtiers, Université Montpellier II, Montpellier, France*: Corresponding authors : Benjamin Marie : email address : benjamin.marie@u-bourgogne.fr ; bijamari@yahoo.com ; Yannick Gueguen : email address : yannick.gueguen@ifremer.fr**Abstract:**

Nacre of the *Pinctada* pearl oyster shells is composed of 98% CaCO₃ and 2% organic matrix. The relationship between the organic matrix and the mechanism of nacre formation currently constitutes the main focus regarding the biomineralization process. In this study, we isolated a new nacre matrix protein in *P. margaritifera* and *P. maxima*, we called *Pmarg*- and *Pmax*-MRNP34 (methionine-rich nacre protein). MRNP34 is a secreted hydrophobic protein, which is remarkably rich in methionine, and which is specifically localised in mineralizing the epithelium cells of the mantle and in the nacre matrix. The structure of this protein is drastically different from those of the other nacre proteins already described. This unusual methionine-rich protein is a new member in the growing list of low complexity domain containing proteins that are associated with biocalcifications. These observations offer new insights to the molecular mechanisms of biomineralization.

Keywords: Matrix protein – Methionine-rich – Biomineralization – Mollusc – Nacre – Calcifying mantle**Abbreviations**

AA	Amino-acid
AIM	Acid-insoluble matrix
ASM	Acid-soluble matrix
EST	Expressed sequence tag
ISH	In situ hybridization
MRNP	Methionine-rich nacre protein
MS	Mass spectrometry
RLCD	Repeated low complexity domain

Introduction

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2 Nacre, also known as mother-of-pearl, is the most extensively studied non-human organo-mineral
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4 biomaterial because of its extremely high fracture toughness (Addadi et al., 2006). Nacre constitutes
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6 the lustrous inner layer of various mollusc shells, such as the pearl oyster from the genus *Pinctada*
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8 (Bivalvia: Pteriidae). Like other mollusc shell microstructures, nacre is built extracellularly under
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10 cellular control by the calcifying epithelium of the mantle. The nacreous layer exhibits a remarkably
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12 regular lamellar microstructure consisting of uniformly thick layers (0.5 μm) of aragonite crystals,
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14 which are densely packed with a small amount of proteins, glycoproteins and polysaccharides (for
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16 review see Addadi et al. 2006). These mantle-secreted biomacromolecules, which constitute the
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18 organic matrix, are believed to control different aspect of the mineral deposition (Lowenstam and
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20 Weiner 1989; Mann 2001). Although, this organic matrix represents only 1-5% of nacre weight
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22 according to the different species (Addadi et al. 2006), it displays key functions such as the local
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24 structuring of the 3-D space in an organic framework just prior to mineral deposition (Sudo et al.
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26 1997; Suzuki et al. 2009), the regulation of the CaCO_3 precipitation (Wheeler et al. 1981), the
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28 selection of the polymorph - aragonite and/or calcite (Falini et al. 1996), and the control of the mineral
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30 shape. Furthermore, at the atomic level, some of these matrix macromolecules exert an effect on the
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32 lattice parameters of CaCO_3 (Pokroy et al. 2006).
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38 Since the first description of the primary structure of a mollusc shell protein by Miyamoto et al.
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40 (1996), 5 and 10 full-length sequences of nacre proteins have been reported from bivalve and
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42 gastropod models, respectively (for a recent review see Marin et al. 2008). These proteins are
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44 multidomain and the domain consist of mucin-type (Marin et al. 2000), of carbonic anhydrase and
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46 GN-rich-type (Miyamoto et al. 1996), of C- and GS-rich-type (Shen et al. 1997), of G- and A-rich-
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48 type (Sudo et al. 1997; Marie et al. 2010a), of WAP or Kunitz-like protease inhibitor-types (Marie et
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50 al. 2010a), and finally, of von Willebrand A, chitin-binding and D-rich-type (Suzuki et al. 2009).
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52 However, although a number of shell proteins have recently been identified (Marin et al. 2008), many
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54 remain to be detected (Jackson et al. 2010; Joubert et al. 2010). Moreover, the relationship between the
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56 components of the organic matrix and the precise molecular mechanism of nacre formation is far from
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58 being understood and constitutes nowadays a hot topic. A deepened analysis of the mechanisms of
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biomineralization requires the identification, the characterization, and the functional analysis of key matrix molecules.

The aim of the present work is to investigate the structure of the nacre matrix of two pearl oyster species that belong to the genus *Pinctada*. Recent works (Jackson et al. 2010; Joubert et al. 2010) have obtained expressed sequence tags (ESTs) from nacre-forming tissues of the pearl oysters *Pinctada margaritifera* and *Pinctada maxima*, two closely related species (Cunha et al. 2011). These works have notably increased the dataset of known mantle-secreted proteins. Here, we have used proteomic methods combined with transcript and protein localization to evidence a new unidentified shell matrix protein of *P. margaritifera* and of *P. maxima*. These homologue forms of methionine-rich nacre proteins (MRNP), called respectively *Pmarg*-MRNP34 and *Pmax*-MRNP34, are novel 34-kDa proteins that are specifically localised in the mineralizing tissues and exclusively in the nacre matrix of these two species.

