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Short communication

# Individual variability of mytimycin gene expression in mussel

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ABSTRACT

The antifungal peptide mytimycin (MytM) is synthesized by hemocytes of the Mediterranean mussel, *Mytilus galloprovincialis*. In addition to sequence and gene structure diversities previously reported from pooled hemocytes, the present report focused on the expression of *mytm* gene in individual *M. galloprovincialis*, before and after challenge. Within untreated mussel, MytM mRNA was observed by ISH in about 42% of circulating hemocytes, characterized by large, diffuse nucleus. Injection with *Fusarium oxysporum* increased such percentage, but in only some of the mussels. Similarly, MytM gene expression increased after injection in only some of the mussels, as measured by qPCR. Responders and not responders are common evidence in any given population of organisms. Nevertheless, even if the use of proper pool size selection has been practised to find out and evaluate the most common response trends, individual analyses must be regarded as optimal.



## 1. Introduction

Several bivalve functions and/or gene expressions showed extended variability of responses according to environmental stressors (reviewed by [11]). When addressing to mussels, physiological responses were routinely measured on pools of 8–10 individuals to minimize such not understood individual variability [2,4,14]. Moving to genomic structure analysis, it was only recently that the nucleotide sequences of the antimicrobial peptides, mytilin B [18] and myticin C [7], were reported as different from one mussel to another. In addition, intra-individual diversity of myticin C [7], C1q domain-containing protein [10] and fibrinogen-related proteins (FREPs) [22] were observed. We previously reported on polymorphism of the antifungal peptide mytimycin (MytM) from the Mediterranean mussel, *Mytilus galloprovincialis*, with the existence of 16 nucleotide sequences translated into 6 amino acid sequences [23]. Among various challenges with bacteria and fungi, only injection of *Fusarium oxysporum* was able to increase the mytm gene expression with maximum expression observed 9h post-challenge, as measured on pools of hemocytes from 10 mussels [24]. In addition to such analyses performed on pools, the present report focused on the expression of MytM gene in individual *M. galloprovincialis*, before and after injection with the filamentous fungus, *F. oxysporum*. Two techniques have been applied, (i) qPCR quantifying MytM mRNA and, (ii) ISH visualizing and counting the MytM-expressing hemocytes.

## 2. Material and methods

### 2.1. Mussels, fungus, in vivo challenge and sampling

Adult mussels, *M. galloprovincialis* (6–7 cm shell length) from Palavas (Mediterranean Sea-France), filamentous fungus, *F. oxysporum*, injection and hemocyte collection 9h post-injection corresponding to maximum MytM expression, were as previously reported [24]. Posterior adductor muscle have been dissected 9h after injection, immediately immerged in liquid nitrogen and grounded into 1 ml of Trizol. For *in situ* hybridization (ISH), freshly collected hemocytes have been fixed by overnight incubation in 10% neutral buffer formalin (NBF: 46 mM $Na_2HPO_4$, 30 mM $NaH_2PO_4$, 3.7% formaldehyde in distilled water, pH 7), washed by

*Abbreviations:* AMP, Antimicrobial peptide; ISH, *In situ* hybridization; MytM, Mytimycin; qPCR, Real-time or quantitative PCR.

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15 min centrifugation at 800×*g*, 4 °C and deposited onto poly-lysine coated glass slides [6].

### 2.2. RNA extraction, reverse transcription and qPCR

Total RNA has been extracted according to the RNeasy Mini kit protocol (Qiagen). Quantity has been evaluated on NanoDrop ND-1000 and quality checked by electrophoresis on Nano LabChip (Agilent Technologies). First strand cDNA synthesis has been performed on 1 μg of total RNA using hexaprimers (Invitrogen) and murine leukemia virus reverse transcriptase (Promega). Primers for house keeping gene 28S rRNA were from [19]. Primers for MytM mRNA were from [24] and designed within conserved sequences regarding mussels from Palavas. Measurement of gene expression using SYBR Green qPCR technique on LightCycler 480 384-wells plate, and quantification by the Livak $2^{-\Delta\Delta CT}$ method expressed as *x*-fold the expression adjusted to 1 in untreated, have been previously reported [24]. A threshold of 4 times the expression in untreated was considered as the limit for significant up-regulation [13]. Statistical tests related to low number of assays, used the Mann–Whitney test. *P* values lower than 0.05 revealed significant differences.

### 2.3. In situ hybridization (ISH) on hemocytes

MytM amplicon has been synthesized by PCR using qPCR primers and GoTaq DNA polymerase (Promega), then cloned using pCR II-TOPO plasmid (Invitrogen). Such amplicon did not discriminate between MytM variants and covered all the different forms. Antisense and sense digoxigenin labeled riboprobes have been synthesized from the plasmid using the digoxigenin-RNA Labeling Kit SP6/T7 (Roche). Proteinase-K (Sigma) treatment, hybridization with 200 ng/μl denaturized riboprobes, incubation with sheep anti-digoxigenin Fab fragments (Roche) conjugated to alkaline-phosphatase, incubation in nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) solution (Promega), counterstaining with Bismarck Brown Y (Sigma), dehydration and mounting have been previously reported for lysozyme [13]. Observations, counting and pictures have been done with a photonic microscope Leica DMR (Leica Microsystems, Wetzlar-Germany). Statistics as in Section 2.2.

