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Functional and molecular immune response of Mediterranean mussel (*Mytilus galloprovincialis*) haemocytes against pathogen-associated molecular patterns and bacteria

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ABSTRACT

The effect of live bacteria (*Micrococcus lysodeikticus* and *Vibrio anguillarum*), and PAMPs (poly I:C, zymosan, LPS, LTA and CpG) on the production of intermediate toxic radicals (respiratory burst activity and production of nitric oxide) and mytilin B, myticin C and lysozyme gene expression was studied *in vivo* and *in vitro*. *In vitro*, bacteria were able to modulate the haemocytes' respiratory burst activity, being significantly increased after 6 h of incubation. The effect of pathogen-associated molecular patterns (PAMPs) was also studied. Zymosan produced an increase of the PMA-mediated response but an inhibition of the zymosan-mediated response. A significant increase of nitric oxide production was found at all the sampled time points (1, 3 and 6 h) in comparison with controls on both, the Gram-positive and Gram-negative bacteria. The *in vivo* responses measured on haemocytes after *M. lysodeikticus* injection were faster than those induced by *V. anguillarum*. However, *V. anguillarum* induced stronger *in vitro* effects. Mytilin B, myticin C and lysozyme *in vitro* gene expression, occurred at short times after infection. The maximum *in vitro* expression was detected 3 h post-infection. The differences between *M. lysodeikticus* and *V. anguillarum* in different measured parameters may suggest that different signalling pathways might be involved. Moreover, among all assayed PAMPs, LPS elicited the highest response.

1. Introduction

Bivalve production and the reports on their pathogens and associated mortalities have increased steadily in the latest decades. High densities, stress or frequent stock transfer together with virus, bacteria and parasites have been reported as the main causes of bivalve diseases [1]. Members of the Gram-negative genus *Vibrio* are the most frequently isolated bacteria from molluscs. Several species belonging to this genus have been related with mortalities and diseases in larvae and juvenile individuals [2–4].

The immune innate system, conserved throughout the animal kingdom [5], is the first line of defense against pathogenic agents and infectious microbes. This system is able to recognize common structures present in microorganisms, known as pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) [6]. Lipoteichoic acid (LTA), lipopolysaccharide (LPS) and DNA (CpG) from bacteria, zymosan and β-1,3-glucan from yeast and double-stranded RNA (poly I:C), mimicking viral infection, have been identified as PAMPs [7].

Traditional approaches to study the immune innate system in mussels consist of functional assays and histological studies to determine the lesions and the interactions between host immune system and pathogens [8]. To date, most of the information on mussel immunity is based on phagocytosis assays [9], chemiluminescence production [10,11] and nitric oxide release [11,12]. However, lack of information about gene sequences has made the analysis of the molecular basis of these processes in mussel haemocytes quite slow.

Among the few known genes with immune function in the Mediterranean mussel, several classes of antimicrobial peptides (AMPs) [13–17], lysozyme [18] and heat shock proteins [19,20] have been described. Several ESTs obtained after the construction of a non-stimulated library, have increased the number of immune-related genes in mussels such as lipopolysaccharide and beta-1,3-glucan binding protein and lectins of different types [21]. We have recently reported a total of 1147 sequences obtained from primary and Suppression Subtractive Hybridization (SSH) libraries made with stimulated mussel haemocytes. A striking high abundance of

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AMPs suggested a potential critical role in the mussel innate immune system [17].

In spite of the high Mediterranean mussel, *Mytilus galloprovincialis*, production in Galicia (NW of Spain) (about 250,000 ton per year), no mortalities, due to pathogenic agents, have been reported as for oysters and clams.

The main goal of the present research was the study of the functional and molecular immune reactions produced after stimulation with live and inert stimuli. The obtained results will contribute to increase our knowledge about the defence mechanisms in this species with a great interest in the aquaculture sector.

2. Material and methods

2.1. Animals

Mediterranean mussels (*M. galloprovincialis*) with a maximum shell length of 6 cm were obtained from a commercial shellfish farm from the Ría de Vigo (NW of Spain) in summer season. Animals were maintained in open circuit filtered seawater tanks at 15 °C with aeration and they were fed daily with *Isochrysis galbana* (12×10^8 cells/animal), *Tetraselmis suecica* (10^7 cells/animal) and *Skeletonema costatum* (3×10^8 cells/animal). Prior to the experiments, bivalves were acclimatized for 1 week.

2.2. Stimuli preparation

Several PAMPs and live bacteria were administered intramuscularly (*in vivo* assays) or to haemocytes primary cultures (*in vitro* assays). Solutions of 1 mg ml^{-1} of poly I:C, zymosan, lipopolysaccharide (LPS) and lipoteichoic acid (LTA) were prepared from a commercial stock obtained from Sigma–Aldrich Co. CpG, was prepared from DNA isolation from a *Vibrio anguillarum* culture. Bacteria were grown in TSA supplemented with 1% NaCl at room temperature (20 °C). The culture was maintained for several days with the aim to obtain a very high cellular density. DNA was isolated using phenol–chloroform [22]. Concentration was adjusted to 1 mg ml^{-1}.

