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Lysozyme gene expression and hemocyte behaviour in the Mediterranean mussel, *Mytilus galloprovincialis*, after injection of various bacteria or temperature stresses

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Abstract The aim of the present study was to evaluate the expression of the *Mytilus galloprovincialis* lysozyme gene in different *in vivo* stress situations, including injection of bacteria *Vibrio splendidus* LGP32, *Vibrio anguillarum* or *Micrococcus lysodeikticus*, as well as heat shock at 30 °C and cold stress at 5 °C. Injection of *V. splendidus* LGP32 resulted in: (i) a general down-regulation of lysozyme gene expression, as quantified by Q-PCR; (ii) reduction in the number of circulating hemocytes; (iii) decrease in the percentage of circulating hemocytes expressing lysozyme mRNA which was now restricted to only small cells, as observed by ISH; and (iv) accumulation of hemocytes expressing lysozyme in the muscle sinus where injection took place. Injection of *V. anguillarum* or *M. lysodeikticus* induced significant up-regulation of lysozyme gene expression, but only 2–3 days post-injection, with no change in the total hemocyte counts but an increased percentage of hemocytes expressing lysozyme mRNA. Neither the control injection of PBS-NaCl nor temperature stress modified the lysozyme expression pattern. Consequently, the hemocyte population appears to be capable of discriminating between stress factors, and even between 2 *Vibrio* species.

Introduction

Lysozymes are small ubiquitous antibacterial enzymes that hydrolyze β-1, 4-linked glycoside bonds of peptidoglycan, a major cell wall component of Gram-positive bacteria.

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Several studies have shown that lysozymes are also able to kill Gram-negative bacteria, demonstrated in bivalves [1,2] and in shrimp [3,4]. Anti-protozoan and anti-fungal activities of lysozymes, mediated by cleaving *N*-acetylglucosamine in chitin, have been also documented [5,6]. In addition, lysozyme can kill bacteria by non-enzymatic activity [7]. Thus, lysozymes appear to constitute an important component of immune defence against diverse microbial infections.

Several types of lysozymes have been purified, from the best-known chicken-type (c-type) and goose-type (g-type) [8], to the more recently identified invertebrate-type (i-type) [1,9]. Such i-type lysozyme has been identified in several bivalve molluscs, including the Icelandic scallop, *Chlamys islandica* [1], the blue mussel, *Mytilus edulis* [10], several mytilids and vesicomyids [11], and the Mediterranean mussel, *Mytilus galloprovincialis*, the hydrothermal-vent mussel, *Bathymodiolus azoricus* and the cold-seep clam, *Calyptogena* sp. [12]. Although numerous reports concern the different types of lysozymes and their activities [10,12–15], only the genes from the blue mussel, *M. edulis* [12], and from the Icelandic scallop, *Chlamys islandica* [16], have been sequenced. Curiously, the *M. edulis* lysozyme gene comprises five exons instead of the classical four exons of the c-type lysozyme gene, such as the *C. islandica* ones.

The Mediterranean mussel, *M. galloprovincialis*, is a filter-feeding bivalve and lysozymes are believed to be involved in digestive processes [17] as well as in host defence [18]. In *M. edulis*, lysozyme has been found localised within granular hemocytes [19] and higher levels of activity have been detected in hemocytes compared with plasma [20] in both *M. edulis* and the carpet shell clam, *Ruditapes decussatus* [21]. However, mRNA transcripts of g-type lysozyme were most abundantly expressed in gills, hepatopancreas and gonad, but only weak expression was evident in hemocytes and mantle from the Zhikong scallop, *Chlamys farreri* [22]. In the Pacific oyster, *Crassostrea gigas*, lysozyme mRNA was expressed in all tissues except the adductor muscle and ISH (*in situ* hybridization) analyses revealed strong expression in basophilic cells from the digestive tubules [23]. Similarly, ISH located lysozyme gene expression in the mantle and gill cells of the eastern oyster, *C. virginica*, with significantly higher mRNA content in labial palps and mantles than in gills, digestive glands and hemocytes [14]. For a long time, hemocytes have been considered as primary mediators of anti infectious defence, yet no work has been done on the kinetics of lysozyme gene expression in response to various challenges.

The aim of the present report was to study the kinetics of expression of the *M. galloprovincialis* lysozyme gene in response to various stress factors: high temperature, low temperature, and bacterial injection. Quantification of lysozyme transcripts was done using Q-PCR (quantitative polymerase chain reaction) with 28S ribosomal RNA as the house keeping gene. Visualisation of circulating hemocytes containing lysozyme mRNA was done by ISH and completed by histological observations of the posterior adductor muscles where injection took place.

Materials and methods

Bacterial growth and mussel challenges

Vibrio splendidus LGP32 is a Gram-negative marine bacterium isolated from juvenile oysters, *C. gigas*, during summer mortalities in 2001 [24], while *V. anguillarum* was 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, Bruz, France) for 4–6 h to ensure bacteria were in the exponential growth phase, then centrifuged for 10 min at 500 × *g*, and adjusted to 10^8 CFU/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 $OD_{600\ nm} = 5 \times 10^8$ CFU/ml. *Micrococcus lysodeikticus* from the Institut Pasteur-France (ATCC 4698) was grown at 37 °C in Luria Broth (Sigma Chemical Co, St Louis, MO, USA) until bacteria were in the exponential growth phase, then centrifuged 10 min at 500 × *g*, and adjusted at 10^8 CFU/ml with PBS-NaCl according to 1 $OD_{600\ nm} = 0.36 \times 10^8$ CFU/ml.

Adult *M. galloprovincialis* were purchased from the marine farm Les Compagnons de Maguelone (Palavas, France) in May–June 2006 and April 2007. They were maintained in the laboratory in oxygenated sea water at 20 °C for 1–3 days prior to experimentation. Four batches of 10 mussels each per sampling end-point were injected with 100 μl (10^7 bacteria) into the posterior adductor muscle, through a hole created by light filing on the shells. After injection, mussels were returned to 20 °C sea water. Control injections consisted of 100 μl of PBS-NaCl. To test temperature stress, four batches of 10 mussels each per sampling end-point were subjected to 90 min immersion in sea water at either 30 °C or 5 °C, with their subsequent return to 20 °C sea water. Four batches of 10 untreated mussels each (referred to as untreated) were sampled simultaneously with each corresponding stress to minimize seasonal variations. Thus, the experiments involved a grand total of 2160 mussels.

Primers for lysozyme and 28S ribosomal RNA

Primers for lysozyme were designed from the *M. galloprovincialis* lysozyme mRNA (AF334665) [12]: forward 5′-ATGTGGAATCTGAAGGACTTGT-3′ (position 140–161) and reverse 5′- CCAGTATCCAATGGTGTTAGGG-3′ (position 486–507), giving an expected amplicon of 368 bp. Presence of a unique amplicon was checked by melting curve analysis (see Section 2.4) and gel electrophoresis on 2% agarose in Tris-borate-EDTA buffer stained with ethidium bromide. Specificity has been confirmed by several complete sequencings performed by Millegen (Labège, France).

28S rRNA was used as the house-keeping gene according to Cellura et al. [25] using the previously reported primer sequences: forward, 5′-AAGCGGAGGAAAAGAAACTAAC-3′ and reverse, 5′-TTTACCTCTAAGCGGTTTCAC-3′, giving an amplicon of 378 bp with a melting temperature of 90.17 ± 0.04 °C, also sequenced for confirmation of identity.