## 3. Results and discussion

### 3.1. Quantification MytM gene expression

#### 3.1.1. In circulating hemocytes

Nine hours after injection with *F. oxysporum*, five mussels did not show significantly different stimulation compared to untreated ($p = 0.73$) (Fig. 1). These mussels, nos. 1, 3–5 and 9, with expression below the threshold of 4, were considered as non-responders. On the opposite, folds of expression dramatically increased in 4 of the challenged mussels with a mean value of $36.6 \pm 17.3$, significantly different from untreated ($p < 0.001$) and from the five non-responders ($p < 0.05$). Obviously these four mussels responded to the challenge by increasing the expression of MytM gene; they were qualified as responders. The present study involved only a restricted number of mussels. Meanwhile, it appeared clearly that only some mussels reacted to the injection with *F. oxysporum* by increasing the expression of MytM gene.

In all the previously reported negative effect of stress and injections on AMP [5,24], lysozyme [13] and HSP70 [4] gene expressions measured on pools, one can hypothesized that we missed the few reacting mussels. Even if such treatments resulted in up-regulation in some mussels, such effect was minimum and not sufficient to increase the fold expression of the pool to a value significantly different from untreated.

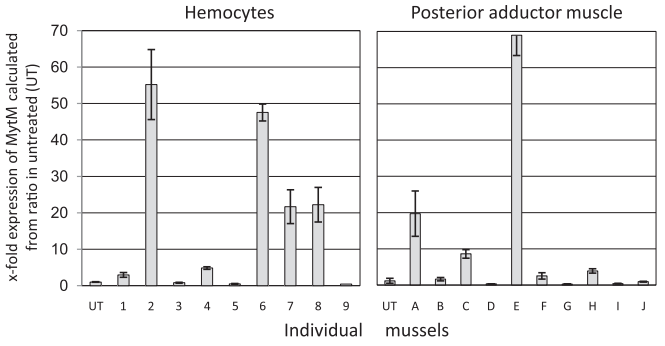


**Fig. 1.** Relative quantification of MytM mRNA in circulating hemocytes (left panel) from 9 mussels (1–9) and in posterior adductor muscle (right panel) from 10 mussels (A–J), 9 h after challenge with one injection of $10^4$ *F. oxysporum* spores into the posterior adductor muscle. Quantifications as x-fold expression compared to 1 in untreated (UT). Values are from the arithmetic mean of 5–7 replicates ± SD (bars) of individual mussel, except for UT which values are from pools of 10 individuals each. Note that only some mussels presented an up-regulation of MytM gene expression: 4 regarding hemocytes and 3 regarding muscle.

#### 3.1.2. In posterior adductor muscle

Although reverse transcriptions have been always performed with 1 μg of total RNA, differences in MytM mRNA quantifications we measured might result from variable numbers of circulating MytM gene expressing hemocytes. Indeed, we previously reported that lysozyme-expressing hemocytes accumulated within sinuses of the posterior adductor muscle where injection took place. Optical observation of ISH revealed enlarged sinuses blocked with aggregated hemocytes [13]. Similarly, considering MytM gene expressing hemocytes would be trapped in muscle sinuses, present quantification of MytM mRNA from circulating hemocytes addressed principally non-MytM gene expressing sub-population of hemocytes. Meanwhile, the needle used for sampling might extract variable numbers of MytM gene expressing sinus-adhering hemocytes, leading to variability in the detected levels of MytM transcripts. To control such hypothesis, MytM transcript levels have been quantified from RNA extracted from the entire posterior adductor muscle 9 h after injection with *F. oxysporum* (Fig. 1). Among the 10 dissected muscles, 7 did not show significant stimulation of MytM gene expression ($1.41 \pm 1.37$, $p = 0.6$) compared to untreated. On the opposite, three mussels presented significant up-regulation ranged from $8.6 \pm 1.2$ ($p < 0.001$) to $68.9 \pm 5.6$ ($p < 0.001$).

Consequently, the variability of MytM gene expression measured in hemocytes was not due to the sampling process, but to the fact that not all the mussels reacted to the challenge with *F. oxysporum*. As we tested only 9 and 10 mussels, any percentage on reacting versus non-reacting mussels will not be valid. In addition, we cannot exclude that technical injection problems resulted in ineffective or partially effective fungal injections, explaining absence or erratic responses. We previously reported that injection of $10^3$ *F. oxysporum* spores did not significantly up-regulated the MytM gene expression [24]. Only larger batch of injected mussels might confirm the existence of non-responders.

### 3.2. Cytology of MytM-expressing hemocytes

Optical microscopy observation of hemocytes from untreated mussels revealed the presence of two cell categories mainly based on nucleus aspect: small, dense with condensed chromatin versus