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Differential gene expression in the mussel *Bathymodiolus azoricus* from the Menez Gwen and Lucky Strike deep-sea hydrothermal vent sites

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Abstract

The deep-sea hydrothermal vent mussel *Bathymodiolus azoricus* is a symbiont bearing bivalve that is found in great abundance at the Menez Gwen and Lucky Strike vent sites and in close vicinity off the Azores region near the Mid-Atlantic Ridge (MAR). The distinct relationships that vent mussels have developed with their physical and chemical environments are likely reflected in global gene expression profiles providing thus a means to distinguish geographically distinct vent mussels on the basis of gene expression studies, fluorescence in situ hybridization (FISH) experiments and 16S rRNA amplicon sequencing, to assess the natural expression of bacterial genes and vent mussel immune genes and the constitutive distribution and relative abundance of endosymbiotic bacteria within gill tissues. Our results confirmed the presence of methanotroph-related endosymbionts in Menez Gwen vent mussels whereas Lucky Strike specimens seem to harbor a different bacterial morphotype when a methane monooxygenase gene specific probe was used. No qualitative differences could be visualized between Menez Gwen and Lucky Strike individuals when tested with sulfur-oxidizing-related nucleic-acid probe. Quantitative PCR (qPCR) studies revealed varied gene expression profiles in both Menez Gwen and Lucky Strike mussel gill tissues for the immune genes selected. Genes encoding transcription factors presented noticeably low levels of fold expression whether in MG or LS animals whereas the genes encoding effector molecules appeared to have higher levels expression in MG gill tissues. The peptidoglycan recognition molecule, encoding gene, PGRP presented the highest level of transcriptional activity among the genes analyzed in MG gill tissues, seconded by carcinolectin and thus denoting the relevance of immune recognition molecules in early stage of the immune responses onset. Genes regarded as encoding molecules involved in signaling pathways were consistently expressed in both MG and LS gill tissues. Remarkably, the immunity-related GTPase encoding gene demonstrated in LS samples, the highest level of expression among the signaling molecule encoding genes tested when expressions levels were compared between MG and LG animals.

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A differential expression analysis of bacterial genes between MG and LS indicated a clear expression signature in LS gill tissues. The bacterial community structure ensued from the 16S rRNA sequencing analyses pointed at a unpredicted conservation of endosymbiont bacterial loads between MG and LS samples.

Taken together, our results support the premise that *Bathymodiolus azoricus* exhibits different transcriptional statuses depending on which hydrothermal vent site it is collected from and within the same collection site while exhibiting differential levels of expression of genes corresponding to different immune functional categories.

The present study represents a first attempt to characterize gene expression signatures in hydrothermal vent animals issued from distinct deep-sea environmental sites based on immune and bacterial genes expressions.

1 Introduction

Deep-sea hydrothermal vent systems are considered extreme environments and yet animals dwelling around the vents form large communities depending almost exclusively on chemosynthesis (Childress and Fisher, 1992; Fisher et al., 1989). High concentrations of hydrogen sulphide and methane provide chemosynthetic microorganisms, known as primary producers, with the chemical elements required to produce organic compounds assuring thus a continuous food supply into the trophic web (Childress and Fisher, 1992; Fisher et al., 1989). The deep-sea vent mussel *Bathymodiolus azoricus* is the dominant species at Lucky Strike (LS) and Menez Gwen (MG) hydrothermal vent sites owing their high biomasses observed at these sites and remarkable adaptation to vent environments to the existence of symbiont chemosynthetic bacteria living in specialized gill epithelial cells. This light-independent survival at different hydrothermal vent sites has been referred as depending on mixotrophy by which metabolic needs are supplemented through the ingestion of particulate organic matter (Page et al., 1991) and/or assimilation of dissolved amino-acids (Lee et al., 1992) in addition to chemoautotrophy by virtue of endosymbiotic bacteria located within the

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gill bacteriocytes (Distel et al., 1995 and Fiala-Medioni et al., 2002). Given the different geophysical and chemical parameters that distinguish the Lucky Strike and Menez Gwen hydrothermal vent sites (Charlou et al., 2000), we surmised that animals living upon the vents would hypothetically be under direct influence of environmental factors that in turn may shape the overall animal's gene transcription activity. With the aim of identifying and performing a primary comparison of gene expression profiles in animals collected from both Lucky Strike and Menez Gwen sites, we have conducted several studies to characterize differences in the expression of genes selected on basis of sequence information obtained from our recent analysis of *B. azoricus* gill transcriptome (Bettencourt et al., 2010). Most genes were designated for their relevance in innate immunity and for corresponding to the immune function categories as defined in Bettencourt et al. (2010). In addition, bacterial genes ensued from our metatranscriptome characterization were also analyzed (Egas et al., 2012). Keeping in line with the assumption that geographically different vent mussels will adopt different physiological statuses in relation to their environmental settings, we also assumed that the relative abundance of methanotrophic and sulfide oxidizing endosymbiotic bacteria would differ between LS and MG mussels as previously reported by other researchers (Duperron et al., 2005, 2006).

In the present study, the specific gene expression levels for both bacterial genes and host-immune related genes, were compared between animals from the shallower Menez Gwen and the deeper Lucky Strike vent sites, to address the hypothesis that distinct hydrothermal vent sites can be experimentally differentiated on the basis of gene expression patterns and in the vent most emblematic species, the mussel *Bathymodiolus azoricus*. A taxonomical structure of the vent mussel gill's microbiome was also assessed in an attempt to characterize the bacterial community composition of Menez Gwen and Lucky Strike gill tissue samples.

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2 Material and methods

2.1 Animal collection

The present study was carried out with mussels collected from the hydrothermal vent fields Menez Gwen (37°50.8–37°51.6 N; 31°30–31°31.8 W) and Lucky Strike (37°15.32 N; 32°26.18 W) in the Mid-Atlantic Ridge (MAR) (Fig. 1) with the French R/V *Pourquoi pas?* using the ROV Victor 6000 (MoMARETO cruise (6 August–6 September 2006). Animals were immediately preserved either in formalin buffered (10 %) for histological preparations or frozen at −84 °C for subsequent gene expression analyses.

