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Running title: *Bathymodiolus azoricus*'s PGRPs and regulation of the symbiosis

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Abstract

The relationship between the deep-sea mussel *Bathymodiolus azoricus* and its thiotrophic (SOX) and methanotrophic (MOX) symbionts has been ecologically and functionally well studied. Endosymbiosis is common in deep-sea hydrothermal vent fauna, yet little is known about the molecular mechanisms underlying the regulation of interactions between host and symbionts. In this study we focused on a group of pattern recognition receptors (PRR), called PGRPs that are able to recognize the peptidoglycan of bacterial cell wall. We first characterised the different PGRPs isoforms in *B. azoricus* gills and identified five paralogs. Among them two displayed a signal peptide. Then, specific probes designed for each paralog were used to perform real-time PCR quantification in gills of individuals showing various bacterial content as a result of *in situ* experimental procedures. Overall we found a decrease of PGRPs expression when symbionts amount decreases, suggesting an implication of PGRPs in the regulation of symbionts in *B. azoricus* gills. We therefore hypothesize that secreted proteins could act as cooperation signals to induce colonisation of symbiotic tissue while non-secreted proteins may regulate the density of endosymbionts within the gill tissue.

Keywords: Chemoautotrophic symbiosis, symbiont recognition, PRR, immune response, bacterial control

1. Introduction

Symbioses between invertebrates and bacteria are a common feature of chemosynthetic ecosystems (Tunnicliffe, 1991; Van Dover and Fry, 1994; Dubilier et al., 2008). In particular, around most deep-sea hydrothermal vents all over the world, bivalves with enlarged gills, a reduced digestive system and symbiotic sulphur-oxidizing and/or methane-oxidizing bacteria are dominant (Le Pennec et al.,

1990; Kyuno et al., 2009; Miyazaki et al., 2010). *Bathymodiolus azoricus*, a mussel species from the Mid-Atlantic Ridge found at vent sites south of the Azores, acquires horizontally and hosts both types of intracellular gamma-proteobacterial symbionts within its specialised gill cells. Methanotrophic symbionts (MOX) use methane and fix its carbon using energy derived from its oxidation whereas sulphur-oxidizing or thiotrophic symbionts (SOX) oxidize hydrogen sulphide to generate energy and fix inorganic carbon into organic carbohydrates (Cavanaugh et al., 1992; Distel et al., 1995; Fiala-Médioni et al., 2002; Duperron et al., 2006). These bacteria receive all metabolites necessary for chemoautotrophy from the surrounding seawater – a mix between deep-sea water and hydrothermal fluid – through the bivalve gills, which maintain a flux usually serving nutrition by filtration. This symbiosis is known to be plastic and highly dependent on environmental conditions (Colaço et al., 2002; Fiala-Médioni et al., 2002; Salerno et al., 2005; Duperron et al., 2006; Boutet et al., 2011). Indeed the lack of hydrogen sulphur or methane can lead one or the other type of symbiont to take precedence on the other. Halary and co-authors showed that a high concentration of hydrogen sulphur, in mussels kept in aquaria, induces a higher level of sulphur-oxidizing symbionts in *B. azoricus* gills (Halary et al., 2008). This flexibility is thought to be a host adaptation to cope with its heterogeneous and highly variable environment (Boutet et al., 2011). Despite the extensive research done on bathymodiolin symbiosis, the underlying molecular mechanisms that govern this symbiosis are largely unknown. Specifically, mechanisms involved in the interaction between host and symbiont at the cellular and/or molecular level have not been explored. To date, only a few studies have examined related question through microscopic investigations or gene expression (Won et al., 2003; Kádár et al., 2005; Boutet et al., 2011; Bettencourt et al., 2014; Barros et al., 2015). As in other symbioses, *B. azoricus* may regulate its symbiont population through apoptosis mechanisms or immunological responses. These mechanisms imply that the host should be able to recognize its symbionts, differentiate them from other bacteria (pathogenic or not) and influence their proliferation. To further our understanding of the molecular mechanism used by bathymodiolin

mussels to interact with their symbionts, we conducted *in situ* experiments and focused on a family of PRRs (Pattern Recognition Receptors): PeptidoGlycan Recognitions Proteins (PGRPs). PGRPs are present in most animals including insects, echinoderms, molluscs and vertebrates, but absent in some other metazoan such as nematodes (Dziarski and Gupta, 2006; Guan and Mariuzza, 2007). PGRPs are innate immunity molecules that contain PGN-binding domain (around 165 amino acids in length) which enable them to recognize and bind the peptidoglycan (PGN) of bacterial cell-wall (Yoshida et al., 1996). A minority of them, called catalytic-PGRPs, possess an amidase activity allowing them to hydrolyse the bond between MurNac and L-Ala in PGN (Gelius et al., 2003; Kim et al., 2003; Wang et al., 2003; Li et al., 2007). PGRPs have been well studied in insects in which they are grouped into two classes - short (S) and long (L) ones - and can be intracellular, extracellular or transmembrane proteins (Werner et al., 2000). These proteins are known to be involved in innate immunity in insects and more precisely in two NF-κB signalling pathways: Toll and Imd (Leulier et al., 2003). Indeed, in *Drosophila*, the secreted PGRP-SA and PGRP-SD recognize Lys-type PGN (Gram-positive bacteria), activate a protease that cleaves an extracellular cytokine-like protein (Spaetzle) which in turn activates the Toll pathway, whereas the transmembrane PGRP-LC recognize DAP-type PGN (Gram-negative bacteria or bacillus) and triggers the Imd pathway (Choe et al., 2002; Takehana et al., 2004; Kaneko et al., 2006). Both pathways lead to the production of anti-microbial peptides to fight against pathogenic bacteria. Finally, PGRPs with amidase activity (PGRP-SC and PGRP-LB) can modulate the immune response to bacterial infection by inhibiting the activation of the IMD pathway (Bischoff et al., 2006; Mellroth et al., 2003; Zaidman-Rémy et al., 2006). The key role of PGRPs in immunity and their high expression in gills of symbiotic bathymodiolin mussels such as *B. azoricus* or *B. platifrons* (Bettencourt et al., 2009, 2014; Martins et al., 2014; Wong et al., 2015) makes them interesting candidates for studying mechanisms underlying symbiosis. In this study we propose to look at the implication of PGRPs in the initiation, breakdown or maintenance of the association between *B.*