Materials and Methods

Materials

The adult *P. margaritifera* and *P. maxima* were collected from the Vairao lagoon (Tahiti, French Polynesia) and from the western coast of Australia, respectively. These two Pteriod bivalves exhibit a bilayered shell composed of an external prismatic calcitic layer, and of an aragonitic nacreous one. The two layers were separated mechanically. In addition, nacreous pearls were obtained from *P. margaritifera* after a classical 18-month grafting procedure of a nucleus and a graft of the mantle tissue in the pearl pocket (Cochennec-Laureau et al. 2010). Nucleus was carefully removed from the pearl prior to proteomic analysis.

Biochemical analysis

The acid-soluble matrix (ASM) and the acid-insoluble matrix (AIM) of the nacreous and the prismatic layers were extracted with cold acetic acid (5%) as previously described (Marie et al. 2007). Shell pieces were mechanically crushed and fragments were immersed in 1% (v/v) NaOCl for 24 h to remove superficial contaminants - as well as the periostracum, prior to matrix extraction. The nacre

1 AIM of *P. margaritifera* was denatured and partly solubilized in Leammli sample buffer before being
2 fractionated on a preparative SDS 12% polyacrylamide gel as described by Marin et al. (2001). Eluted
3 fractions containing the purified *Pmarg*-MRNP34 detected with a polyclonal antibody (Marin et al.
4 1994), then were tested by SDS-PAGE with CBB (Coomassie Brilliant Blue) staining, before being
5 pooled, thoroughly dialyzed against MilliQ water and freeze-dried.
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10 Prior to the proteomic analysis, the trypsin digestion of the matrices and of the purified MRNP34
11 samples was performed in solution or in gel (Marie et al. 2010b). Mass spectrometry was performed
12 using a Q-Star XL nanospray quadrupole / time-of-flight tandem mass spectrometer, nanospray-qQ-
13 TOF-MS/MS (Applied Biosystems, France), coupled to an online nano liquid chromatography system
14 (Ultimate Famos Switchos from Dionex, The Netherlands). One μL of samples were loaded onto a
15 trap column then separated at a flow rate of $0.300 \mu\text{L}\cdot\text{min}^{-1}$ with a linear gradient of 5 to 50%
16 acetonitrile in 0.1% formic acid over 120 min. MS data were acquired automatically using Analyst QS
17 1.1 software (Applied Biosystems). Following a MS survey scan over m/z 400–1600, MS/MS spectra
18 were sequentially and dynamically acquired for the three most intense peptide molecular ions over m/z
19 65-2000. Proteins identification was performed using the Paragon algorithm of the ProteinPilot™
20 software (Applied Biosystems, Foster city, CA, USA; version 2.0.1) against *P. margaritifera* and *P.*
21 *maxima* EST dataset obtained from the calcifying mantle cells (Jackson et al. 2010; Joubert et al.
22 2010). The different peptide hits for *Pmarg*-MRNP34 and *Pmax*-MRNP34 were manually confirmed
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44 *Sequence analysis*

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46 Protein sequence identification was attempted using BLASTp and tBLASTn analysis performed
47 against Swiss-Prot, GenBank's nrdb and dbEST using the online tool provided by UniProt
48 (www.uniprot.org) and NCBI (www.ncbi.nlm.nih.gov/blast.cgi). Signal peptides were predicted using
49 SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>), and conserved domains were investigated using
50 SMART (<http://smart.embl-heidelberg.de/>) and InterProScan
51 (<http://www.ebi.ac.uk/Tools/InterProScan/>). A hydrophobic plot was obtained with the Kyte and
52 Doolittle calculation (Kyte and Doolittle 1982) using ProtScale tool
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(<http://www.expasy.org/tools/protscale.html>). Following peptide signal removal, the theoretical mass and *pI* were determined with ProtParam tool (www.expasy.org/tools/protparam.html). The putative glycosylation and phosphorylation sites were investigated using DictyOGlyc, NetCGlyc, NetNGlyc, YinOYang and NetPhos tools from EXPASY online server (www.expasy.org/tools/). Putative cysteine bonds were predicted with Scratch Protein Predictor (www.expasy.org/tools/).

Localisation of the Pmarg-MRNP34 expression

Around 300 mg of each tissue (*i.e.* adductor muscle, hemocytes, gills, ungrafted and grafted pearl pocket, mantle distal and marginal zones, comprising all 3 mantle folds) were collected from six adult oysters *P. margaritifera* and total RNAs were extracted using the TRIzol[®] Reagent (Invitrogen). One µg of pooled total RNAs was reverse-transcribed using the iScript[™] cDNA synthesis Kit (Bio-Rad), and cDNAs were amplified using the iQ[™] Supermix kit (Bio-Rad) with the *Pmarg-MRNP34* specific primers (MRNP34_1_S GCGGTATGAGTCTATCCAGCC / MRNP34_1_AS TCCAAAGCCTCCCATCATAC) designed from the *Pmarg-MRNP34* ORF (188 bp amplicon), on a Mastercycler (Eppendorf). Specific *EF-1* primers (EF1_S ATGCTGCCATGGTTGATATG / EF1_AS GTGGCCTCAGCTTTCTCAAC) were used as a standardisation control. PCR products were analyzed on a 2% agarose gel electrophoresis.

