Marine Genomics xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Marine Genomics

Cells to Shells

Method paper

A shell regeneration assay to identify biomineralization candidate genes in mytilid mussels

Anne K. Hüning [a,b,1], Skadi M. Lange [b,1], Kirti Ramesh [b], Dorrit E. Jacob [c], Daniel J. Jackson [d], Ulrike Panknin [b], Magdalena A. Gutowska [b], Eva E.R. Philipp [e], Philip Rosenstiel [e], Magnus Lucassen [a], Frank Melzner [b,*]

[a] Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Bremerhaven, Germany
[b] GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany
[c] Macquarie University, Sydney, Australia
[d] Georg-August Universität, Göttingen, Germany
[e] Christian-Albrechts Universität, Kiel, Germany

ARTICLE INFO

Article history:
Received 7 December 2015
Received in revised form 28 March 2016
Accepted 30 March 2016
Available online xxxx

Keywords:
Biomineralization
Shell regeneration
Mytilus
Transcriptomics

ABSTRACT

Biomineralization processes in bivalve molluscs are still poorly understood. Here we provide an analysis of specifically expressed sequences from a mantle transcriptome of the blue mussel, *Mytilus edulis*. We then developed a novel, integrative shell injury assay to test, whether biomineralization candidate genes highly expressed in marginal and pallial mantle could be induced in central mantle tissue underlying the damaged shell areas. This experimental approach makes it possible to identify gene products that control the chemical micro-environment during calcification as well as organic matrix components. This is unlike existing methodological approaches that work retroactively to characterize calcification relevant molecules and are just able to examine organic matrix components that are present in completed shells. In our assay an orthogonal array of nine 1 mm holes was drilled into the left valve, and mussels were suspended in net cages for 20, 29 and 36 days to regenerate. Structural observations using stereo-microscopy, SEM and Raman spectroscopy revealed organic sheet synthesis (day 20) as the first step of shell-repair followed by the deposition of calcite crystals (days 20 and 29) and aragonite tablets (day 36). The regeneration period was characterized by time-dependent shifts in gene expression in left central mantle tissue underlying the injured shell, (i) increased expression of two tyrosinase isoforms (TYR3: 29-fold and TYR6: 5-fold) at day 20 with a decline thereafter, (ii) an increase in expression of a gene encoding a nacrein-like protein (max. 100-fold) on day 29. The expression of an acidic Asp-Ser-rich protein was enhanced during the entire regeneration process. This proof-of-principle study demonstrates that genes that are specifically expressed in pallial and marginal mantle tissue can be induced (4 out of 10 genes) in central mantle following experimental injury of the overlying shell. Our findings suggest that regeneration assays can be used systematically to better characterize gene products that are essential for distinct phases of the shell formation process, particularly those that are not incorporated into the organic shell matrix.

1. Introduction

Biomineralization is one of the most fascinating processes in the animal kingdom. Mineral skeletons made of silica and calcium carbonate ($CaCO_3$) can be traced back until the terminal Proterozoic (Knoll, 2003). Since the Proterozoic-Cambrian transition, the mollusc phylum experienced a broad evolutionary success that can partly be attributed to its ability of building protective shells, made of different $CaCO_3$ polymorphs. The mytilid shell consists of two $CaCO_3$ polymorphs with the inner shell layer (nacre, mother-of-pearl) being composed of tabloid aragonitic crystals, the outer layer of calcitic prisms (Uozumi and Suzuki, 1979; Marin et al., 2007). It is externally covered by the periostracum, a protective organic layer of varying thickness on the 1–100 μm scale (Harper, 1997). The tissue responsible for biomineralization is the mantle, situated underneath the shell. Three mantle zones have been identified to be protagonists in $CaCO_3$ polymorph formation: While the outer mantle (marginal and pallial zone) is involved in calcite and aragonite formation, the inner mantle (central zone) is responsible for formation of the aragonitic shell-layer, primarily during secondary growth in shell thickness (Owen et al., 1953; Kadar et al., 2009). The periostracum evolves from the inner epithelial cells of the outer mantle fold and serves as surface for the deposition of calcium carbonate. (Beedham, 1958; Beedham and Trueman, 1968). It is built from periostracin, a DOPA-containing precursor-protein (Waite et al., 1979) that undergoes a tanning process during which DOPA is oxidized by tyrosinases to quinones (Waite, 1983), a reaction followed

* Corresponding author at: Hohenbergstr. 2, D-24105 Kiel, Germany.
E-mail address: fmelzner@geomar.de (F. Melzner).
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.margen.2016.03.011
1874-7787/

by subsequent cross-linking of the quinones to nucleophilic groups (Waite and Andersen, 1980).