2.2 Total RNA extraction

Total RNA from freshly collected *B. azoricus* gills tissues was extracted with the RiboPure™ kit (Ambion®) according to manufacturer's instructions and re-suspended in nuclease-free, DEPC-treated water. Total RNA quality preparations were assessed by agarose gel electrophoresis using the standard formaldehyde denaturating system with MOPS buffer and determining the $A_{260/280}$ and $A_{260/230}$ spectrophotometric ratios using the NanoVue spectrophotometer (General Electric, Healthcare Life Sciences) for concentration and purity assessments. mRNA was further purified from total RNA using the MicroPoly(A) Purist™ kit (small scale mRNA purification kit, Ambion®) and subsequently used for ds cDNA synthesis.

2.3 Polymerase chain reaction

Based on transcriptome sequencing and analyses results (Bettencourt et al., 2010) and from cDNA library screening results, primers were designed using the Primer-Blast functionality from NCBI (http://www.ncbi.nlm.nih.gov/), specifying an expected PCR product of 200–300 bp and primer annealing temperatures between 56 °C and

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58 °C (Table 1). 25 µL PCR volume reactions were set with 1 µL of each forward and reverse primer (0.5 µM final concentration) and using a 2X PCR mix from PROMEGA. PCR conditions were according to Bettencourt et al. (2009). PCR products were examined by agarose gel electrophoresis using standard Tris-boric acid-EDTA buffer and ethidium bromide for DNA visualization.

2.4 Quantitative PCR (qPCR)

Quantitative PCR was used to assess and quantify the expression of genes selected on the basis of results obtained from cDNA library screenings and whole transcriptome sequencing of *Bathymodiolus azoricus* for detecting systematically putative genes involved in innate immunity (Bettencourt et al., 2010) . The cDNA from (MG) and (LS) samples was obtained as previously described, using 2 µg of total RNA extracted from gill tissues and the ThermoScript™ RT-PCR system (Invitrogen). mRNA was reverse-transcribed with the oligo (dT) according to manufacturer's instructions. Resulting cDNAs were used in Real-Time PCR reactions performed on the CFX96™ Real Time PCR System mounted onto the C1000 Thermal Cycling platform (Bio-Rad). Amplifications were carried out using 0.5 µL specific primers (10 µM) as in Table 1 and 2 and mixed to 10 µL of SsoFast™ Eva Green SuperMix (SYBR based system, Bio-Rad) and 50 ng of cDNA in a final volume of 20 µL. PCR cycling conditions were 95 °C for 3 min, followed by 35 cycles of 95 °C 10 s, 58 °C 15 s and 72 °C 30 s. 6 replicates were performed for each gene tested in real time PCR reactions. Melt curves profiles were analyzed for each gene tested. The 28S rRNA gene was used as the housekeeping gene and for normalization of expression of gene of interest or immune-related target genes. The comparative C_T method ($\Delta\Delta C_T$) for the relative quantification of gene expression was used for assessing the normalized expression value of immune-related genes using the 28S rRNA as the control transcript (CFX Manager™ Software, Bio-Rad). Data were transferred to Excel files and plotted as histograms of normalized fold expression of target genes.

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2.5 Fluorescence in situ-hybridization (FISH)

The presence of endosymbiont-related genes in *B. azoricus* was determined by FISH according to Bettencourt et al. (2008). Tissues sections were incubated with the hybridization solution containing specific single-stranded oligo-probes, consisting of the Alexa Fluo-488 $^{5'}$GCTCCGCCACTAAGCCTA$^{3'}$ and Alexa Fluo-532 $^{5'}$CGAAGGTCCTCCACTTTA^{3} fluorescent probes (Molecular Probes®, Invitrogen) to target respectively methanotrophic and thiotrophic bacterial symbionts in FISH experiments. Fluorescent oligo sequences corresponding to universal 8F Alexa Fluo-555 TCTCAAACTAGKACCGAGTC and 1492 Alexa Fluo-555 CGGYTACCTTGTTACGACTT bacterial 16S sequences were also used in FISH experiments to assess the natural distribution and relative abundance of bacteria in gill tissues. Control experiments were carried out with oligos corresponding to non-complementary sequences to the mRNA or to mitochondrial 16S rDNA. Gill filaments were visualized under fluorescent light and differential interference contrast (DIC) microscopy using a Leica DM6000 digital microscope (Leica Microsystems CMS GmbH, Germany).

2.6 Gills' microbiome structure

The bacterial community structure was characterized by deep sequencing of the 16S ribosomal RNA amplicon library of the V6 hypervariable regions of the rRNA by massive parallel 454 sequencing. 2 µg of total RNA, from MG and LS gill samples, were initially used to generate the transcriptome library, were reverse transcribed using the ThermoScript™ RT-PCR system (Invitrogen, CA, USA) following manufacturer's instructions. Using the cDNA as template, the V6 region of the 16S rRNA gene was amplified, using bar-coded fusion primers with the Roche-454 A or B Titanium sequencing adapters, a six-base barcode sequence and forward 5′-ATGCAACGCGAAGAACCT-3′, 5′-AATTGGABTCAACGCC-3′, 5′-GAGGWGGTGCATGGC-3′ and reverse 5′-TAGCGATTCCGACTTCA-3′ primers (Wang and Qian, 2009). Each primer pair

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generated amplicons of different sizes. Two replicate PCR were amplified from the same sample for each primer set, quantified by fluorimetry with PicoGreen (Invitrogen, CA, USA), pooled at equimolar concentrations and sequenced in the A direction with GS 454 FLX Titanium chemistry, according to manufacturer's instructions (Roche, 454 Life Sciences, Brandford, CT, USA). The raw data from 454 pyrosequencing were processed as described in Egas et al. (2012). The high quality sequences were clustered together by uclust v2.1 (Edgar et al., 2010) with a similarity of 97 %. The clustered sequences were then assembled by Cap3 (Huang et al., 1999) to produce OTU (Operational Taxonomic Units). The OTU were searched by NCBI BLAST against RDP, release 10 update 24 (Ribosomal Database Project) with a cut-off of $1e^{-50}$ to identify the taxa (16S RNA sequences of closely related microorganisms) as in Egas et al. (2012).