azoricus and SOX/MOX bacteria. To do so, we characterised PGRPs paralogs present in the gills of *B. azoricus*. Then, after the induction of a gradual loss of symbiont in mussels during *in situ* experiments, we analysed expression of the different PGRPs in gills of individuals with few or a very few symbionts and compared these expressions with those of individuals from natural population. Thereby, we observed that four of the PGRPs paralogs are down-expressed with the loss of symbionts suggesting a potential role of the corresponding protein in the maintenance of symbiotic relationship.

2. Materials and methods

2.1. Animal collection: field sampling, in situ experiments and quantification of symbionts

Deep-sea mussels *Bathymodiolus azoricus* were collected from one hydrothermal vent field (Lucky Strike) at the Montsegur site (37°17,286' N; 32°16,530' W; 1700 m depth) located on the Mid-Atlantic Ridge during the BioBaz and MoMARSAT cruises (July/August 2013). The sampling was done using the Remote Operating Vehicle (ROV), Victor 6000 on the Research Vessel "Pourquoi pas?". Around thirty mussels representing natural population were collected at the same time, and brought on board. Then gills were dissected, frozen in liquid nitrogen, and stored at -80°C until further analysis. This population correspond to the control of the *in situ* experiment that were previously described (Détrée et al., 2016).

Briefly, between 15 and 30 mussels were put in several wire cages and 5 of these cages were placed a dozen meters away from the diffuse fluid emission. This condition was called "basalt". Cages were brought back on board in three steps: after 6 days on basalt (BT1), 20 days (BT2) and 27 days (BT3). Moreover, after six days on basalt (BT1), two cages were translocated from basalt back to the mussel bed, at their original site (next to hydrothermal fluid) and brought back to the research vessel after respectively 14 days (called TR1.2) and 21 days (TR1.3) on mussel bed (Figure S1). Immediately

after collection, gills were dissected, frozen in liquid nitrogen and stored at -80°C until further analysis. Then the whole DNA was extracted and a relative quantification of symbiont was performed by qPCR according to Boutet and co-authors (Boutet et al., 2011). Results from Détrée and co-authors showed that MOX symbiont content was very low in mussels from natural population and almost no MOX bacteria were detected in experimental samples. Moreover, the quantification of SOX content revealed a decrease in SOX content in a time dependent way in individuals kept on basalt (BT1, BT2 and BT3), and maintenance of SOX population in gills of translocated individuals (either TR1.2 or TR1.3) (Détrée et al., 2016).

2.2. PGRPs characterisation: sequences, structure and phylogeny

All *B. azoricus* PGRP sequences used in this study have been obtained from previous existing transcriptome databases deposited in DNA Data Bank of Japan (DDBJ) Sequence Read Archive (DRA, http://trace.ddbj.nig.ac.jp/dra/) with accession number DRA 004082. The amino acid sequences of Ba-PGRPs were determined using the Expert Protein Analysis System (http://www.expasy.org/tanslate/). Multiple alignments were conducted with the ClustalW program (http://npsa-prabi.ibcp.fr/). Signal peptides were predicted by SignalP 4.1 server (http//www.cbs.dtu.dk/services/SignalP/). A phylogenetic tree of mollusc PGRPs, based on the alignment of 102 residues within the PGN domain, was constructed with Seaview 4.5.4 software using the neighbour-joining (BioNJ) method with 1000 bootstrap resampling of the data set. The three-dimensional models of Ba-PGRPs were constructed by comparative modelling with Modeller 9v13 program (Webb et al., 2006). The structures of the PGRP-LE, crystallized with the tracheal cytotoxin as substrate (pdb: 2CB3), isolated from *Drosophila melanogaster*, was used as template. One hundred models were generated for each PGRPs and their quality assessed using the Modeller Objective Function parameter. The reliability of modelled structures was validated by Ramachandran

plot analysis using PROCHECK (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html).

