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Vibrio diabolicus challenge in *Bathymodiolus azoricus* populations from Menez Gwen and Lucky Strike hydrothermal vent sites

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ABSTRACT

Menez Gwen (MG) and Lucky Strike (LS) deep-sea hydrothermal vents are located at 850 m and 1730 m depths respectively and support chemosynthesis-based ecosystems partially differing in heavy metal concentration, temperature range, and faunistic composition. The successfully adapted deep-sea vent mussel *Bathymodiolus azoricus* is found at both vent locations. In such inhospitable environments survival strategies rely on the establishment of bacteria-vent animal symbiosis In spite of the toxic nature of deep-sea vents, the problem of microbial threat and the need for immunity exist in *B. azoricus*. This study aims at investigating the immune system of *B. azoricus* from MG and LS populations by comparing immune gene expressions profiles using the deep-sea vent-related *Vibrio diabolicus*. Expression of nineteen immune genes was analyzed from gill, digestive gland and mantle tissues upon 3 h, 12 h and 24 h *V. diabolicus* challenges. Based on quantitative-Polymerase Chain Reaction (qPCR) significant gene expression differences were found among MG and LS populations and challenge times MG mussels revealed that gill and digestive gland gene expression levels were remarkably higher than those from LS mussels. Expression of Carcinolectin, Serpin-2, SRCR, IRGs, RTK, TLR2, NF-κB, HSP70 and Ferritin genes was greater in MG than LS mussels. In contrast, mantle tissue from LS mussels revealed the highest peak of expression at 24 h for most genes analyzed. The activation of immune signaling pathways demonstrated that gene expression profiles are distinct between the two mussel populations. These differences may possibly ensue from intrinsic immune transcriptional activities upon which host responses are modulated in presence of *V. diabolicus*. mRNA transcript variations were assessed during 24 h acclimatization taking into account the partial depuration to which mussels were subjected to. Additionally, gene expression differences may reflect still accountable effects from the presence of vent remaining microfluidic environments within the tissues analyzed.

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1. Introduction

Deep-sea hydrothermal vents and cold seeps are submarine hot springs where nutrient-rich fluids emanate from the sea floor. They are characterized by uncommon chemosynthetic-based ecosystems linked to geographic areas where plate tectonic movements and deep ocean volcanism are active [1]. The deep-sea hydrothermal vents animals not found anywhere else, their survival and prevalence at these environments, depend on energy derived from chemoautotrophic bacterial activity using reduced chemicals present in the vent fluids, mainly hydrogen sulfide [2]. Since the discovery of hydrothermal vents, the establishment of dense faunal communities dwelling around the vents has been the subject of intense biological investigations [3]. The vent mussel *Bathymodiolus azoricus*, represents the predominant endemic faunal community at the Mid-Atlantic Ridge (MAR), south-west of the Azores, where they have been sampled from different geographical locations, ranging from the Menez Gwen (37°51′N, 31°31′W, 850 m), Lucky Strike (37°17′N, 32°16′W, 1700 m) and Rainbow (36°13′N, 33°54′W, 2300 m) hydrothermal vent sites [4–7]. These mussels withstand extreme environmental conditions including higher heavy metals concentrations, pressure and temperature,

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acidic pH and deep-sea darkness while coping successfully with environmental microbes [8]. Under these extreme conditions, *B. azoricus* requires the development of specialized gill epithelial cells harboring methanotrophic and thiotrophic endosymbiont bacteria. This is regarded as an adaptation strategy to chemosynthetic environments and a means to overcome energy consumption requirements [9–12]. Genetics population has demonstrated the phylogenetic conservation between the MG and LS *B. azoricus* populations, unlike the more Southern MAR *Bathymodiolus puteoserpentis* population which presents genetic divergence with the Northern MAR vent mussels [13,14].

The innate immune system functions as host defense mechanisms against pathogens including viruses, fungi, protozoa and bacteria. This system acts as the first line of defence against foreign microorganisms [15–17]. Pattern Recognition Receptors (PRRs) recognize and trigger defense reactions in response to conserved structures known as Microbial-Associated Molecular Patterns (MAMPs) present on the surface of microbes [18,19]. The anatomical and protective barriers such as the shell, cuticle and mucus layer constitute the first lines that the pathogens and parasites need to breach [8]. Upon self-non-self-recognition, the innate immune system is engaged and ensued by defense mechanisms consisting of humoral and cellular components. These components act together in a concerted fashion protecting the host against infection, while maintaining homeostasis [20–22].