The *in situ* hybridization was performed as previously described (Joubert et al. 2010). The digoxigenin-labelled sense or anti-sense probes were synthesized using two sets of *Pmarg-MRNP34* specific primers (MRNP34_1_S/AS and MRNP34_2_S GTATGATGGGAGGCTTTGGA / MRNP34_2_AS TTGTGCGTACAGCTGAGGAG). The sections of *P. margaritifera* mantle tissues were hybridized with a pool of the two probes. The detection steps were performed according to the manufacturer's instructions (Dig nucleic acid detection kit, Roche Molecular Biomedicals) and the slides were counter-stained with a solution of Bismark Brown Yellow and observed using a DM-4000B Leica microscope.

For quantitative RT-PCR analysis of *Pmarg-MRNP34* the forward and reverse primers used, were MRNP34_2_S and MRNP34_2_AS, respectively. A universal set of primers for the 18S rRNA gene sequence was used as a first reference gene, and was originally designed based on the alignment of

1 different bivalve species (Uni1304F TTAGTTGGTGGAGCGATTT / Uni1670R
2 TAGCGACGGGCGGTGTG) (Larsen et al. 2005). A second reference gene was used and chosen
3 based on its ubiquitous and constitutive expression pattern (REF1S AGCCTAGTGTGGGGGTTGG /
4 REF1AS ACAGCGATGTACCCATTTCC). First strand cDNA was synthesized using 300 ng total
5 RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche) and a mix of poly(dT) and random
6 hexamers primers. PCR amplifications were carried out with the Stratagene MX3000P in the presence
7 of SYBR-Green using 400 nM of each primer and 1 μ L of cDNA template. The following run protocol
8 was used: initial denaturation at 95°C for 10 min; 95°C for 30 s; 60°C for 1 min; 72°C for 30 s with a
9 two fluorescence measurements; melting curve program (45-95°C with a heating rate of 0.1°C.s⁻¹ and
10 a continuous fluorescence measurement). The copy ratio of each analyzed cDNA was determined from
11 3 pools of 10 different animals. All measures were done in duplicate. The relative expression ratio of
12 *Pmarg-MRNP94* was calculated based on the delta-delta method for comparing relative expression
13 results that is defined as: ratio = 2^{-[Δ Ct sample - Δ Ctcalibrator]} = 2^{- $\Delta\Delta$ Ct} (Pfaffl 2001). Here the “ Δ Ctcalibrator”
14 represent the mean of Δ Ct values obtained for all tissues for the target gene.
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33 *Immunolocalisation of Pmarg-MRNP34 protein*

34 In order to localize *Pmarg-MRNP34*, we used a polyclonal antibody developed in a previous study
35 (Marin et al. 1994). This antibody specifically recognized a protein fraction around 34-kDa in the
36 nacre AIM of *P. margaritifera* separated on SDS-PAGE. To assess the similarity between the
37 recognized 34-kDa fraction and the MRNP34 protein, this antibody was tested on Western-blot as
38 previously described (Marie et al. 2008), with the ASM and AIM of both the nacreous and prismatic
39 layers of *P. margaritifera* (1/2000 dilution). The fraction targeted by the antibody was excised from
40 the gel and analyzed further by proteomics.
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51 For immunolocalisation, deparaffinized 5 μ m sections of *P. margaritifera* mantle tissues, previously
52 fixed for 24 h in Davidson fixative, were permeabilized for 10 min in phosphate buffered saline (PBS,
53 pH 7.4)-0.5% triton X100. Tissues were then incubated for 1 h in saturating medium (1% BSA, PBS)
54 at RT before incubation with the anti-MRNP34 (1/100 dilution) for 1 h in PBS-BSA 1%-0.5 g.L⁻¹
55 Tween 20 at RT. After rinsing in saturating medium, samples were incubated for 2h at RT with a
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1 FITC-coupled secondary antibody (Sigma, F9887; goat anti-rabbit, 1/320 dilution). Finally, samples
2 were briefly stained with Evans blue dye (0.5 g.L⁻¹ in PBS), mounted in Vectashield® medium and
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4 observed with a DM-4000B Leica microscope. Control experiments were performed similarly without
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6 the first antibody step.
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8 9 10 **Results and Discussion**

