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Comparative study of immune responses in the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus* and the shallow-water mussel *Mytilus galloprovincialis* challenged with *Vibrio* bacteria

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ABSTRACT

The deep-sea hydrothermal vent mussel *Bathymodiolus azoricus* and the continental European coast *Mytilus galloprovincialis* are two bivalves species living in highly distinct marine habitats. Mussels are filter-feeding animals that may accumulate rapidly bacteria from the environment. Contact with microorganism is thus inevitable during feeding processes where gill tissues assume a strategic importance at the interface between the external milieu and the internal body cavities promoting interactions with potential pathogens during normal filtration and a constant challenge to their immune system.

In the present study *B. azoricus* and *M. galloprovincialis* were exposed to *Vibrio alginolyticus*, *Vibrio anguillarum* and *Vibrio splendidus* suspensions and to a mixture of these *Vibrio* suspensions, in order to ascertain the expression level of immune genes in gill samples, from both mussel species. The immune gene expressions were analyzed by means of quantitative-Polymerase Chain Reaction (qPCR). The gene expression results revealed that these bivalve species exhibit significant expression differences between 12 h and 24 h post-challenge times, and between the *Vibrio* strains used. *V. splendidus* induced the strongest gene expression level in the two bivalve species whereas the NF-κB and Aggrecan were the most significantly differentially expressed between the two mussel species. When comparing exposure times, both *B. azoricus* and *M. galloprovincialis* showed similar percentage of up-regulated genes at 12 h while a marked increased of gene expression was observed at 24 h for the majority of the immune genes in *M. galloprovincialis*. This contrasts with *B. azoricus* where the majority of the immune genes were down-regulated at 24 h. The 24 h post-challenge gene expression results clearly bring new evidence supporting time-dependent transcriptional activities resembling acute phase-like responses and different immune responses build-up in these two mussel species when challenged with *Vibrio* bacteria.

High Pressure Liquid Chromatography (HPLC)-Electrospray ionization mass spectrometry (ESI-MS/MS) analyses resulted in different peptide sequences from *B. azoricus* and *M. galloprovincialis* gill tissues suggesting that naïve animals present differences, at the protein synthesis level, in their natural environment. *B. azoricus* proteins sequences, mostly of endosymbiont origin, were related to metabolic, energy production, protein synthesis processes and nutritional demands whereas in *M. galloprovincialis* putative protein functions were assumed to be related to structural and cellular integrity and signaling functions.

1. Introduction

The invertebrate immune system distinguishes self from non-self, resulting in physiological responses mediated through cellular and humoral processes that effectively fight against

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invading agents subsequently leading to their removal from the host [1]. This natural immunity is formed by anatomical and chemical protective barriers [2]. In the case of *Bivalvia*, the first line of defense includes protective barriers such as the cuticle, shell and mucus layer [3]. Once these protective barriers are breached allowing pathogen entry, sensitive recognition of microbial surface components commonly referred as Microbe Associated Molecular Patterns (MAMPs) will ensue, followed by a rapid activation of the cellular component of the hemolymph, the circulating hemocytes or macrophage-like cells, and the participation of humoral factors present in the hemolymph with antimicrobial, cytotoxic properties or with opsonin-like properties, facilitating phagocytosis [4]. Through the open circulatory system of bivalves the hemolymph flows into the hemocoel, bathing all the organs and diffusing through a series of tissues sinuses where infective agents may be disseminated. The hemolymph assumes thus an important role in the immune system circulating around the body of mussels reaching the gills and mantle where potential pathogens interact with innate immune factors [5–7]. The *Bivalvia* mussels are widely distributed around the world including the *Bathymodiolus azoricus* and *Mytilus galloprovincialis* species. *B. azoricus* (Cosel & Comtet, 1999) is frequently the dominant species in numerous chemosynthesis-based communities such as cold (methane) seeps and hydrothermal vent ecosystems [8,9]. The genus *Mytilus* includes the Mediterranean mussel *M. galloprovincialis* (Lamarck, 1819) that is an endemic species to the Mediterranean Sea and Atlantic Ocean, from Morocco to Ireland [10].

Vibrio species are Gram-negative opportunistic pathogens indigenous to aquatic environments. They are ubiquitous and abundant in marine coastal waters, estuaries, ocean sediment and also in deep sea hydrothermal vents [11]. The *Vibrio diabolicus* strain was first isolated from a deep-sea vent animal, from the Pacific [12] and has been phenotypically related to pathogenic *Vibrio* species of which *Vibrio anguillarum* and *Vibrio alginolyticus* *Vibrio parahaemolyticus* are associated to human foodborne illnesses due to the consumption of fish and shellfish animals [13]. These *Vibrio* species demonstrated high genomic similarity to pathogenic *Vibrio* species in human [11].