2.3. PGRPs expressions: RNA extraction and qPCR

The mRNA expression of PGRPs paralogs was analysed by real-time PCR. Total RNA was extracted from mussel gills by using Tri-Reagent (Sigma) according to the manufacturer's instructions. Both quantity and quality of RNA were assessed *via* UV absorbance (OD260/280/230) using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Delaware, USA). Two µg of total RNA were reverse transcripted using M-MLV reverse transcriptase (Promega), an anchor-oligo(dT) primer (5′-CGCTCTAGAACTAGTGGATCT-3′) and random hexamers (Promega). A volume of 2 µL of each diluted reverse transcription product (1:200) was subjected to real-time PCR in a final volume of 5 µL containing 40 nM of each specific primer and 2×Lightcycler®480 SYBR Green I Master mix (Roche Diagnostics, Mannheim Germany). The amplification was carried out as follows: initial enzyme activation at 95°C for 15 min, then 45 cycles at 95°C for 10 sec and 60°C for 30 sec. A dissociation curve was generated and PCR efficiency was estimated for each primer pair. All primer pairs tested generated a single peak in the dissociation curve and a PCR efficiency of 95 to 100%. Relative expression of each gene (fold change) was calculated according to the comparative Ct method using the formula: Ratio = $2^{-\Delta Ct}$ (with ΔCtgeneX = CtGeneX-CtRpL15) for the comparison of PGRPs expression in natural population, while the formula used for the *in situ* experiment was Ratio = $2^{-\Delta\Delta Ct}$ (with $\Delta Ct_{geneX} = Ct_{GeneX}\text{-}Ct_{RiboL15}$ and $\Delta\Delta Ct = (\Delta Ct_{geneX} - \text{mean}\Delta Ct_{geneX})$. The later formula was chosen to standardize the levels of gene expression to a mean of zero and consequently give the same weight to all response variables. A fragment of ribosomal protein L15 gene (RpL15) from the host was used as the internal PCR control (Table 1). We validated the RpL15 after having observed very low (less than 5%) variation of its expression in all samples (both experimental and field individuals) for gill tissue and three technical replicates were performed.

2.4. Statistical analysis

Data from quantitative real-time PCR experiments were analysed with a Kruskal-Wallis test GraphPad Prism, USA (www.graphpad.com) followed by a Dunn's Multicomparison Test Differences were considered statistically significant when $p \leq 0.05$.

3. Results

3.1. Characterisation and phylogenetic position of Ba-PGRPs

The analysis of the EST database of *Bathymodiolus azoricus* revealed the existence of five paralogs of PGRPs, referred to as Ba-PGRP 1 to 5. The five PGRPs are encoded with paralogous genes and contain from 191 to 435 residues (Figure S2, Table 2). Ba-PGRPs 2 to 5 are short (S) and contain one PGRP domain whereas Ba-PGRP 1 is a long one (L) with also only one PGRP domain in its C-terminal part. Ba-PGRP 3 and 5 possess a predicted signal peptide in their N-terminal part indicating that these two paralogs are possibly secreted into the extracellular space where they may recognize and bind PGN. Sequence analysis suggest that Ba-PGRP 2 and 5 recognize specifically DAP-type peptidoglycan residues responsible for this binding selectivity being conserved (Gly 89, Trp 90 and Arg 109 in *Drosophila melanogaster* PGRP LB) (Figure 1) (Lim et al., 2006; Swaminathan et al., 2006). For Ba-PGRP 1 and 3 the arginine and tryptophan residue are conserved and the third position is variable, while for the Ba-PGRP 4 only the arginine residue is conserved. This suggests that Ba-PGRP 1, 3 and 4 may recognize DAP-type PGN with lower affinity, or other PGN domains. In the case of the four short paralogs (Ba-PGRP 2 to 5), the amino acid residues involved in amidase activity (*N*-acetylmuramoyl-L-alanine amidase) are conserved: Zn^{2+}-binding site (His 59, Tyr 95, His 169 and Cys 177) and Thr175 (in Dm-PGRP LB) (Kim et al., 2003). In the long paralog, Ba-PGRP1, the cysteine residue (Cys 177 in Dm-PGRP LB) is replaced by a glycine. This kind of mutation was already observed in human, leading to a loss of the amidase activity (Michel et al., 2001; Wang et al.,

2003), but also in *Drosophila* leading to an abolishment of its ability to activate the Toll pathway (Wang et al., 2003). Sequence alignment suggests that the four short paralogs, Ba-PGRPs 2 to 5 contain one disulphide bridge (Figure 1). The structure of the complex between Ba-PGRP 2 and the tracheal cytotoxin (TCT) was predicted by homology modelling (Figure S3). Ba-PGRP 2 in the 3D model has three main α-helices and one central β-sheet composed of six β-strands and the TCT occupies the potential PGN-binding cleft. The phylogenetic tree of mollusc PGRPs show that the different Ba-PGRPs are clustered with those of other Mytilidae, *B. thermophilus, B. platifrons* as well as *M. galloprovincialis* in agreement with the classical taxonomy (Figure 2). Moreover, their distribution within five distinct clades support the fact that these PGRPs sequences are actual paralogs.