11 *Identification of a novel methionine-rich nacre protein from Pinctada spp.*

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13 In order to investigate nacre proteins, we extracted the organic matrix from the different shell and
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15 pearl layers of *P. margaritifera* and *P. maxima*. The nacre or prisms AIMs, representing 90% of the
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17 whole matrices, were analyzed by mass spectrometry (MS) just after trypsin cleavage. The matching
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19 amino-acid (AA) sequences were then retrieved from the EST databases available for these organisms
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21 (Jackson et al. 2010; Joubert et al. 2010). Among the numerous protein identifications, we detected the
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23 presence of a novel putative protein that was called 34-kDa methionine-rich nacre protein (MRNP34),
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25 in the nacreous shell and pearl layers of both *P. margaritifera* and *P. maxima*, but not in prismatic
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27 layers (Fig. 1A).
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33 The analysis of the different protein fractions from the nacre AIM of *P. margaritifera*, obtained by 1-
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35 D SDS-PAGE separation or by preparative purification (Fig. 1B), indicates that *Pmarg*-MRNP34, was
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37 primarily detected in 34-kDa bands (Fig. 1A). Indeed, when 6 and 5 different peptides, corresponding
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39 to tryptic fragments of MRNP34, were observed in shell and pearl nacre AIM *P. margaritifera*, 8 and
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41 16 MRNP34 peptides were detected in the 34-kDa SDS-PAGE band and in the fraction enriched in
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43 MRNP34 purified by preparative electrophoresis (Fig. 1A-B). It is interesting to note that we also
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45 detected in the purified fraction of *Pmarg*-MRNP34 the presence of peptide fragments of other nacre
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47 proteins, e.g. *Pmarg*-MSI60, *Pmarg*-N66 and *Pmarg*-Pif177 likely corresponding to cleaved or
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49 degraded forms of the these proteins (data not shown). According to this result, the *Pmarg*-MRNP34
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51 enriched fraction obtained by purification using preparative electrophoresis appeared impure, hence
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53 not proper for further specific biochemical characterization using *in vitro* tests, like those generally
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55 performed with nacre purified proteins (Marie et al. 2008). Similarly, Lao and co-workers have
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57 observed that N66 protein fractions, purified by chromatography from *P. fucata* nacre matrix, were not
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1 pure and contain fragments of different proteins (Lao et al. 2007). These observations highlight the
2 difficulty in this model to extract pure protein fractions from direct separation of nacre matrix
3 components, whatever the purification technique used.
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8 *MRNP34 structural characterization* 9

10 The complete cDNA sequence of *Pmarg-MRNP34* (Genbank HQ625028) and *Pmax-MRNP34*
11 (GenBank EZ420111) were obtained by clustering of EST sequences from calcifying mantles as
12 previously described (Jackson et al. 2010; Joubert et al. 2010). The deduced amino acid sequences
13 revealed that these cDNAs encode 263 and 262 AA-long proteins, respectively (Fig. 2A). They both
14 exhibit signal peptides indicating that they are likely secreted proteins. Moreover, sequence alignment
15 indicates that *Pmarg-* and *Pmax-MRNP34* share 96% of AA sequence identity, suggesting that these
16 are conserved proteins of the nacre. Indeed, no homolog could be found, neither in other bivalve nor in
17 other organisms, using BLASTp and tBLASTn searches against UniProtKB, GenBank or nrdbEST.
18 This fact is not unique, since most known biomineralization proteins do not present known homologs.
19 Furthermore, about 62% of *P. margaritifera* and more than 70% of *P. maxima* ESTs sequences do not
20 present significant hits with the known protein database (Jackson et al. 2010; Joubert et al. 2010).
21 Taken together, this suggests - but not demonstrates - that MRNP34 may be a protein, which was
22 recently recruited among the Pteriidae bivalves, as homolog was not detected in some other large
23 genomic database of pteriomorph bivalve (Fleury et al. 2009).
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42 Following signal sequence removal, *Pmarg-* and *Pmax-MRNP34* are characterized by a theoretical *pI*
43 of 10, and a theoretical molecular weight around 26 kDa. In contrast, the proteomic analysis of SDS-
44 PAGE bands indicates that *Pmarg-MRNP34* migrates around 34 kDa (Fig. 1). This discrepancy
45 between the theoretical and the apparent molecular weight of *Pmarg-MRNP34* might be explained by
46 specific enrichment of the protein composition in some specific amino acid, as MG-repeats, that can
47 quantitatively influence the binding of SDS to the protein core, then its PAGE migration.
48 Alternatively, it may also traduce the putative presence of post-translational modifications in *Pmarg-*
49 MRNP34 mature protein, when it is secreted and integrated within the biomineral (Marie et al. 2007).
50 Numerous putative glycosylation and phosphorylation sites could indeed be found within the protein
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sequence, supporting that hypothesis (Fig. 2). Both *Pmarg*- and *Pmax*-MRNP34 protein sequences exhibit a noteworthy high amount of methionine (12%), together with an important amount of glycine (12%) and arginine (8%), but is depleted in acidic residues (2 and 3% of aspartic and glutamic acids, respectively). Mature MRNP34 proteins contain 8 conserved cysteine residues that potentially form four disulfide bridges between Cys₁₂₅/Cys₂₄₇, Cys₁₇₁/Cys₁₈₁, Cys₂₁₈/Cys₂₃₄, Cys₂₂₆/Cys₂₄₈, as analyzed for *Pmarg*-MRNP34 and Cys₁₂₄/Cys₁₇₀, Cys₁₈₀/Cys₂₄₆, Cys₂₁₇/Cys₂₃₃, and Cys₂₂₅/Cys₂₄₇ for *Pmax*-MRNP34 (Fig. 2A). The two methionine-rich domains present between 80-104 and 192-215 AA positions in *Pmarg*-MRNP34 are noteworthy. Although their function is unknown, they may be putatively involved in protein/protein interactions, by analogy with other proteins, like Calmodulin (CaM), which exhibits two hydrophobic methionine-rich domains that bind to the target protein (Siivari et al. 1995). Interestingly, the nacre matrix of the gastropod *Haliothis asinina* comprises also an uncharacterized 13-kDa methionine-rich protein, previously called ML6A10 (Marie et al. 2010a), but no reliable alignment can be performed with MRNP34. The different searches for known protein domain failed to detect any already described domain. Although previous works report the presence in different nacre-associated proteins of numerous repeated low complexity domains (RLCDs) (Jackson et al. 2010) - poly-Ala, poly-Asp, Poly-Ser, poly-Gly or Gly/Asn-repeats (Miyamoto et al. 1996; Shen et al. 1997; Sudo et al. 1997; Marin et al. 2008; Suzuki et al. 2009; Jackson et al. 2010; Joubert et al. 2010; Marie et al. 2010a) - the presence of such methionine-rich domain constitutes an entirely new feature of mollusc nacre matrix proteins that seems to be shared by different mollusc classes. Finally, a hydrophobic plot shows the overall hydrophobic character of *Pmarg*-MRNP34, except at four short internal regions (Fig. 2B) suggesting that this protein is predominantly hydrophobic and/or insoluble. Taken together, these results show that pearl oyster MRNP34 proteins share very close structures and further suggest that MRNP34 are insoluble proteins that interact with other matrix macromolecular components in the nacre organic framework.