Bivalves are filter-feeding animals constantly being exposed to pathogenic bacteria and environmental pollutants. Consequently, bivalves have been used as sentinels in ecotoxicological studies to monitor the quality of the aquatic environment [14]. The foodborne infections in humans cannot be predicted unless we know how they are transmitted. Therefore studying how the immune system of marine mussels work may lead to a better understanding of the physiological defense systems and to the elucidation of the physiological principles underlying the cellular and molecular mechanisms involved in specific adaptation processes of *B. azoricus* and *M. galloprovincialis* to very distinct natural habitats. Comparative immunological studies between these two mussel species may shed light into survival strategies of other marine bivalve species enduring unbalanced marine habitats and detrimental effects of climate change. These two mussel species represent valuable models to investigate their strategic adaptation to the surrounding environment and bring insight into the evolution of their innate immune system living under highly divergent environmental conditions. The progressive adaptation of *Bathymodiolinae* mussels to deep-sea environments [15] during evolution, might be now reflected in *B. azoricus* capacity to react to *Vibrio* infections in ways dissimilar to their Mytilid ancestors capacity to withstand *Vibrio* infections. The presence of endosymbiont bacteria in *B. azoricus* gills also constitutes another evolutionary feature that confer deep-sea vent mussels the hability to adapt to chemosynthesis-based environment while potentially driving host-immune gene expression [16].

2. Material and methods

2.1. Mytilidae samples collection

B. azoricus mussels were collected from the hydrothermal vent field Menez Gwen (850 m depth, 37°50.8–37°51.6N, 31°30–31°31.8W), with the French R/V "Pourquoi Pas?" using the Remote Operated Vehicle (ROV Victor 6000) (MoMARSAT cruise, 28 June–23 July 2011) and the *M. galloprovincialis* mussels were obtained from a commercial shellfish farm (Vigo, Galicia, Spain).

2.2. Vibrio preparations and mussel challenges

B. azoricus and *M. galloprovincialis* mussels were acclimatized to aquarium conditions for 24 h, to avoid stress after collection and transportation, prior to the experimental challenges with *Vibrio* bacteria. The mussels were then maintained in 2 L filtered seawater beakers with aeration at 8 °C and 15 °C respectively, and grouped into five sets of eight animals from each species and used for experimental *V. alginolyticus*, *V. anguillarum* or *Vibrio splendidus* and a mixture of all these Vibrios hereafter referred as "pool" challenges. 25 mL of overnight *Vibrio* cultures grown in a TCBS (Thiosulfate Citrate Bile Sucrose) medium were used as suspensions with an $OD_{600} = 1.5$ and 1×10^{10} CFU/mL for the subsequent challenges. After *Vibrio* challenge, four animals from each experimental conditions were dissected at 12 h post-challenge time and four mussels dissected later at 24 h post-challenge time.

2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted from gill tissues with TRIzol Reagent (Invitrogen) and purified with RNeasy mini kit (Quiagen) following the manufacturer's instructions and resuspended in nuclease-free, DEPC-treated water. Total RNA quality and concentrations were assessed by the $A_{260/280}$ and $A_{260/230}$ spectrophotometric ratios using the NanoDrop 1000 spectrophotometer (Thermo Scientific). The cDNA was synthesized with SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's specifications using 2 μg/mL total RNA per sample and its concentration measured using the NanoDrop 1000 spectrophotometer as above. The 12 h and 24 h gill cDNA samples were prepared from a mixture of 4 RNA gill purifications from each of the challenged and unchallenged (control) mussel species and from each *Vibrio* challenges including the pool challenge.

2.4. Gene expression analyses

Gene expression analyses were conducted by means of qPCR following the MIQE guidelines [17] using the 12 h and 24 h cDNA samples from both challenged and unchallenged mussel species. The immune genes selected were Galectin, Peptidoglycan Recognition Protein (PGRP), Aggrecan, Lipopolysaccharide Binding Protein (LBP) and Bactericidal/Permeability-Increasing Protein (BPI) (LBP-BPI), Myeloid Differentiation primary response gene-88 (MyD88), Toll-Like Receptor 2 (TLR2), Lipopolysaccharide (LPS)-induced Tumor necrosis factor-alpha TNF-α factor (LITAF) Jun-like, Nuclear-Factor kappa B (NF-κB), Heat Shock Protein 70 (HSP70) and Glutathione peroxidase I (Gpx1) for *B. azoricus* (Table 1) and *M. galloprovincialis* (Table 2).

The specific primers were designed based on sequences retrieved from the DeepSeaVent [19], MytiBase [20] and Genbank databases for both species using the Oligo Analyzer 1.0.2 program [21]. The primer pair efficiencies for *B. azoricus* and *M. galloprovincialis* were analyzed in consecutive cDNA dilutions through the regression line of the Cycle thresholds (Ct) versus the