3 Results

The expression of immune and stress-related genes in Menez Gwen and Lucky Strike vent mussels was investigated by ways of quantitative PCR (qPCR) after preliminary validation, by standard PCR, of candidate genes whose cDNA sequences were obtained through cDNA library screens and on-going transcriptome analyses in our laboratory. Validation also took into consideration the inducibility of immune genes upon bacterial challenge using the marine bacteria *Vibrio parahaemolyticus* which for most candidates genes induced an up-regulation (data not shown).

qPCR was carried out using the 28S rRNA as the housekeeping gene and as control gene in all the normalized gene expression analyses. The results indicated variable expression levels within and between the two sets of MG and LS gill samples analysed and for the different genes tested corresponding to the immune recognition, signalling, transcription and effector functional gene categories as defined in Bettencourt et al. (2010). Quantifiable differences were observed among genes included within the signalling category for LS animals such as the immune-related GTPase, LPS-induced

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TNF and Toll-like receptor whereas gene expression in MG gills was quantifiably lower within the same category except for Myd88, a common adaptor protein in the downstream signaling pathways of mammalian and *Drosophila* Toll-Like Receptors (TLRs), which presented a higher expression level in MG animals. However, for some of the genes tested, specific patterns of expression were recorded both for MG and LS exclusively. For instance, the pattern recognition molecule, PGRP was highly induced in MG animals as well as the carbohydrate-binding lectin, carcinolectin in comparison to other immune recognition genes whether in MG or LS animals. On the other hand, effector genes such as Big cytolysin Defensin, metallothionein, matrix metalloproteinase and macroglobulin thioester revealed higher expression levels in Menez Gwen animals as compared to LS specimens (Fig. 3). In addition, qPCR was carried out using primers aimed at targeting bacterial genes ensued from our metatranscriptome analyses (Egas et al., 2012) and thus bacterial gene were also compared between MG and LS gill samples aiming at the detection of differentially expressed genes between animals collected from distinct hydrothermal vent sites. Results indicated a clear expression signature in LS samples whose bacterial genes were mostly at a higher level of expression than MG gill samples (Fig. 4). Out of the 14 genes tested, only the β-glucuronidase and xylose isomerase genes revealed higher level of expression in MG animals whereas the remaining genes exhibited higher expression levels in LS animals (Fig. 4).

Vent mussels from the two MG and LS hydrothermal vent fields were subjected to a comparison study in which the distribution and prevalence of endosymbiont bacteria, immediately after animal deep-sea retrieval, were assessed. Both methanotrophic and sulphur-oxidizing bacteria were specifically detected in FISH experiments utilizing gill sections from MG mussels whereas LS mussels revealed a different pattern of endosymbiont distribution with the same fluorescent probes (Fig. 2). The characteristically enclosed methanotrophic bacteria within epithelial gill bacteriocytes was not evidenced in gill sections from LS animals. The occurrence of methanotrophic-like endosymbiont bacteria assumed a more random distribution and a lesser fluorescence

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intensity across epithelial tissue in LS animals as compared to MG animals. To confirm that the signal obtained in gill tissues from LS animals was indeed of bacterial origin, the 8F and 1492R-based 16S rRNA fluorescent probes were used in FISH experiments. Expectedly, the fluorescence signal was confined to bacteria and coincides to that of methanotrophic bacteria in MG and LS animals as detected with the respective methanotrophic targeting fluorescent probe (Fig. 2).

Results from the ribosomal RNA amplicon sequencing of the V6 hypervariable regions, by massive parallel 454 pyrosequencing, indicated that the percentage of sequences obtained were from endosymbiont bacteria at nearly the same proportion between MG and LS samples. Moreover, comparative analyses based

on BLAST searches in the RDP database, using the 16S rRNA OTU sequences, revealed that the thiotrophic endosymbiont represented 90 % of all the sequences and methanotrophic endosymbiont almost 5 % of the sequences from vent mussel samples originated from the distinct Menez Gwen and Lucky Strike hydrothermal vent fields (Fig. 5).

4 Discussion

The hydrothermal vent mussel *Bathymodiolus azoricus* is commonly found in dense populations at the Mid Atlantic Ridge and appears to be well adapted to its atypical environment. Its functional adaptability to extreme environments (Gustafson et al., 1998; Von Cosel et al., 1999) is explained, to a great extent, by the presence of thiotrophic and methanotrophic bacterial symbionts harbored in specialized gill epithelial cells providing thus a substantial nutritional advantage to the mussel and enhancing its aptitude to oxidize sulfide and methane at the vent sites (Fiala-Médioni et al., 2002; Charlou et al., 2000; Duperron et al., 2006). Moreover, the existence of symbioses between the dominant fauna and bacteria was proven to be the key adaptation explaining the high biomasses observed at deep-sea hydrothermal vent sites (Duperron et al., 2009).

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A qualitative comparison of gill sections from *B. azoricus* individuals collected from two geographically different hydrothermal vents revealed a distinct distribution and prevalence of methanotroph-like symbiont bacteria in all the tissue sections analyzed as illustrated in FISH representative experiments in Fig. 2. While a specific methane monooxygenase (MMO) gene-based fluorescent probe detected methanotrophic-related symbiont bacteria within the bacteriocytes in Menez Gwen animals, a mixture of 16S-based fluorescent probe revealed the same pattern of bacterial distribution and bacterial size, presumably within the bacteriocytes in the same gill tissues and targeting the same methanotrophic bacteria. However, Lucky Strike animals revealed a different pattern of bacterial distribution when the MMO fluorescent probe was used. Distribution and relative bacterial abundance coincided with that of 16S-based probes in LS animals revealing an expression towards a more basal localization within gill tissues. These results suggest that two different bacterial morphotypes were detected by the same MMO probe whether MG or LS gill tissue sections were used. It is possible that chemosynthetic, carbon-fixing bacterial symbionts located in LS gill tissue are not methanotrophic but rather methylotrophic, while the MMO probe was used, given the high degree of nucleic-acid conservation that MMO genes may share between methanotrophic and methylotrophic bacteria. This is further corroborated by our latest results on the transcriptome and metatranscriptome analyzes of gill tissues from LS vent mussels, bringing evidence for the presence of methylotrophy-related gene encoding enzymes such as the methanol dehydrogenase (MeDH) and the ribulose-1,5-biphosphate carboxylase (RuBisCO) (Bettencourt et al., 2010; Egas et al., 2012). As expected, thiotrophic bacteria were detected in both LS and MG animals with a sulphur oxidation specific fluorescent probe, to the extent of that 16S rRNA based probe in FISH experiments.