3.2. Relative expression of Ba-PGRPs in natural population and in situ experiments

The relative expression of the five Ba-PGRPs was measured in gill tissue by using qPCR in different conditions, corresponding to different symbiotic state (Table 3). First, the relative expression of each paralog in gills of individuals from natural population was investigated (Figure 3). Results showed that Ba-PGRP 3 and 5 have a significantly higher expression in gills than Ba-PGRP 1, 2 and 4. From this first analysis, it appears that Ba-PGRP 3 and 5 are the two paralogs that are particularly expressed in gills. Gills are directly exposed to the environment due to the water flux they entertain and, because of the main function of PGRPs it is possible that these two paralogs may be implicated in the response to environmental pathogens and/or in the regulation of symbiosis.

To go further we investigated the relative expression of the 5 paralogs in individuals translocated away from the venting area and maintained on basalt for 6, 20 and 27 days (*i.e.* conditions BT1, BT2 and BT3) and compared it to the relative expression in natural population individuals (Figure 4). Note that, mussels translocated to basalt have lost partially (BT1) or almost completely (BT2 and BT3) their symbionts (Table 3). Interestingly the 4 paralogs Ba-PGRP 1, 2, 3 and 4 have the same

expression pattern in BT1 and BT2. Indeed, their relative expression decreased significantly in BT2 (compared to natural population) *i.e* when mussels were facing a massive loss of symbiont. In BT3 the down-regulation of Ba-PGRP 2 and 3 remains significantly different from the natural population while in the case of Ba-PGRP 4 a slight up-regulated in BT3 was observed. The expression pattern of Ba-PGRP 5 is similar to that of Ba-PGRP 4, but not significantly different to the natural population. Thus, a massive lost of symbionts (BT2) seems to induce a significant down-regulation of the expression of four Ba-PGRPs paralogs.

The analysis of the relative expression of the five paralogs in individuals that were translocated away from venting area during 6 days, and then re-exposed to hydrothermal fluid in their original mussel bed (TR1.2 and TR1.3) (Figure 4) show no significant differences for 4 paralogs (Ba-PGRP 1, 2, 3, 5), while Ba-PGRP 4 is significantly down-regulated in TR1.2 and TR1.3 compared to natural population. Comparison between the control of translocation (*i.e.* BT1) and TR1.2 and TR1.3 show no significant effects of a return to mussel bed on paralogs expressions. However, the expression of Ba-PGRP 3 and 5 seems to increase after a return to mussel bed while the expression of Ba-PGRP 4 seems to diminish.

Discussion

Numerous studies have focused on the mechanisms of symbiosis from its physiology, to the nutrient exchange or to the specific molecular communication between host and symbionts. Indeed, the ability of the host to choose between symbiotic bacteria and other bacteria, especially in symbiosis with horizontal transmission, connote the presence of a very efficient system of recognition from both host and symbionts as was shown for the well-documented association between Rhizobium/leguminous plant (Cooper, 2007).

In this study, we investigated the putative role of a family of protein, PGRPs, in the symbiotic association between the deep-sea mussel *Bathymodiolus azoricus* and its symbionts. Paralogs of

PGRPs were identified from *B. azoricus* transcriptome and their expression was measured by qPCR in gills of individuals hosting different amount of symbionts. Thus, the expression of PGRPs in natural population individuals (average amount of 800 U) was compared with individuals that experimented a partial loss of symbionts (between 370 and 70 U), and individuals that experimented a partial loss of symbionts and were brought back to mussel bed (between 350 and 400 U) (Détrée et al., 2016).

PGRPs have been highlighted in several bivalves' species (*e.g.* mussels, scallops, oysters, razor clams) and proved to play a key role in the defence against pathogenic bacteria (Itoh and Takahashi, 2008; Martins et al., 2014; Ni et al., 2007; Wei et al., 2012). Grippingly, only three paralogs of PGRPs were reported in mytilids (*Mytilus galloprovinciallis*), among which two are predicted to be bound to the membrane and one to be secreted (Gerdol and Venier, 2015) while in a cold seep symbiotic mussel eleven transcripts matching PGRP sequence were identified. These last can be clustered in three groups according to their sequence which suggest the presence in this specie of three paralogs of PGRPs with at least one predicted to have a signal peptide (Wong et al., 2015). In our study, five paralogs of PGRPs in *Bathymodiolus azoricus*' gills were identified. Among these, no membrane-bound paralogs were identified but two secreted paralogs were reported (Ba-PGRP 3 and Ba-PGRP 5). A previous study on *Bathymodiolus azoricus* transcriptome highlighted only one paralogs of PGRP (Bettencourt et al., 2010) that corresponds to the Ba-PGRP 3 in the present one. The transcriptome used by Bettencourt's group in their studies have been obtained from gills while our data were obtained from gills, mantle and digestive gland. As, in our study, three paralogs (Ba-PGRP 1, 2 and 4) have a very low expression in gills of individuals from natural population, it is likely that their identification has been possible by the sequencing of three different tissues including gills. Nevertheless, the absence of membrane-bound PGRP in the five paralogs we identified suggest that the list of reported PGRP in this study might still not be completely exhaustive. However, the presence in *Bathymodiolus azoricus'* gills of two secreted proteins instead of one in other non-

