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Site-related differences in gene expression and bacterial densities 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 hydrothermal vent sites and in close vicinity of the Azores region near the Mid-Atlantic Ridge (MAR). The physiological relationships that vent mussels have developed with their physical and chemical environments are likely to influence global gene expression profiles providing thus the means to investigate distinct biological markers predicting the origin of *Bathymodiolus* sp. irrespectively of their geographical localization. Differences found at gene expression levels, and between fluorescence *in situ* hybridization (FISH) and 16S rRNA amplicon sequencing results provided experimental evidence for the distinction of both Menez Gwen and Lucky Strike vent mussel individuals based on bacterial and vent mussel gene expression signatures and on the constitutive distribution and relative abundance of endosymbiotic bacteria within gill tissues.

Our results confirmed the presence of methanotroph 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 a sulfur-oxidizing-related probe. Quantitative PCR (qPCR) studies revealed different 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 Menez Gwen or Lucky Strike animals whereas the genes encoding effector molecules appeared to have higher levels expression in gill tissues from Menez Gwen animals. The peptidoglycan recognition molecule encoding gene, PGRP, presented the highest level of transcriptional activity among the genes analyzed in Menez Gwen mussel 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 Menez Gwen and Lucky Strike mussel gill tissues. Remarkably, the immunity-related GTPase encoding gene demonstrated, in Lucky Strike samples, the highest level of expression among the signaling molecule encoding genes tested when expressions levels were compared between Menez Gwen and Lucky Strike animals. A differential expression analysis of bacterial genes between Menez Gwen and Lucky Strike mussels indicated a clear expression signature in the latter animal gill tissues. The bacterial community structure ensued from the 16S rRNA sequencing analyses pointed at an unpredicted conservation of endosymbiont bacterial loads between Menez Gwen and Lucky Strike samples.

Taken together, our results support the hypothesis that *B. azoricus* exhibits different transcriptional statuses while living in distinct hydrothermal vent sites may result in distinct gene expressions because of physico-chemical and/or symbiont densities differences.

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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 [9,21]. High concentrations of hydrogen sulfide 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 [9,21]. The deep-sea vent mussel *Bathymodiolus azoricus* is the dominant species at Menez Gwen (MG) and Lucky Strike (LS) 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 [30] and/or assimilation of dissolved amino-acids [27] in addition to chemoautotrophy by virtue of endosymbiotic bacteria located within the gill bacteriocytes [12,20]. Given the different geophysical and chemical parameters that distinguish the Menez Gwen and Lucky Strike hydrothermal vent sites [7], we surmised that animals living upon the vents would hypothetically be under the direct influence of abiotic and biotic environmental conditions that in turn would shape the overall animal's gene transcription activity providing the means to test the hypothesis whether living in distinct hydrothermal vent sites may result in distinct gene expressions because of physico-chemical and/or symbiont densities differences.

We have conducted several analyses to characterize differences in the expression of host immune genes whose sequence information were obtained from our recent analysis of *B. azoricus* gill transcriptome [3]. Most genes were designated for their relevance in innate immunity, as defined in Ref. [3], and regarded in this study, as suitable physiological markers to evaluate the influence of abiotic and biotic environmental conditions on *B. azoricus* transcriptional activity. In addition, bacterial genes ensued from our metatranscriptome characterization were also analyzed [19] as a means to evaluate symbiont density differences in both Menez Gwen and Lucky Strike animals respective to their environmental settings. Keeping in line with the assumption that geographically distinct vent mussels will adopt different physiological statuses in relation to their environmental conditions, we also surmised that the relative abundance of methanotrophic and sulfide oxidizing endosymbiotic bacteria would differ between Menez Gwen and Lucky Strike mussels as previously reported by other researchers [13,14,6].

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 geographically distinct *B. azoricus* individuals may be experimentally traced back to their original hydrothermal vent site on the basis of their transcriptional activity and bacterial gill densities and/or activities at the time animals were retrieved from the vent sites. A taxonomical structure of the vent mussel gill's microbiome was also assessed to determine the bacterial community composition of Menez Gwen and Lucky Strike gill tissue samples to infer the symbiont densities differences between animals from both vent sites.

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°51,6N; 31°30–31°31,8W, 800 m depth) and Lucky Strike (37°15,32N; 32°26,18W, 1700 m depth) in the Mid-Atlantic Ridge (MAR) (Fig. 1) with the French R/V *Pourquoi pas*? using the ROV Victor 6000 (MoMARETO cruise (August 6th–September 6th 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 RiboPureTM 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) PuristTM 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 [3] 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 58 °C (Tables 1 and 2). 25 μl PCR volume reactions were set with 1 μl of each forward and reverse primer (0.5 μM final concentration) and using a 2× PCR mix from PROMEGA. PCR conditions were according to Bettencourt et al., 2009 [4]. 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 putatively involved in innate immunity and identified from our previous cDNA library screenings and whole transcriptome sequencing of *B. azoricus* [2]. The cDNA from Menez Gwen and Lucky Strike 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 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 CT 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.