Vent fields in the Mid-Atlantic Region (MAR) exhibit varied environmental conditions, resulting from depth variation of the water column and associated physical and chemical parameters and different source or bed rocks (Desbruyères et al., 2000). For this reason, factors affecting the composition of biological vent communities in the MAR

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are likely to determine the physiological status of vent animals and the overall transcriptional activity of genes involved in adaptation processes to extreme environments. This opens the possibility of finding gene expression signatures between animals living at distinct hydrothermal vent conditions. Hence, we looked into the expression of genes selected on the basis of their putative functional involvement in immune and/or stress-related reactions including recognition; signalling; transcription and effector functional categories (Bettencourt et al., 2010). Results indicated that there was no clear overall difference in gene expression among all the immune genes tested regardless of the origin of vent mussels. This made it difficult to attribute immune gene expression traits to a particular hydrothermal vent site, even though, a few immune genes whose expression were markedly higher in MG than in LS gill tissue samples could be evidenced as this was the case for peptidoglycan recognition protein (PGRP) and carcinolectin, MyD88, Iκ-B and the signal transducer and activator of transcription STAT SH2, Big Defensin, Metallothionein, Matrix Metalloproteinase and cytolysin. Furthermore, PGRP, carcinolectin, MyD88 and Iκ-B are distinctive innate immunity molecules present in several animal phyla and frequently upregulated in response to peptidoglycan (PGN) from bacteria or other stimulants which in turn activate the Toll-like receptors both in Drosophila and mammals, leading to the production of antimicrobial compounds (Dziarski and Gupta, 2006; Swaminathan et al., 2006).

There are, nevertheless, noteworthy expressions of genes whose fold expression remained higher in LS in comparison to MG animals. That is the case for immune-related GTPase, Toll-like receptor, LPS-induced Tumor Necrosis Factor (TNF), the transcription factors AP1, JUN and p43 and ferritin, lysozyme and HSP70 genes whose expression in LS gill tissues are quantitatively higher than MG animals. These differences observed in gene expression levels during qPCR analyses point to differences of mRNA levels for the genes referred in this study at the time animals were collected and their genetic material preserved. The explanation for this apparent divergence remains a biological challenge that cannot be fully comprehended under the scope of this study. The immunity-related GTPases (IRGs) are a family of proteins induced by interferon-γ

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that play a crucial role in innate resistance to intracellular pathogens (Traver et al., 2011). Based on their putative role during signalling and transcription processes, the elevated expression of the immune-related GTPase, a family member of Interferon-induced proteins that are required for resistance to intracellular bacteria and protozoa, the Toll-like receptor and LPS-induced-Tumor Necrosis Factor (TNF) genes, could indicated that the LS animals are undergoing high cellular activity including innate resistance to intracellular pathogens. LPS is a major integral structural component of the outer membrane of Gram-negative bacteria, and one of the most potent initiators of inflammation known. LPS activates monocytes and macrophages to produce cytokines as for the inflammatory cytokines such as TNF, IL-1, and IL-6 that, in turn, serve as endogenous inflammatory mediators (Tang et al., 2006). Such effectors confer on the host cells the ability to more easily eradicate invading pathogens through diverse mechanisms. Based on our comparative gene expression results evidence seem to suggest that LS animals are more likely to express genes involved in innate immunity against intracellular microorganisms whereas MG animals seem to display increased expression levels of genes involved in the "classical "bacterial recognition and Toll signaling pathway. Interestingly, cross-talk between signaling pathways may occur in *Bathymodiolus azoricus* individuals originated from distinct hydrothermal vent sites. That is, same immune or pro-inflammatory signaling molecules may serve different signaling pathways whether they are more conspicuous in MG or LS animals. The pleiotropic activities of TNF represent an interesting example of how a cytokine involved in systemic inflammation is also involved in promoting apoptosis of certain tumor cells, while keeping its primary role in the regulation of immune cells (Chen and Goeddel, 2002). The "effector" immune genes, Thioester containing α-macroglobulin and Matrix metalloproteinase (MMP) revealed higher expression in MG gill tissues. The former is an evolutionarily conserved element of the innate immune system in invertebrates whose best-characterized function is the clearance of active proteases from the tissue fluids by ways of its association with α-macroglobulin receptor to form a protein complex found in the blood and involved in the inhibition of proteinases (Armstrong

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and Quigley, 1996). The latter is involved in tissue remodeling associated with various physiological and pathological processes such as morphogenesis, angiogenesis, tissue repair, arthritis, and metastasis. MMPs are transcriptionally regulated by growth factors, hormones, cytokines, and cellular transformation. The proteolytic activities of MMPs are precisely controlled during activation from their precursors and inhibition by endogenous inhibitors, α-macroglobulins, and tissue inhibitors of metalloproteinases (TIMPs) (Nagase and Woessner, 1999). In addition, recent findings indicate that matrix metalloproteinases act on pro-inflammatory cytokines, chemokines and other proteins to regulate varied aspects of inflammation and immunity (Parks et al., 2004). Taken together, these results suggest that gill tissues, from which this comparative mRNA expression study was based upon, are likely under greater cellular transformation and tissue reorganization in Menez Gwen animals than Lucky Strike individuals.