Shell-formation involves the secretion of an organic matrix framework comprising proteins and polysaccharides (e.g. silk-fibroin and ß-chitin) (Weiner and Traub, 1980; Lowenstam and Weiner, 1989; Levi-Kalisman et al., 2001, Addadi et al., 2006). Polymorph formation, size and shape depend on specific interactions with the framework of constituents (Lowenstam and Weiner, 1989; Addadi and Weiner, 1985; Levi-Kalisman et al., 2001; Zhang et al., 2006). *In vitro* studies confirmed that aspartic acid-rich shell matrix proteins specifically regulate and induce biomineralization (Addadi and Weiner, 1985; Gotliv et al., 2003). It has furthermore been demonstrated that aspartic acid-rich hydrophilic macromolecules, extracted from calcitic or aragonitic shell-layers of mytilid shells, respectively, determine the formation of $CaCO_3$ polymorphs *in vitro*, provided that ß-chitin and silk fibroin were present (Falini et al., 1996). The detection of an insoluble, acidic matrix protein (Pif), in the pearl oyster *Pinctada fucata* (Suzuki et al., 2009) and similar proteins in mytilid shells (Marie et al., 2012) altered the existing hypotheses for aragonite formation. Pif apparently contains a chitin-binding domain as well as an aragonite-binding domain and regulates the aragonitic crystal orientation during growth (Suzuki et al., 2009). This observation suggested a new model for aragonite formation, in which a hydrophobic protein complex, together with the protein-bound chitin, provides the core of a three-dimensional nucleation site for aragonite (Weiss, 2010). It is evident from these studies and the numerous specific gene transcripts with unknown function that have been identified in various mollusc mantle transcriptomes (e.g. Jackson et al., 2010; Clark et al., 2010; Bai et al., 2010) that a complex and species specific mixture of carbohydrates and proteins is interacting with the calcium carbonate moiety of the shell. For example, more than 100 proteins have recently been extracted from mytilid nacre, myostracum and prismatic layers, many of them layer-specific (Gao et al., 2015). It is a great challenge to identify and characterize those unknown transcripts that are essential for shell formation. In the absence of functional RNA knockdown/knockout techniques for molluscs to study gene function (however, see Suzuki et al., 2009 and recent successes using CRISPR/Cas techniques, Perry and Henry, 2015), we chose to use the ability of mytilid bivalves to regenerate fractured parts of the shell to identify genes related to specific phases of the biomineralization process.

Within a natural environment, the bivalve shell is continuously exposed to biotic and abiotic stressors that can lead to shell fracture or injury, e.g. during boring activity (Owada, 2009), human dredging and digging activities (Vasconcelos et al., 2011; Ambrose et al., 1998) or via iceberg scours (Harper et al., 2012). Mytilid mussels e.g. from the North Sea are often heavily infested by the shell boring spionid worm *Polydora ciliata* (Ambariyanto and Seed, 1991). To counteract shell injury, repair mechanisms have evolved to prevent the organism from subsequent vulnerability to predation and to defend the extracellular compartment against microbial, protozoan or metazoan intruders. Repair processes in bivalve molluscs have been studied in a number of species and common mechanisms have been identified in mytilid and other bivalves: analogous to the normal biomineralization process, a periostracum-like layer is formed to seal the damaged shell area from the outside medium, followed by calcite secretion onto the organic layer, and, finally, aragonite formation. Circumstantial evidence points at the involvement of the underlying epithelial cells in repairing the injured shell region: histological studies indicated strong changes in cell morphology (e.g. mitochondrial proliferation, modifications in cell size and microvilli abundance, cytoplasm appearance, calcium positive deposits) and histochemical studies demonstrated modifications in RNA and alkaline phosphatase abundance (Kawaguti and Ikemoto, 1962; Beedham, 1965; Uozumi and Suzuki, 1979, Kadar et al., 2009; Tsujii, 1976, Saleuddin, 1967). Thus, it appears that mantle regions that are normally not secreting specific shell constituents can be induced to do so. We therefore hypothesized that by inducing shell injury in central parts of the mytilid shell, gene expression patterns in the underlying central mantle tissue should be evoked that resemble characteristic expression patterns for the mantle margin region, the main area of biomineralization in young mytilid mussels. Up-regulation of biomineralization candidate genes in mantle tissue of bivalves following shell damage has been demonstrated in a range of bivalve species (Wang et al. (2013); Sleight et al. (2015)). However, these changes in gene expression have not been related to different shell repair stages.