The Pmarg-MRNP34 expression and translation is specific to nacre mineralising cells

To better understand the physiological role of *Pmarg*-MRNP34 in pearl oyster, the expression pattern of its transcript has been investigated. RT-PCR analyses led on total RNAs extracted from adult main

1 organs showed that *Pmarg-MRNP34* gene is primarily expressed in the truly calcifying tissues, *i.e.* the
2 mantle distal zone (mantle tissue excluding the marginal zone), the mantle margin (mantle folds) and
3 the grafted pearl pocket (Fig. 3A). No or weak expression was detected in other tissues (adductor
4 muscle, hemocytes, gills, and ungrafted pearl pocket). Additionally, our *in situ* analysis of the mantle
5 further revealed that the *Pmarg-MRNP34* transcript is specifically expressed in the mineralizing outer
6 epithelial cells of the mantle. ISH observations suggest that *Pmarg-MRNP34* is preferentially
7 expressed in the dorsal part of the mantle external epithelium (Fig. 3B), which is known to contain
8 mineralizing cells responsible of nacre formation (Joubert et al. 2010). Indeed, quantitative expression
9 analyses conducted on total RNAs extracted from two parts of the mantle (full mantle and mantle
10 margin) confirmed that *Pmarg-MRNP34* gene is primarily expressed in the nacre forming zone
11 beneath the calcifying external fold of the mantle (Fig. 3C). Those analyses also confirmed that the
12 weak transcript expression detected using RT-PCR in other tissues were negligible (adductor muscle,
13 hemocytes, gills). Furthermore, the protein localization revealed that *Pmarg-MRNP34* is exclusively
14 synthesized in the nacre secreting external epithelium of the mantle (Fig. 4A) and is detected in nacre
15 organic matrix only, and not in the prisms (Fig. 4B).

35 **Conclusion**

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37 Taken together, our results indicate that MRNP34 is secreted mineralization-associated protein, as it
38 transcript a nacre specific of the epithelial mantle calcifying cells. Furthermore, it is a nacre-specific
39 protein, as it is only produce in nacre forming cells, and present only in nacre. However, because of
40 the lack of sequence similarity with already characterized proteins, no assumption on its biological
41 function can be proposed, and the precise contribution of MRNP34 in nacre formation remains
42 undetermined. This study illustrates the efficiency of combine transcriptomic/proteomic approach for
43 identifying of novel shell proteins. The challenge that now faces the field of biomineralization is the
44 characterization of the functions of the growing list of novel biomineral associated proteins (Marin et
45 al. 2008; Jackson et al. 2010; Joubert et al. 2010; Marie et al. 2010a) that typically do not present
46 sequence homology with already known proteins. Our results also give new insight into calcium
47 carbonate biomineralization, by emphasizing the cellular control of the process. Plans to elucidate the
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1 physiology and the molecular mechanism of the nacreous formation by combining *in vivo* regulation
2 of protein expression (*e.g.* “knock-down” approach) and *in vitro* crystallization experiments using
3 recombinant protein and synthetic peptides are clearly the next step of our analysis.
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26 **Conflict of interest**

27 None declared
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Figure legends

Fig. 1. Detection of MRNP34 in nacre acid-insoluble matrix of *P. margaritifera* and *P. maxima*. A) Detection of MRNP34 by proteomics in the different matrices and the purified proteins from *P. margaritifera* and *P. maxima*. AIM fractions were analyzed on mass spectrometry after trypsin cleavage, and the resulting MS/MS spectra were compared *in silico* to amino-acid sequences derived from EST dataset from the mantle cells of *P. margaritifera* [14] and *P. maxima* [15] using ProteinPilot software. Total protein score indicates the sum of individual peptide scores. Respective numbers of matching peptides are indicated in the right column. B) 12 % SDS-PAGE stained with CBB of AIM shell nacre and purified MRNP34 from *P. margaritifera* nacre AIM. The molecular weight markers are indicated on the left. The arrow localizes the nearby 34-kDa band excised for MS analysis.