The existence of immune and stress-related genes was validated by the evidence gathered using qPCR to assess gene expression in MG and LS animals, and thus corroborate our previous reports on the expression of immune genes in the deep sea hydrothermal vent mussel, *Bathymodiolus azoricus* (Bettencourt et al., 2010) It also points to the possibility of using specific immune or stress-related genes to characterize transcriptional statuses in deep sea hydrothermal vent animals particularly when aiming at *Bathymodiolus* mussel's acclimatization studies in land based aquaria systems. However, no clear immune gene expression signature was able to be depicted from our study given the variability of expression observed within and between the different functional immune gene categories for both MG and LS samples (Fig. 3).

In sharp contrast with host-mussel genes, bacterial gene expression analyses clearly indicated a greater overall gene expression prominence in LS gill tissues when compared to MG samples. Out of 14 bacterial genes analyzed only xylose isomerase and β-glucuronidase showed higher levels of expression than LS samples as demonstrated by qPCR (Fig. 4). The increased levels of bacterial gene expression in LS gill samples could indicate a higher load of bacteria in gill tissues or an increased transcriptional activity of the genes tested, from a relatively constant amount of bacteria associated

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to the gills. Whether the amount of bacteria or its transcriptional activity that resulted in higher gene expression quantifications in qPCR experiments was under geochemical and hydrostatic pressure conditions from the environment is still under investigation. To help understanding the dynamic view of the microbial community in MG and LS gill tissues given the environmental conditions in which vent mussels are found or from the sample collection sites, we conducted an analysis of the bacterial 16S ribosomal RNA by means of amplicon pyrosequencing from PCR amplification of the V6 hypervariable region with three different primer pairs to maximize the diversity coverage. A comparative analysis at the sequence level unravelled a surprising conservation of percentage of sequences from thiotrophic and methanotrophic endosymbionts between MG and LS samples, the thiotrophic sequences representing nearly 90 % of all the sequences analysed (Fig. 5)

Taken together, attempts at finding gene expression signatures in vent mussels living in such distinct hydrothermal vent systems proved inconclusive when considering vent mussel (host) immune genes to look at differences in gene expression level whereas bacterial genes clearly revealed a signature expression in Lucky Strike samples. Whether this gene expression signature corresponds to a bacterial fingerprint in Lucky Strike animals could not be fully elucidated at this point. The characterization of the gill bacterial community structure by massive parallel pyrosequencing of the 16S ribosomal RNA amplicon library indicated that the natural distribution of both thiotrophic and methanotrophic endosymbiont bacteria is comparable between MG and LS animals (Fig. 5), being the thiotrophic endosymbiont the most prominent phylotypes (88.8 % and 89.1 % for LS and MG respectively). Therefore the highest level of gene expression seen for most of the bacterial genes tested must rely on a higher transcriptional activities likely induced by environmental factors found at the Lucky Strike vent site.

The dynamics of symbiont populations may be influenced by environmental conditions (Halary et al., 2008; Duperron et al., 2009), thus modulating the amount of endosymbionts according to environmental factors. A scenario, where fluctuations of

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methane and hydrogen sulphide (H_2S) emissions occur and gradients of methane and H_2S concentrations are formed across a field of vent systems, can be easily pictured and could be a plausible explanation for dissimilar metatranscriptome observations. In this case, the expression of genes involved in methane and suphur oxidation may not correlate with in situ methane and sulphur concentrations.

However, it is not possible to rule out, at this point of our investigations, the effect of decompression on Lucky Strike animals when retrieved from 1700 m depth in comparison to Menez Gwen animals retrieved from 800 m depth. Menez Gwen animals have been known to survive well to decompression and withstand long periods of maintenance in aquaria at atmospheric pressure (Bettencourt et al., 2008). In the absence of more suitable methodologies to preserve deep-sea vent animals upon in situ collection, immediate tissue preservation and appropriate RNA handling, once animals reach the surface, seem adequate for comparative gene expression studies.

We conclude that the same specimens of *Bathymodiolus azoricus* may present different transcriptional activities most likely at the level of the gill's microbiome, which is presumably under direct influence of the hydrothermal vent environment from which mussels were originated.

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Table 1. Forward and reverse primer sequences used in quantitative PCR analyses of immune and stress-related genes in *Bathymodiolus azoricus* vent mussels. Based on sequences retrieved from the DeepSeaVent database, primers were designed and previously tested, in RT-PCR experiments, to confirm the physical counterpart of *B. azoricus* genes putatively associated with immunity and inflammatory reactions. Primers were subsequently used to study gene expression profiles in Menez Gwen and Lucky Strike gill samples. Contig references are indicated and accessible through the DeepSeaVent database at: http://transcriptomics.biocant.pt:8080/deepSeaVent/.