symbiotic mussels may suggest that one of these proteins might be link to the presence of symbiont. Numerous studies, including one on *B. azoricus* focused on PGRP's expression after an exposure to pathogenic bacteria. Martins and co-authors focused on the response of *Mytilus galloprovinciallis* and *B. azoricus'* immune related receptors and effectors in response to the injection of different *Vibrio* strains. Interestingly while *M. galloprovinciallis'* PGRP is up-regulated 12 and 24 hours post-injection, very little effects are observed on *B. azoricus'* PGRP (Ba-PGRP 3) which basal expression in the control is similar to the one of *M. galloprovincialis*. Thus, on the contrary of *M. galloprovincialis*, the only PGRP paralog identified in *B. azoricus* was not responding to these pathogenic bacteria (Martins et al., 2014). The same research group performed another challenge of *B. azoricus* with, this time, *Vibrio parahaemolytics*, flavobacterium and seawater as control. Surprisingly, they observed an important up-regulation of PGRP (Ba-PGRP 3) only 24h after a seawater challenge (Bettencourt et al., 2014) while no response was observed with bacterial challenge, suggesting that the paralog reported was not involved in the defence against these bacteria but may be responding to the presence of others in seawater.

In our study, we analysed the expression of the five paralogs of PGRP in natural population and in individuals depleted of part of their symbionts. The high expression of the secreted paralogs Ba-PGRP 3 and 5 in natural population followed by their down-regulation in individuals that lost part of their symbionts raised our awareness and led to two main hypotheses. Due to the key role of PGRPs in the immune response of bivalves and invertebrates in general (Gerdol and Venier, 2015), we can suggest (*i*) that these two secreted paralogs are highly expressed in gills to defend the organism against pathogenic bacteria. As gills are one of the first barriers between the host and environment, the host to prevent an infection could produce Ba-PGRP 3 and 5. Upon infection, the amidase activity of these paralogs could weaken the bacteria cell wall having, in synergy with others innate immune response actors such as lysozymes, a putative bactericidal effect. As no membrane-bound PGRP were reported, the possibility of triggering an immune cascade is less likely. Thus, the loss of

symbionts, may lead to a concomitant loss of energy supply (as symbiont content is small after 21 and 27 days) that will induce a diminution of energy allocated to the defence against pathogens and may lead to the down-regulation of these PGRPs. However, PGRPs have been shown to be highly involved in symbiotic relationships. In several symbioses such as the association between the squid *Euprymna scolopes* and *Vibrio fischeri* or the cold seep mussel *Bathymodiolus platifrons* with its MOX bacteria, the expression of PGRPs have been shown to be higher in symbiotic tissue or bacteriocytes (Anselme et al., 2006; Nyholm et al., 2012; Wong et al., 2015). Some hypothesis followed these observations, with authors suggesting the putative role of PGRP in the acquisition and regulation of symbionts and proposed PGRP as a candidate to study immunomodulation. In *Bathymodiolus azoricus'* symbiosis few informations are available so far. Nevertheless, gill colonisation by endosymbiotic bacteria seems to be a continuous process throughout mussels lifetime (Wentrup et al., 2014), suggesting a necessity for the host to produce constantly a signal of cooperation to indicate the correct location for the colonisation of future symbionts as it has been suggest in the association between the weevil *Sitophilus zeamais* and its symbionts (Reynolds and Rolff, 2008). Therefore we may postulate that (*ii*) Ba-PGRP 3 and 5 would rather be used as a signal of cooperation in natural population to acquire constantly new symbionts. Thus, on bare basalt, where less free-living bacteria are observed the cooperation signal would be down regulated. This hypothesis is supported by the up-regulation of the two secreted paralogs when mussels were brought back to mussel bed. However as the down-regulation is significant after a loss of symbiont only for Ba-PGRP 3 we can suppose that only this paralogs is involve in this process. This hypothesis may seem more likely as there is a growing amount of evidence that mussels are able to digest there symbionts when the later are in excess, having as a consequence access to enough energy to produce an immune response (Détrée et al., 2016; Fiala-Médioni et al., 2002). More, symbiotic bacteria (SOX) in deep-sea mussels have been shown to be able to defend host against

pathogens (Sayavedra et al., 2015). Thus it seems more likely that when the amount of symbionts is low, the host would be able to take over and protect itself.

In the case of other PGRPs paralogs, their down-regulation correlated with the loss of symbionts can also suggest their implication in the regulation of symbiosis. Nonetheless their low expression in natural population may suggest that they would rather be involve in the defence against pathogenic bacteria. Thus, we suggest that the expression of these paralogs may be inducible upon infection but that the basal expression stay low not to interfere with symbiotic bacteria.

Concluding remarks

Overall our results show a high correlation between the loss of symbionts and the down-regulation of PGRPs paralogs in the gills of the deep-sea mussel *Bathymodiolus azoricus*. In such a specific and intimate association, the stability and homeostasis of the holobiont are thought to require a tight regulation through complex molecular mechanisms. An important speculation from this work is that PGRP might be involve in the regulation of symbionts through the constant acquisition of SOX and MOX bacteria. Although the correlation between the amount of symbiont and expression of PGRP is clear, their key role in immunity prevent us from assigning a specific role to these proteins in gills of *B. azoricus*. Future investigations should focus on a functional role of PGRPs in the deep-sea mussel by analysing the putative interaction between these five paralogs and microbe associated molecular pattern (MAMPs) to confirm or not our main speculation.