Micrococcus lysodeikticus and *V. anguillarum* were used as Gram positive and Gram negative stimuli respectively. *M. lysodeikticus* was growth in LB medium at 37 °C and *V. anguillarum* was cultured as previously described. Once grown, bacteria were resuspended in sterile filtered seawater in order to obtain an OD_{620} of 0.033 (1.6×10^7 cfu of *Vibrio* ml^{-1} and to 1.2×10^6 cfu of *Micrococcus* ml^{-1}).

2.3. In vivo and in vitro stimulation

A total of 20 naïve mussels were used to perform the *in vitro* experiments using haemocytes primary cultures. Four pools of haemolymph from 5 naïve animals per pool were collected using the methodology previously described, pooled and dispensed into 96-well plates (ROS release and NO production) or into 24-well plates (phagocytic activity). The remaining volume of each pool (between 2 and 5 ml) was used to perform the molecular studies (Q-PCR). Mussel haemocytes were incubated with a PAMPs solution (final concentration of 50 μg ml^{-1}) or live bacteria (OD_{620} 0.033) in order to evaluate their ability to modulate the production of oxygen and nitrogen radicals, the phagocytic activity (only determined in the case of bacterial stimulation) and the immune-related genes expression. All measurements were performed after 1, 3 and 6 h post-inoculation. For each experiment four pools of haemolymph from five animals were used.

All the experiments were performed at 15 °C and they were carried out twice at least.

In order to determine the *in vivo* effects of several PAMPs and live bacteria on mussel immune functions, eight groups, of 48 naïve animals each, were inoculated intramuscularly (i.m.) on the posterior adductor muscle, with 100 μl of a solution of each PAMP (poly I:C, zymosan, LPS, LTA or CpG; 1 mg ml^{-1}) or live bacteria (*V. anguillarum* or *M. lysodeikticus*; OD_{620} 0.033). The remaining group was inoculated with an equivalent volume of FSW and it was used as control. All individuals were maintained out of the water for 20–30 min before and after the injection. Each treatment group was individually maintained in tanks with aeration until the sampling moment (3, 6 and 24 h post-inoculation). Haemolymph from the adductor muscle was collected using a disposable syringe without anti-clotting agent, pooled and the cell concentration adjusted to 2×10^5 cells ml^{-1} with FSW. A total of 4 pools of 4 individuals were used in each treatment and each time. Haemolymph belonged to each pool was used to analyse several functional parameters such as measurement of oxygen and nitrogen radicals release and phagocytic ability (measured only in the case of bacteria stimulation). In addition the remaining volume of haemolymph (between 2 and 5 ml) from each pool was used to measure gene expression.

2.4. Respiratory burst

The haemocytes' respiratory burst was determined by measuring the chemiluminescence (CL) of mussel haemocytes in 96-well plates. The emission of relative luminescence units (RLU) was determined after the stimulation of the cells with a phorbol myristate acetate (PMA, Sigma–Aldrich, Co) or zymosan A (Sigma) and amplified by the addition of 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol, Sigma). A stock solution of luminol 0.1 M was prepared in dimethyl sulphoxide (DMSO, Sigma) just before use and this was diluted in FSW (working solution) to obtain a final concentration of 10^{-4} M.

Zymosan A was previously boiled for 30 min, washed twice in FSW, resuspended in FSW (20 mg ml^{-1}) and diluted in the luminol working solution to get a final concentration of 1 mg ml^{-1}. PMA stock (1 mg ml^{-1} in ethanol) was also diluted in the luminol working solution to get a final concentration of 1 μg ml^{-1}.

Haemolymph was dispensed into 96-well plates (100 μl per well) and after 30 min of incubation at 15 °C, 50 μl of the working solution of luminol alone or with PMA or zymosan A was added per well. Just after the addition of the solutions into the wells, generation of CL was measured in a luminometer (Fluoroskan Ascent, Labsystems, Vantaa, Finland) six times at intervals of 5 min with an integration time of 1000 ms in each measure. In each individual experiment, several pools of haemolymph were assayed, and triplicate wells were used for all the treatments.

Several PAMPs and two types of live bacteria solutions, previously described, were incubated with a primary culture haemocytes (*in vitro* experiments) or injected into the adductor muscle (*in vivo* experiments) during 1, 3 and 6 h or 3, 6 and 24 h respectively, to determine the following parameters:

1) The direct effect of the incubation/injection with PAMPs and live bacteria on the triggering of the respiratory burst.
2) The effect of the incubation/injection with PAMPs and live bacteria on the PMA or zymosan-mediated respiratory burst, in order to determine the putative modulatory ability of PAMPs and bacteria on the known response of the PMA or zymosan on the haemocytes ROS production.

2.5. Analysis of NO production

The haemocytes NO production was assayed by the Griess reaction [23] which quantifies the supernatants' nitrite content, since NO is an unstable molecule and degrades into nitrite and