Candidate gene	Contig reference from DeepSeaVent database	5′-3′ sense	5′-3′anti-sense
Recognition			
Carcinolectin	mussel_c2366	CGGATACAGTGGCACGGCAG	TGATACCAACGAGCACCAGCAC
LBP/BPI	mussel_c39362	GCTTCACTGATACTGCTTGCCC	CCACGGTGGAGCAGCATGGA
PGRP	mussel_c1910	TCACACGGAAGGAGGAGCGT	AGGGCTGCCTTGGATGGTGT
C-Lectin-1	mussel_c8619	TGCCCAGGGTTTAAGGGTAGCA	GGACCATGGTTTGCTGCAGAGA
Serpin-2	mussel_c6158	AGGGTTGTGCGTGAAGTGGA	TCTCAAAGCGAGGCTGCCAGA
Galectin	mussel_c179	CTCCGGCGGGAGGGAATCCA	AGTGGAAGCTGGGGTTCCGAGG
Sialic ac. bind lectin	mussel_rep_c71109	GGTTGTGGAGTTGTAAAGCGTGC	GGGCGGTCCAAGCAAGTTCAAT
Aggrecan	mussel_lrc83347	TGCAAGCGGATACCCGGTAAA	ATCAACGCAGAGTGGCCAG
Signaling			
Immune GTPase	mussel_c1444	ATTACGGCCGGGGGACACAC	TTCGGCATCCTGGCACTTCG
TNF factor	mussel_c12211	GGGATTAGGCAACACCCAAGCC	CCGCCACAGTACAGCCAACC
Toll-like 2	mussel_c3962	AGGAGGACTCGGATGACACAGC	ACTCCGGAACTTGGAGAGCACG
MyD88	mussel_c3721	CTGCCACACCCAACAACGCA	TCGAGACTGAGGTTCTCGCACA
MAPK7	mussel_rep_c28417	TTACGGCTTGCTTTAGGAAACAGAGT	ACTCGATGGTACAGAGCCCCA
TRAF 6	mussel_c9675	GTTCATGTCTCGCCAAACTC	CGCCAAGCCATACATATAACAA
LPS-induced TNF-α factor	mussel_c1273	TGCGTACCAGGTGTCCAGCA	TCAACGACACGCTTGACCTGC
VEGF receptor	mussel_c29359	AGCTGCATGGAGACTTGAACCAGA	AGGTGGGGTGGTACTTGCTCC
Immune lectin receptor	mussel_rep_c70917	TGGACACTGCTACCATTATGGGACC	CGATTGGTCATAGCTCCAACGCC
Transcription			
Jun	mussel_c14202	CGCCAACACCGACACAGTTCA	AACCCCCGGGGAGTGTTGTT
AP-1	mussel_c963	TGCAGCTACACGGTTTCTGGC	TCGGCAACAACACTCCCC
IkB	mussel_c2112	TGAGGCAGCACTGAACGGAC	CGCAGAGTGTGCCAACAGCA
STAT (SH2 motif)	mussel_c5862	AGCGTCAAACACGACAGACGA	AGACCACGCCCTGTTTCAGC
P43	mussel_c25175	AGTGGAACACCAGATGCCATGC	TCACATGCATTGTCTGTCGGCC
Effector			
Cytolysin	mussel_rep_c32842	GATCACCCCTTCTCTATCGG	AAGAGTTCCGGCCATTACG
Glutathione perox	mussel_c23951	TTAACGGCGTCGTCGCTTGG	TGGCTTCTCTCTGAGGAACAACTG
Metallothionein IB	mussel_c72489	TCGGCACTGTCCACACAAAACC	CAACCGGAAGCGGATGTGGC
Big Defensin	mussel_c8746	CCGGGGGCGATTGCCTTTC	ACCAAGGCCCAAAATGCAGC
Ferritin	mussel_rep_c23342	GTCGGCGATCTTATGAGGTCAAGG	GGCCTACTATTTTGACCGTGACG
HSP70	mussel_c9170	TGGGTGGTGAAGATTTTGAC	CCCTCGAACAGAGAGTCAAT
TIMP	mussel_c3901	CAGGATCAGACGCTGCTTGTGG	TCATTATTCCACCGGGGCTCCC
Matrix mettaloproteinase	mussel_c33576	CTTGCCCATGCCTTCTTCCCAC	GGTGGTCTTGTTGGTGGTGGTG
Thioester macroglobulin	mussel_c8269	CTGGCTCTCTCAGCTCGGCA	GGGCACTCTCCGGTCTTGGT
Lyzozyme	mussel_rep_c24145	TCTTACACTTTAAAGTCAACA	TTTTCATCATACATAATATAT
Reference gene			
28S		AAGCGAGAAAAGAAACTAAC	TTTACCTCTAAGCGGTTTCAC

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Table 2. Forward and reverse primer sequences used in quantitative PCR analyses of bacterial gene expression in Menez Gwen and Lucky Strike gill samples. Sequences were retrieved from the DeepSeaVent database and subjected to bioinformatics analyses as described in Bettencourt et al. (2010) and Egas et al. (2012) and subsequently used for primer design for RT-PCR and qPCR experiments.

Candidate gene	DeepSeaVent accession	5′-3′ sense	5′-3′anti-sense
Xylose isomerase	mussel_rep_c66800	TGTCGTCCAGAGGCTACTGATGG	GGTGGGATACAGCCTGGTGGT
Rubisco activation CbbQ	mussel_c53956	TTCTAGCGCGGCGTGCGATT	AGGCGCGCCAAGATACCACA
Sulfur oxidation protein SoxB	mussel_rep_c38890	GGCTTCTGGCGAATCTGGCT	GCCATTACGGCCGGGGTCAG
Sulfate thiol esterase SoxB	mussel_c24142	TGGGCGGGCATCGTATTGCAG	CCGACCACACGCGATGCCAT
Sulfur oxidation protein SoxY	mussel_c3834	AGGTGCAAGAGGTGCTGGGAAC	GCTACCACAGCTGATGCTGCACA
Transketolase	mussel_c24501	TTCGTTTCCCAAAGGGGCGC	TGGAGAAAGGCGTTCCGGGT
Methane monooxygenase A	mussel_c5320	AGCTTGCACAGCAGCAGGGA	TCGCCATCTACGGGTGGCGA
Methane monooxygenase B	mussel_c29697	TACCGTGTTCCGGGTCGTGC	CTCACCTGGAGCTATAGGGCTGT
Methane monooxygenase C	mussel_c589	GCTTGCACAGCAGCAGGGAGA	TCGCCATCTACGGGTGGCGA
Metallophosphoesterase	mussel_c762	AGTGAGGCGTGCCATTGTGTGT	TGCGGGTTGAAACCGTGGCA
Methanol dehydrogenase	mussel_c40905	CCAGCCCCAAGTTGTACCGCC	ACGGCCGGGGCTTAACAGGT
Hydrox-methyl-diphosphate synthase	mussel_c10712	GCCAATTGCCCGCAAGCACC	GCAGGCACACAAGAGCGCCA
Diphosphocytidyl-methyl-D-erythritol kinase	mussel_c58553	TCCCCAGTGTCCACGTGACA	GGGCTAGGGGGAGGGAGCTC
Ribose 5-phosphate isomerase	mussel_c26206	GGCGGCACCGTGAAACTCAGA	GCCGTTACCATGCGAACACCA
Beta-glucuronidase	mussel_rep_c68125	TCCAGCAACGGTATCAGCACCAT	GGGGGTCCGCACTTAATTATCCTG