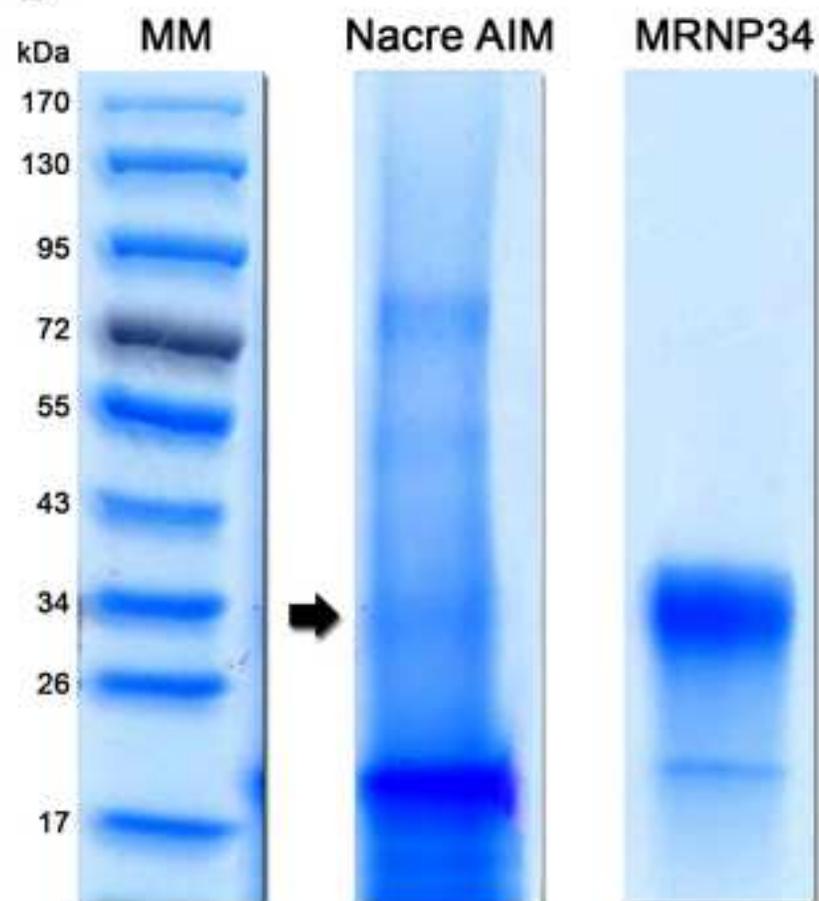
Fig. 2. Amino acid sequence analysis of MRNP34. A) Amino acid (AA) sequence alignment of *Pmarg*-MRNP34 and *Pmax*-MRNP34 (SwissProt numbers are P86872 and P86871, respectively). Conserved AA positions are indicated by (*). The putative signal peptides are underlined. The peptides observed by MS/MS are indicated in italic bold face. The two MG-rich domains are shaded in grey. The putative O-glycosylation sites are shaded in black and the putative phosphorylation sites are boxed. The eight cysteine residues potentially involved in disulfide bridges are indicated by “[”]. B) Hydrophobicity “Kyte and Doolittle” plot of *Pmarg*-MRNP34, performed with ProtScale tool (<http://www.expasy.org/tools/protscale.html>). Similar, if not identical, plot was obtained when using *Pmax*-MRNP34 (not shown).

1 Fig. 3. *Pmarg-MRNP34* transcript localisation in oyster tissues. A) RT-PCR analysis using RNA
2 extracted from adductor muscle (AM), gills (G), hemocytes (H), ungrafted pearl pocket (UPP), grafted
3 pearl pocket (GPP), mantle distal zone (MDZ), mantle margin (MM), transcribed and amplified with
4 *Pmarg-MRNP34* and *EF-1* (as control) specific primers. Negative controls were performed without
5 cDNA (-). Positive amplification control with MRNP34 cDNA was also performed (not shown). B)
6 Localization of *Pmarg-MRNP34* gene transcripts in *P. margaritifera* mantle tissue by *in situ*
7 hybridisation. Paraffin-embedded sections of oyster tissues were hybridised with antisense or sense
8 single stranded cDNA probes, which were labelled with digoxigenin and revealed using alkaline
9 phosphatase-conjugated antibodies. Positive cells are stained in dark blue, sense probes showed no
10 hybridisation (inset). Stained cells enlargements are shown in 6 insets. Results are representative of 4
11 independent experiments realized on 4 different tissues. if: inner fold; mf: middle fold; of: outer fold;
12 pg: periostracal groove; OE: outer epithelium; IE: inner epithelium; oec: outer epithelial cell; iec: inner
13 epithelial cell. C) Real-time PCR analysis of *Pmarg-MRNP34* transcript accumulation in different
14 oyster tissues (mantle external fold, full mantle, adductor muscle, gills, hemocytes). The values
15 represent relative expression ratios of 10 samples for 3 different pools of animals. All measures were
16 done in duplicate.

