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Expression of *Mytilus* immune genes in response to experimental challenges varied according to the site of collection

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ABSTRACT

Mussels live in diverse coastal environments experience various physical, chemical and biological conditions, which they counteract with functional adjustments and heritable adaptive changes. In order to investigate possible differences in immune system capabilities, we analyzed by qPCR the expression levels of 4 immune genes (*defensin*, *mytilin B*, *myticin B, lysozyme*) and *HSP70* in the Mediterranean mussel, *Mytilus galloprovincialis* collected in 3 European farming areas {Atlantic Ocean-Ría de Vigo-Spain (RV), French Mediterranean Gulf of Lion-Palavas-Prévost lagoon (PP) and Northern Adriatic Sea-Venice-Italy (VI)} in response to one injection of one of the 3 bacterial species (*Vibrio splendidus* LGP32, *Vibrio anguillarum*, *Micrococcus lysodeikticus*), and to heat shock or cold stress. We confirmed that the 5 genes are constitutively expressed in hemocytes, *defensin* being the less expressed, *myticin B* the highest. As suspected, the same gene resulted differently expressed according to mussel group, with the biggest difference being for *HSP70* and *lysozyme* and lowest expression of all the 5 genes in mussels from RV. In addition, gene expression levels varied according to the challenge. Most frequent effect of bacterial injections was down-regulation, especially for *mytilin B* and *myticin B*. Heat shock enhanced transcript levels, particularly in mussels from RV, whereas cold stress had no effect. *In situ* hybridization of labelled probes on mussel hemocytes indicated that bacterial injections did not change the mRNA patterns of *defensin* and *myticin B* whereas *mytilin B* mRNA almost disappeared. In conclusion, these results demonstrated that constitutive level, nature and intensity of immune gene expression regulations strongly depended from mussel group, and support the concept of gene–environment interactions.

1. Introduction

Mussels are bivalve species commonly found throughout world seashores, including estuaries. Broad resilience of mussels to environmental variations and their ability to accumulate toxins [1,2], as well as human pathogens [3–6], has made them popular and launched their use as indicators of environmental quality in many bio-monitoring programmes. According to the Mussel Watch concept, most of these programmes investigate contaminant levels and early-induced biological alterations in mussels from reference and contaminated areas [7]. Relevant to understand bivalve functional responses were studies aimed to ascertain immunological vigor and immune-related parameters such as total hemocyte counts, phagocytosis and lysosomal functions, expression or activity of metallothioneins and lysozyme, production of intracellular superoxide and cytotoxic effects. More recently, constructions of suppression subtractive cDNA libraries [8–11] and cDNA libraries based on random clone sequencing [12–14] revealed large panels of genes involved in immunity/stress response. EST sequencing approach contributed to the discovery of numerous immune-related genes in pathogen-challenged oysters [15,16] and mussels [17], such are α_2-macroglobulin, MyD88, Toll-like receptors and high sequence variability of antimicrobial peptide myticin C.

According to the purposes of the European Project IMAQUANIM (http://imaquanim.dfvf.dk/info/), we completed the first homogeneous comparison of immune-related gene expression in mussels collected in 3 European farming areas (Atlantic Ocean-Ría de Vigo, Spain; Mediterranean Gulf of Lion-Palavas-Prévost lagoon, France;

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Northern Adriatic Sea-Venice lagoon, Italy) in response to one injection of 3 bacteria species and critical temperature changes. Selected genes were *defensin*, *mytilin B*, *myticin B* and *lysozyme*. *HSP70* has been included to the study, its expression usually triggered by a variety of insults, not specifically related to immunity. In addition, ISH has been used to visualize the corresponding mRNAs within circulating hemocytes.

2. Material and methods

2.1. Geographic origins of mussel groups

Adult mussels, *Mytilus galloprovincialis* (6–7 cm shell length), were collected in 3 different locations (Fig. 1). Mussels from Ría de Vigo, referred as RV, were obtained in June–July 2006 from the shellfish farm Mariscos Ría de Vigo S.L. (Vigo, Galicia-Spain). Mussels from the French Mediterranean coast were obtained in June–July 2005 and June–July 2006 from the marine farm Les Compagnons de Maguelone (Prévost lagoon, Palavas-France) and referred as PP. Mussels from the Adriatic Sea acclimatized for one week in the Venice lagoon were obtained in June–July 2007 from the marine farm Mitilpesca (Alberoni, Venice-Italy) and referred as VI. All the mussels were acclimated for 24 h in corresponding co-author's laboratory (Vigo, Montpellier and Padua) in a flow-through system of oxygenated sea water before any treatment to eliminate the stress resulting from collection and travel as routinely used.