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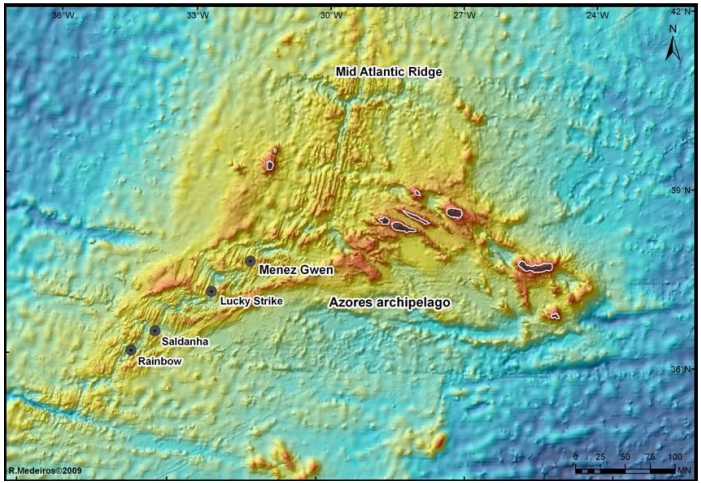

	T ºC	P (bar)	pH	CH4 (mM)	H2S (mM)	Cu (µM)	Fe (µM)	Zn (µM)
Lucky Strike 1700m depth 37º 18'N; 32º 26W	4.3	170	7.4	0.645	2.45	1.07	0.22	0.51
Menez Gwen 850 m depth 37º 50'N; 31º 30W	8.5	84	7.4	1.7	1.6	0.60	0.30	1.20

Fig. 1. Geographic collection sites of deep-sea mussels used in the present study. Geographic coordinates, water temperature (*T*), pH, pressure (*P*), concentrations of methane, sulphide and micro-essential metals are indicated (Charlou et al., 2000; Colaço et al., 2002; Duperron et al., 2006; Kádár and Costa, 2006).

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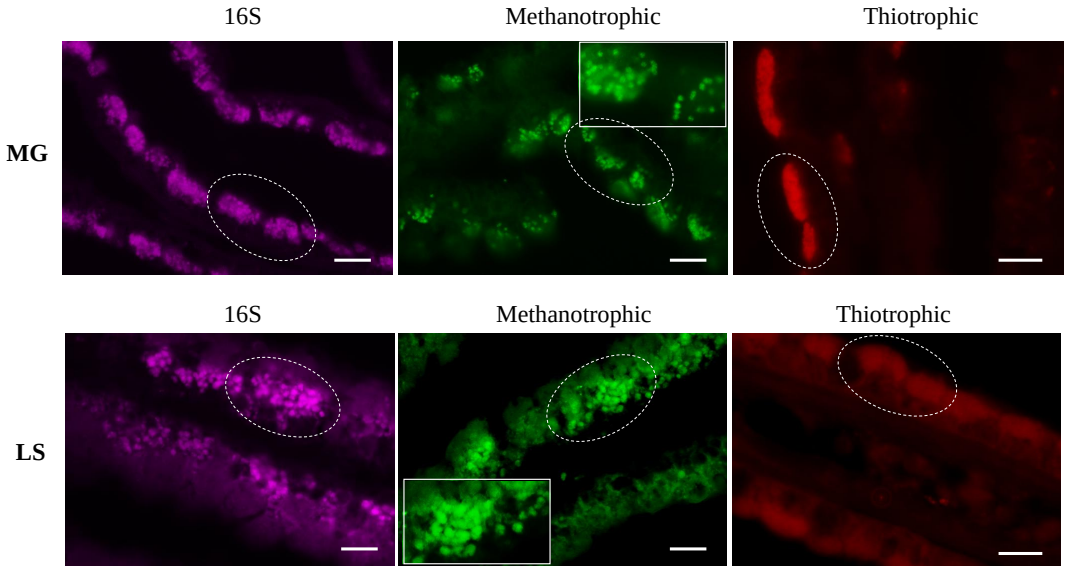

Fig. 2. Fluorescence in situ hybridization. Methane monooxygenase-, Sulfur oxidation SoxB- and 16S rRNA-based fluorescent probes were used to detect methanotrophic and thiotrophic bacteria, in gill tissues of Menez Gwen (MG, upper panels) and Lucky Strike (LS, lower panels) vent mussels. Broken white circle indicate the epithelial localization of endosymbiont in both methanotrophic and thiotrophic bacterial visualizations. Insets represent higher digital magnifications to enhance bacterial visualization. Scale bar indicate 20 µm.

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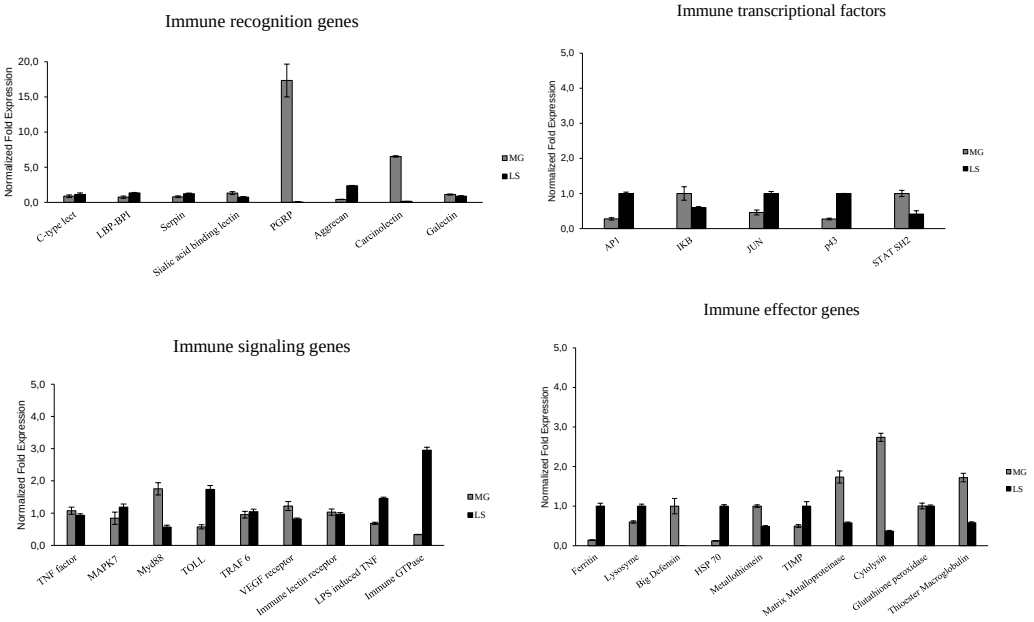