Pathogens of the Vibrionaceae family are gram-negative bacteria naturally occurring in marine environments often posing a constant challenge to the bivalve innate immune system. The pathologies caused by vibrios in bivalves have been described since the 1960s. High mortality has been recorded due to these virulent microorganisms causing high losses in hatcheries and in the aquaculture sector [23]. As human pathogens, the *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus* species have been linked to human consumption [24–26]. Deep-sea hydrothermal vents may also be home to *Vibrio* species such as the *Vibrio diabolicus* strain first isolated from *Alvinella pompejana* vent polychaete annelids [27]. Virulence-related genes occur at high frequencies in non- *V. parahaemolyticus* Vibrionaceae species of which *V. diabolicus* is of particular interest as several strains recovered showed that the large majority (>83%) contained virulence-related genes [26]. Recently, a study relating occurrence, seasonality and infectivity of *Vibrio* strains has been reported in natural *Mytilus galloprovincialis* population [28]. This study is particularly interesting as it demonstrates the presence of *V. diabolicus* in sediments, water samples and oysters [28].

While *V. diabolicus* is naturally occurring in a deep-sea vent related animal, defense reactions to this proteobacterium remain largely uncharacterized. Although *V. diabolicus* has not being reported as causing pathologies to vent mussels nor symbiotic associations with this gammaproteobacterium have been described in this mussel, this bacterium was chosen for its relatedness to hydrothermal vent environments and putative immuno-stimulant properties. The present study aims at elucidating *V. diabolicus* interactions with *B. azoricus* immune system during an experimental challenge immediately performed after mussels were retrieved from the Menez Gwen and Lucky Strike hydrothermal vent sites. This study holds the potential for demonstrating the vent mussel immune response specificity toward the presence of *V. diabolicus* known to secrete a novel exopolysaccharide [27] Immune gene expression analyses were carried out with gill, digestive gland and mantle tissues, from MG and LS mussels. Significant differences were found between mussels from the two vent sites bringing insight into immune response discriminatory specificities at the tissue level. In addition to gill tissues, we provide further evidence supporting unique immune transcriptional activities in tissues not commonly studied in *B. azoricus*. Unlike previous acclimatization studies conducted in our group with *B. azoricus*, the present study takes into consideration the partial depuration and short acclimatization time to which mussels were submitted and experimentally challenged with *V. diabolicus* during 24 h. Both deep-sea vent MG and LS mussels are likely to show similar immune response when challenged with *V. diabolicus* bacterium. However, intrinsic immune transcriptional activities may differ in MG and LS mussels and between gill, digestive gland and mantle tissues due to different usage of transcription factors at the promoter region of immune genes and possibly by the presence of remaining vent microfluidic cellular environments not completely replenished from short depuration time in aquaria.

2. Material and methods

2.1. Sampling of biological specimens

The mussels were collected from the Menez Gwen (MG) hydrothermal vent field (MG4 site: 850 m depth, 37°50.70′N, 31°31.20′W) and the Lucky Strike (LS) hydrothermal vent field (Montségur site: 1730 m depth, 37°17.31′N, 32°16.55′W) with the French R/V "*Pourquoi Pas?*" using the Remotely Operated Vehicle (ROV) Victor 6000 during the BioBAZ mission between 2 and 20 August 2013 (Fig. 1). A group of 32 mussels from each hydrothermal vent site was selected to carry out the experimental work (Fig. 2) at atmospheric pressure. The mussels used to perform our experiments did not show any physiological deterioration given their remarkable resilience to decompression effects. Mussels were immediately processed aboard for subsequent RNA extraction as for the T0 mussel control group. Mussel size (mean length ± SD) was 6.0 ± 1.54 cm and 5.5 ± 1.38 cm for MG and LS samples respectively. Incubations and experiment settings were performed on board cold facilities.

2.2. V. diabolicus preparation and challenge

MG and LS mussels were maintained separately in the same 6 L shared 0.22 μm filtered seawater-containing plastic vessel. Then, the two groups of mussels were challenged with *V. diabolicus* HE800 strain suspension was obtained from Dr. Valerie Cueff (IFREMER, France). A 30 ml bacterial suspension was prepared from an overnight culture grown at room temperature in Marine Broth (Difco™ Marine Broth 2216) at an $OD_{600} = 1.2$ and 2.6×10^{10} CFU/mL and added to the mussels during incubation experiments. The experimental *V. diabolicus* challenge was kept at 4 °C inside a ventilated cold room. Given the limited number of individuals at our disposal and the short incubation time period considered for this experiment (24 h), gene expression studies were standardized comparatively to expression levels obtained from animals immediately recovered from vent sites (T0). This standardization method ensures that changes in gene expression levels were attributed to bacterial challenge as both control conditions T0 and T24 and *V. diabolicus* exposed mussels endured the same background (noise) expression levels caused by environmental stress and decompression effects. No significant gene expression differences were found between T0 and T 24 h in both MG and LS control mussels (Fig. S1). For this reason, animals maintained in plain seawater were not considered at 3 h, 12 h and 24 h for comparison gene expression studies. Both MG and LS mussel populations were sampled during the same time of the year (August, 2013) and during same oceanographic campaign. Eight mussels from MG and LS were dissected at 3 h, 12 h and 24 h over the course of *V. diabolicus* experimental challenges. Gill, digestive gland and mantle tissues were dissected, RNA processed and analyzed

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