In our pilot study, we thus combined a structural approach, using stereo microscopy, SEM and Raman spectroscopy to 'phenotype' the shell regenerate following induced injury of shell overlying the central mantle while studying gene expression patterns in the underlying tissue. We used quantitative real-time PCR to study the expression of a subset of candidate genes that were highly or specifically expressed in mantle margin/pallial regions during normal biomineralization, yet not or lower expressed in central mantle regions. Candidate genes were derived using transcriptomes of different mussel tissues (Philipp et al., 2012). Our hypotheses were that (i) as a response to shell damage, expression of candidate genes mainly expressed in outer mantle tissue could be induced in the central mantle parts (e.g. close to the site of damage), and that (ii) the phenotypic appearance of shells would reflect the underlying gene expression patterns through time.

2. Materials and methods

2.1. Animals and experimental design

Mytilus individuals with a mean shell length of 49 ± 2 mm were collected at three time points (04/07/11, 04/11/11 and 04/12/11; min. 15 mussels each) in Kiel Fjord (54°19.8′N, 10°9.0′E). Baltic mytilids are *Mytilus edulis* × *Mytilus trossulus* hybrids with an increasing fraction of *M. trossulus* alleles towards the eastern parts of the Baltic Sea. Kiel mytilids are genetically very similar to *M. edulis* (Stuckas et al., 2009). Nine holes were evenly drilled per animal into the center of the left valve (see Fig. 1) using a multifunctional drill (N 62/E, Proxxon, Niersbach, Germany) with a drilling head of 1 mm diameter. The site of drilling was centered directly above the central mantle tissue in an orthogonal 3 × 3 design ensuring that mantle tissue was not harmed during the procedure. Mussels were suspended in net cages (mesh width of 15 mm) in Kiel Fjord (54°19.8′N, 10°9.0′E) at depths of 2 m. Six animals were sampled each on days 20, 29 and 36. The net cages were checked three times a week, and fouling organisms were removed to enable maximum water flow and plankton supply to the experimental animals.

2.2. Water chemistry parameters

Salinity (WTW Cond 315i, WTW GmbH, Weilheim, Germany), surface water temperature (WTW Cond 315i, WTW GmbH, Weilheim, Germany) and pH_{NBS} (WTW pH 340i, WTW GmbH, Weilheim, Germany) were measured 1–5 times a week during the entire experimental duration at positions close to the net cages containing the experimental animals (see Fig. S1).

2.3. Structural shell analysis

Shell regeneration states were assessed by classifying newly deposited shells according to four repair stages (Table 1). At the first stage (stage I), no signs of repair were detectable. The following stages were characterized by initial formation of an organic sheet (stage II), enhancement of sheet rigidity and formation of scattered calcite crystals on the organic layer (stage III) and formation of calcite and aragonite crystals on the sheet (stage IV). For each stage and point of time, one photograph of a representative inner shell layer was chosen for the illustration of the shell regeneration progress. Detailed images of the

Please cite this article as: Hüning, A.K., et al., A shell regeneration assay to identify biomineralization candidate genes in mytilid mussels, Mar. Genomics (2016), http://dx.doi.org/10.1016/j.margen.2016.03.011