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38 Fig. 4. Immunolocalisation of *Pmarg-MRNP34* in the organic shell matrices and the calcifying mantle
39 of *P. margaritifera*. A) Immunolocalisation of *Pmarg-MRNP34* in mantle tissue with the MRNP34
40 primary antibodies reacting against MRNP34-containing band, using FITC-coupled secondary
41 antibodies. Images of the mantle outer face and details of outer epithelial zones are shown (1,2). A
42 mantle tested without primary Ab (no AbI control) showing no staining is shown (3, 4). mf: middle
43 fold; of: outer fold; pg: periostracal groove; OE: outer epithelium. Results are representative of 2
44 independent experiments performed on 2 different tissues. B) Western blot analysis of AIM and ASM
45 prisms (Pr) and nacre (N) shell extracts using the primary antibodies reacting against the MRNP34-
46 containing PAGE band. *Pmarg-MRNP34* is indicated by an arrow. The molecular weight markers are
47 indicated on the left.

Figure[Click here to download high resolution image](#)**A**

Organism	Total protein scores	Matching peptides
<i>Pinctada margaritifera</i>		
AIM of shell prisms	0	0
AIM of shell nacre	12	6
AIM of pearl nacre	10	5
34-kDa band	16	8
Purified MRNP34	29.9	16
<i>Pinctada maxima</i>		
AIM of shell prisms	0	0
AIM of shell nacre	6	3

B

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Pmarg-MRNP34 MSEMRRILCLAVVIFIINDVSSQGLGSNKNWKKSQMSLSSPGNKKPTGNNNAVPQKSKMN 60
Pmax-MRNP34  MSIMRRILCLAVVIFIINDVSSQGLGNNKNWKKNGMSLSSPGNKKPTG-NNAVPQKSKMN 59
  ** *****

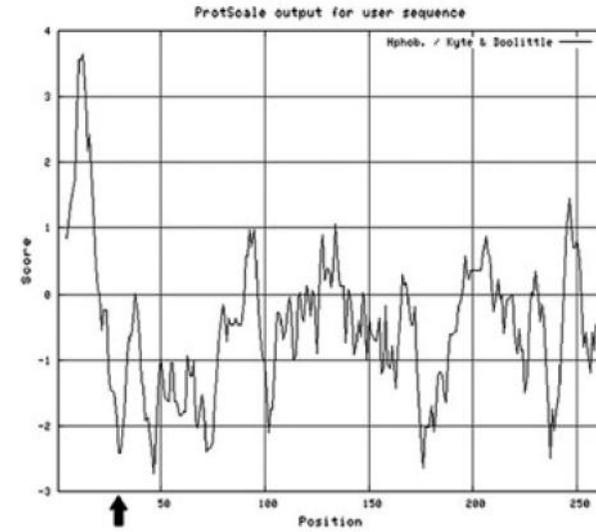
Pmarg-MRNP34 NVNQNSLSQPKRPSHPGNSMYFMGNQGPMMGGFGMGMNNKQMREFMLAKRTHGVSPFL 120
Pmax-MRNP34  NMNQNSLSQPKRSPPGNSMYNMANQGPMMGGFGMGMNNKQMREFMIARRTHGVSPFL 119
  * *****

Pmarg-MRNP34 KKKICHMAKVAPPVNGIMPSPQLYAQGFKIRRIQKWFSDLDWSEGVAMCHNKEMEHRG 180
Pmax-MRNP34  KKKICHMAKVAPPVNGQMPSPQLYAQGFKIRRIQKWFSDLDWSEGVAMCHNKEMEHRG 179
  *****

Pmarg-MRNP34 CEKTPSAKWGRMFPGMGGMGGMGGMGGMMGSRPMASCDVTKPNSCGNPALMKCSKYHKD 240
Pmax-MRNP34  CEKTPSAKWGRMFPGMGGMGGMGGMGGMMGGNRPMASCDVTKPNSCGNPALMKCSKYHKD 239
  *****