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Tables

Table 1. Sequence of primers used for real-time PCR amplification

Genes	Primers sequence 5'-3'
RpL15	For 5'-TATGGTAAACCTAAGACACAAGGAGT-3' Rev 5'-TGGAATGGATCAATCAAAATGATTTC-3'
Ba-PGRP 1	For 5'-GTCTTCATACATCATACTGCCATGAGTTA-3' Rev 5'-CCATTATAGAAACGCTACTGCAACGTC-3'
Ba-PGRP 2	For 5'-GCTGTGATGATGCATGTAGCAGCCCCATC-3' Rev 5'-CTTGTATGGCACTCTGCATCAGTACAACC-3'
Ba- PGRP 3	For 5'- ATGATATGGGCTATTCTTCCGCTCGTTGC-3' Rev 5'-TAATTGTAACACCGGAACAATCACCGGT-3'
Ba-PGRP 4	For 5'- GCCTTAAGTGTGACATCCACCCGACG-3' Rev 5'-AAGCATGCCGGTAGCCACTCTACA-3'
Ba-PGRP 5	For 5'- ACAGGGTCTCACACACCACAGCTGGGTGTCC-3' Rev 5'-GTACATCCACCCGACGCCCCATGATGGAT-3'

Table 2. Molecular properties of the five PGRPs paralogs of *Bathymodiolus azoricus.*

Name	Length	Signal peptide	MW (Da)*	Theoretical p*I**	Potential amidase activity
Ba-PGRP 1	431	No	49367	6.55	No
Ba-PGRP 2	195	No	22274.9	6.35	Yes
Ba-PGRP 3	235	1-17	25983.1/24158.7	9.89/9.89	Yes
Ba-PGRP 4	223	No	24795.4	6.66	Yes
Ba-PGRP 5	265	1-17	28555.9/26764.7	7.7/8.05	Yes

*Molecular weight and p*I* were determined with or without the signal peptide

Table 3: Relative quantification of SOX symbionts in the different *in situ* experimental conditions (from Détrée et al., 2016).

	Experimental conditions	SOX relative quantification (U)	Standard deviation
Natural population	/	805	400
BT1	6 days on basalt	374	175
BT2	20 days on basalt	79	32
BT3	27 days on basalt	73	18
TR1.2	6 days on basalt, brought back to mussel bed for 14 days	358	122
TR1.3	6 days on basalt, brought back to mussel bed for 21 days	407	125

Legends

Figure 1. Multiple alignment of the Ba-PGRPs domain.

The black and grey regions indicate positions where amino acid residues are conserved or highly similar, respectively. *: Residues implicated in the DAP-type PGN selectivity. ◆: Zn^{2+} binding site, ○: residues contributing to amidase activity, s: cysteine residues implicated in the disulphide bridge. Dm-PGRP LB: *Drosophila melanogaster* PGRP LB (access number Uniprot Q8INK6).

Figure 2. Phylogenetic analysis of PGRPs among molluscs

The distance tree was obtained by neighbor joining with 1000 bootstrap resampling of the data set (value below the branches, Seaview 4.5.4). The scale bare refers to 10% sequence variation. Red boxes indicate the *Bathymodiolus azoricus* paralogus PGRPs. The UniProt accession no. and the species are as follows : B2DEU5 (*Crassostrea gigas* PGRP-S2), B2DEU3 (*Crassostrea gigas*

PGRP-S1S), B2DEU6 (*Crassostrea gigas* PGRP-S3), K1RK66 (*Crassostrea gigas* PGRP-SC1), K1S629 (*Crassostrea gigas* PGRP-SC2), K1RGZ5 (*Crassostrea gigas* PGRP-1), X2KW21 (*Hyriopsis cumingii* PGRP-L), X2KPZ1 (*Hyriopsis cumingii* PGRP-S), A0A023I759 (*Hyriopsis cumingii* PGRP), T1YDG3 (*Hyriopsis cumingii* PGRP-S1), Q32S46 (*Euprymna scolopes* PGRP-1), Q32S45 (*Euprymna scolopes* PGRP-2), Q32S44 (*Euprymna scolopes* PGRP-3), Q32S43 (*Euprymna scolopes* PGRP-4), A0A089WX51 (*Euprymna scolopes* PGRP-5), A0A0C5PIX8 (*Mytilus galloprovincialis* PGRP-1), A0A0C5PT93 (*Mytilus galloprovincialis* PGRP-2), A0A0C5PTA0 (*Mytilus galloprovincialis* PGRP-3), CL8290, and CL2060, PGRPs from *Bathymodiolus platifrons* (Wong et al., 2016), Bt *Bathymodiolus thermophilus.*

Figure 3. Relative expression of Ba-PGRPs paralogs in *B. azoricus* gills in natural population. Bars shows the expression of each paralog compared with the reference gene RpL15 according to comparative Ct method using the formula: Relative expression= $2^{-\Delta Ct}$ (with $\Delta Ct_{geneX} = Ct_{GeneX} - Ct_{RiboL15}$) a: significantly different from Ba-PGRP 5 and b: significantly different from Ba-PGRP 3. ($p < 0.05$), (Kruskal-Wallis test, Dunns post test), n=10.