	Geographic coordinates	Sea water temperature	salinity
RV	42°14'32 N – 08°48'26 O	17°C	33
PP	43°31'15 N - 03°54'33 E	20°C	37
VI	45°13'47 N - 12°15'59 E	25°C	32

Fig. 1. Geographic sites of collection of the mussel groups used in the present comparative study: Ría de Vigo-Spain (RV), Palavas-Prévost-France (PP) and Venice lagoon-Italy (VI), with geographic coordinates, typical sea water temperature and salinity at the time of collection in June–July.

2.2. Bacterial cultures

Vibrio splendidus LGP32 is a Gram-negative marine bacterium isolated from juvenile oysters, *Crassostrea gigas*, during 2001 summer mortalities [18]. *Vibrio anguillarum* was obtained from the Institut Pasteur-France (ATCC 19264). Both *Vibrio* species (50 µl of overnight-cultured inoculum) were grown at 20 °C in 10 ml trypsin-casein-soya (TCS, AES Laboratoire) for 4–6 h to ensure bacteria were in exponential growth phase, centrifuged for 10 min at 500 × g, then adjusted to 10^8 bacteria/ml with phosphate buffered solution isotonic to sea water (PBS-NaCl: 2 mM KH_2HPO_4, 10 mM Na_2HPO_4, 3 mM KCl, 600 mM NaCl in distilled water, pH 7.4) according to 1 $OD_{550nm} = 5 \times 10^8$ bacteria/ml as established by counting the number of colony forming unit (CFU). Gram positive *Micrococcus lysodeikticus* from Institut Pasteur-France (ATCC 4698) was grown at 37 °C in Luria Broth (LB, Sigma) until the bacteria were in exponential growth phase, centrifuged for 10 min at 500 × g, then adjusted to 10^8 bacteria/ml with PBS-NaCl according to 1 $OD_{550nm} = 0.36 \times 10^8$ bacteria/ml as established by counting the number of CFU. The 3 bacterial species were from the same stock maintained in Montpellier's lab and freshly prepared in location immediately before injection.

2.3. Experimental mussel challenges and hemolymph collection

Four pools of 10 mussels per challenge and end-point were injected into the posterior adductor muscle with 100 µl (10^7 bacteria) through a hole created by light shell filing. After injection, mussels were returned into sea water at corresponding temperature (Fig. 1). Control injections consisted of 100 µl of PBS-NaCl. Thermal shocks consisted in mussel immersion for 90 min in sea water at either 30 °C or 6 °C, then returning mussels into sea water at local temperature for recovery. Four pools of 10 untreated mussels each were sampled in parallel to experimental treatments to minimize batch variations. Complete set of challenges involved 2160 mussels per location. Only the complete set of challenges on VI mussels was performed twice in June–July 2007, increasing the total number of sampled mussels to 8640.

Hemolymph (0.8 ml per mussel) was collected from the posterior adductor muscle at 1, 3, 6, 9, 12, 24, 48 and 72 h post-injection (p.i.), or immediately after the temperature shock (time 0) and after 3, 6, 9, 12, 15, 18 and 24 h of recovery, with a 1 ml disposable syringe containing 0.2 ml of anti-coagulant modified Alsever's solution [19]. Hemolymphs from 10 mussels were pooled and hemocytes were pelleted by 10 min centrifugation at 800 × g, 4 °C, then carefully resuspended in 500 µl RNALater (Sigma) and stored at 4 °C for the PP samples or shipped to Montpellier' lab for the RV and VI samples. Four pools of 10 mussels each, as replicates, were used for each sampling end-point.

2.4. cDNA synthesis and quantitative PCR (qPCR)

All the 864 samples were processed in the Montpellier's lab and by the same scientist for rigorous comparison. RNAs were purified according to manufacturer's Trizol instructions, resuspended in 45 µl of Tris-EDTA buffer (TE: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and concentrations were measured on spectrophotometer ND-1000 (NanoDrop Technologies). First strand cDNAs were synthesized on 5 µg of total RNA using hexaprimers (Invitrogen) and murine leukemia virus reverse transcriptase (Promega), purified through Wizard SV gel and PCR Clean-up System (Promega), and then kept in nuclease-free water at −20 °C until use.

To maximize reproducibility, all qPCRs were performed in the Montpellier's lab using SYBR Green chemistry on the same Light-Cycler 480 384 well-plate (Roche). Primer sequences were