Fig. 3. Quantitative expression of immune-related genes in gills tissues from deep-sea mussels collected at Menez Gwen (grey bars) and Lucky Strike (black bars) vent sites. 6 replicates were performed for each gene tested in real time PCR reactions. Melt curves profiles were analyzed for each gene tested. The 28S rRNA gene was used as the housekeeping gene and for normalization of expression of gene of interest or immune-related target genes. The comparative CT method (ΔΔCT) for the relative quantification of gene expression was used for assessing the normalized expression value of immune-related genes using the 28S rRNA as the control transcript and normalization gene (CFX Manager™ Software, Bio-Rad). Data were transferred to Excel files and plotted as histograms of normalized fold expression of target genes.. Vertical bars represent the normalized fold expression of genes (means ±SD, *N* = 6).

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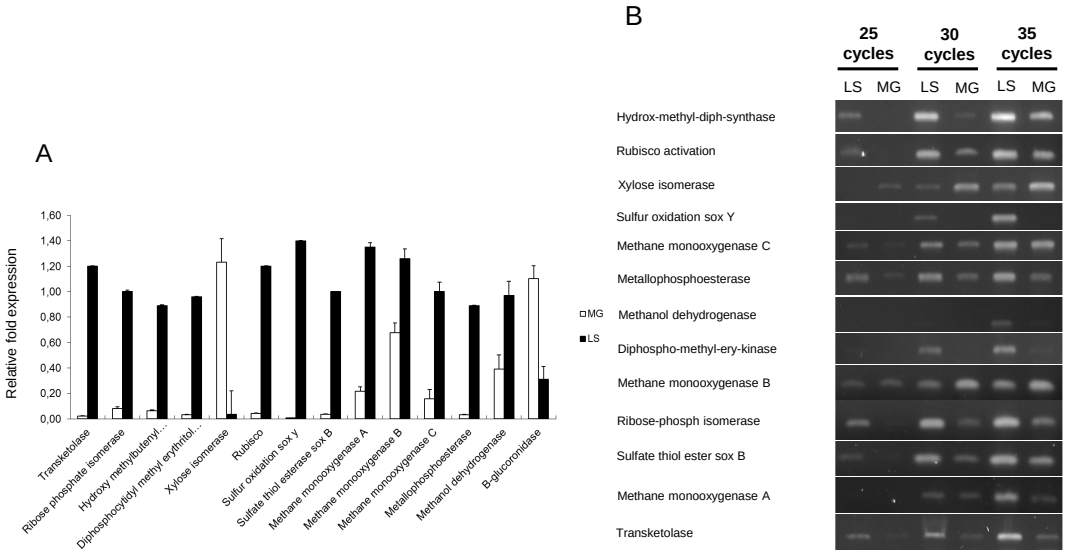

Fig. 4. (A) Comparative expression analyses of bacterial genes, as identified from previous metatranscriptomic studies (Bettencourt et al., 2010; Egas et al., 2012), between Menez Gwen (white bars) and Lucky Strike(black bars) gill samples Data were transferred to Excel files and plotted as histograms of relative fold expression of target genes. Vertical bars represent the relative fold expression of bacterial genes using the host 28S gene to normalize the total RNA as loading control during reverse transcriptase reactions and the cDNA used in qPCR [equal-amounts of host cDNA as evaluated by 28S amplicon obtained for each reaction] (means ±SD, $N = 3$). **(B)** Semi-quantitative Reverse Transcription-PCR (RT-PCR) of bacterial genes. Non-normalized cDNA obtained from reverse transcription of mRNA was used as template for PCR amplifications. Aliquots were taken from both Menez Gwen and Lucky Strike PCR reactions at 25, 30 and 35 cycles and analyzed by agarose gel electrophoresis.

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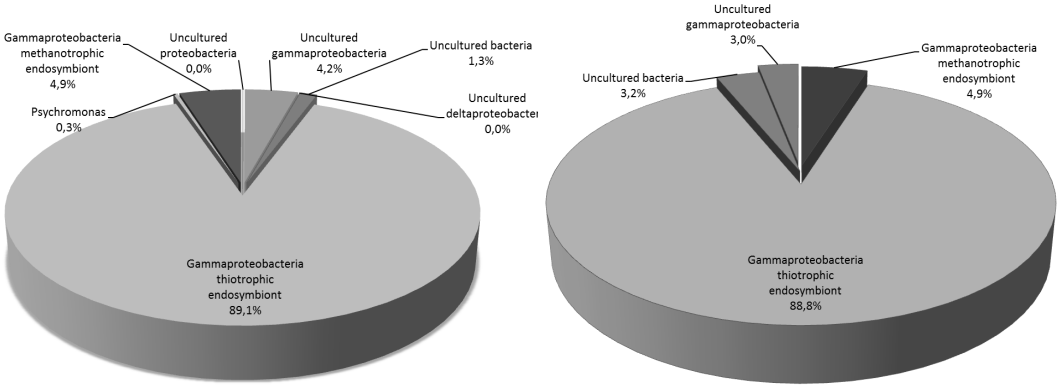

Fig. 5. Bacterial fingerprint of mussel gills as determined by 16S rRNA sequencing. The bacterial community structure from Menez Gwen and Lucky Strike gill samples was characterized by 454 deep-sequencing of the 16S ribosomal RNA amplicon library of the V6 hypervariable regions of the rRNA. Percentages refer to the number of OTU found for each taxa.

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