Figure 4. Relative expression of PGRPs paralogs in *B. azoricus* gills after symbiont loss and translocation. Relative expression level of mRNA (Ratio) is indicated according to comparative Ct method using the formula $2^{-\Delta\Delta Ct}$, with $\Delta Ct_{geneX} = Ct_{GeneX} - Ct_{RiboL15}$ and $\Delta\Delta Ct = (\Delta Ct_{geneX} - mean\Delta Ct_{geneX})$ and measured in natural population (n=10), BT1 (n=15), BT2 (n=19), BT3 (n=22), TR1.2 (n=10) and TR1.3 (n=11). * Significantly different from Nat. population, # significantly different from BT2, ($p<0.05$), (Kruskal-Wallis test, Dunns post test).

Legends to supplementary figures

Supplementary figure S1. Experimental design of *Bathymodiolus azoricus* sampling and in situ experiment at Lucky strike (-1700 m)

Supplementary figure S2. Amino acids sequences of Ba-PGRPs 1 to 5. Peptide signals are highlighted in grey

Supplementary figure S3. Ribbon representation of the 3D model of Ba-PGRP 2.

This model was obtained by homology modeling (Modeller 9v8, Template PGRP-LE from *Drosophila melanogaster*, cristallized with the tracheal cytotoxin as substrate pdb: 2CB3). Dots : tracheal cytotoxin**.**