Pmarg-MRNP34 RFGMPVCCATSEMTANQLENMGF 263
Pmax-MRNP34  RFGMPVCCATSEMTANQLENMGF 262
  *****

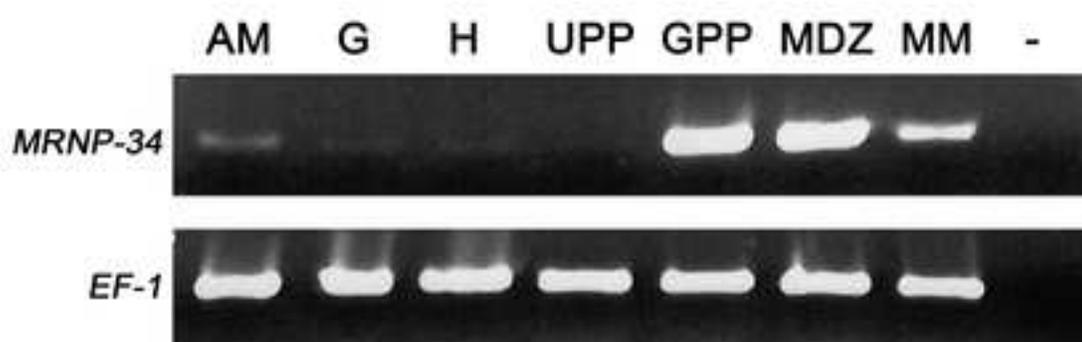
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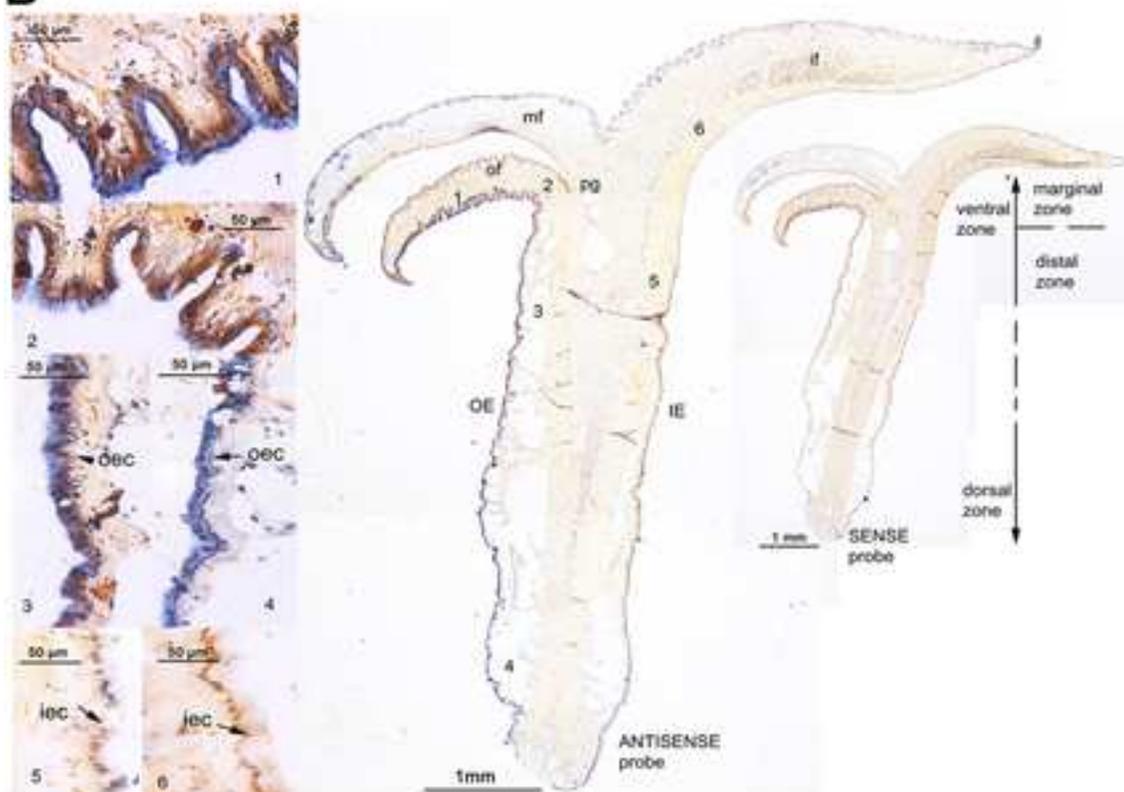
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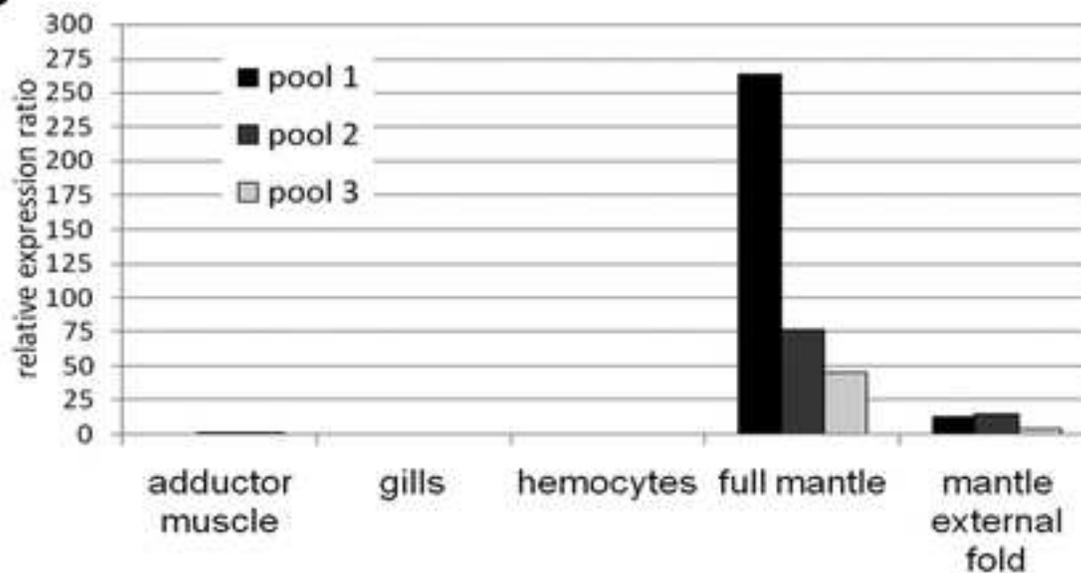


Figure
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