Figure 1

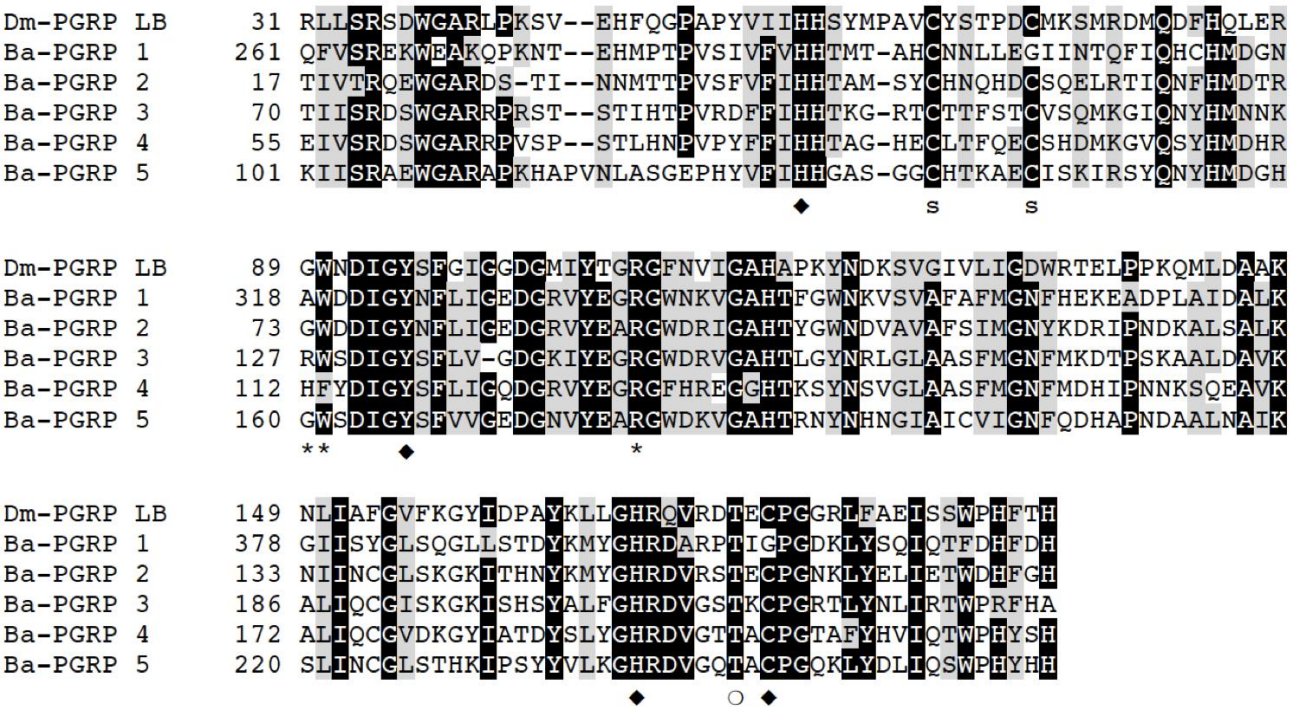

Figure 2

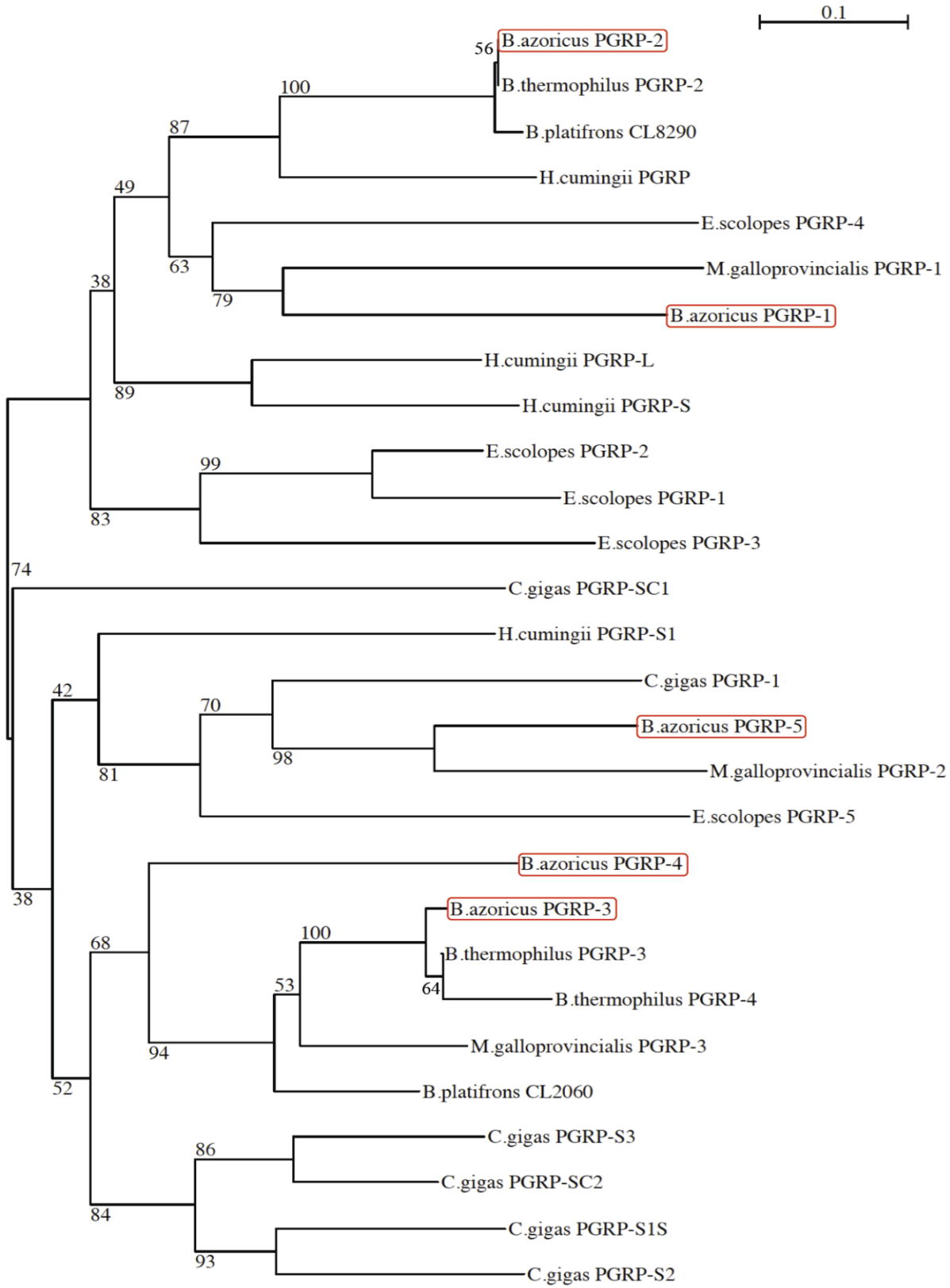

Figure 3

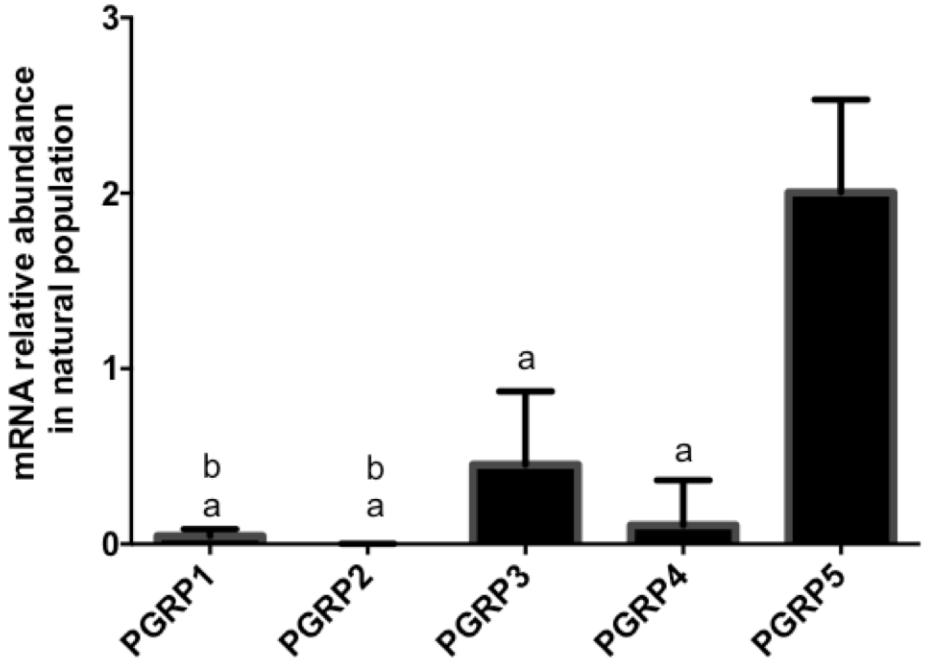

Figure 4