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Gene expression specificity of the mussel antifungal mytimycin (MytM)

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ABSTRACT

We previously reported the nucleotide sequences and diversity of mytimycin (MytM) from the Mediterranean mussel, *Mytilus galloprovincialis*. Using real-time PCR (q-PCR), we observed that the *MytM* gene was mainly expressed in circulating hemocytes and to a less extent in the mantle. *In vivo* challenge with bacteria or with the yeast, *Candida albicans*, did not increase the expression as measured by q-PCR in hemocytes. By contrast, injection of the filamentous fungus, *Fusarium oxysporum*, induced a sudden and strong increase of expression at 9h p.i. (stimulation index of 25.7 ± 2.1). Optimum stimulating dose was 10^4 spores of *F. oxysporum* per mussel. In the same samples, AMP mytilin and myticin showed no stimulation. Consequently, we hypothesized the existence of 2 different signal transduction pathways, one activated by bacteria and yeast, the other triggered by filamentous fungi. A second challenge performed with *F. oxysporum* 24 h after the first challenge induced an increase of *MytM* gene expression (stimulation index of 3.5 ± 1.7). However, this second increase was significantly lower than the first, suggesting less efficient response rather than significant protection.

1. Introduction

Mytimycin (MytM), the first strictly antifungal protein from mollusks, has been partially identified from the blue mussel, *Mytilus edulis*, in 1996 using biochemical techniques and Sanger's sequencing [7]. Recently, we isolated 16 nucleotide sequences related to MytM from a *M. galloprovincialis* EST library [37] and established the full cds of the precursor [34]. It possesses the same construction as other antibacterial peptides: defensin, mytilin and myticin, i.e. a signal peptide followed by the mature MytM of 54 aa, and a C-terminal extension. In addition, 2 major deduced aa precursor sequences were observed, one shared by the mussels from Venice-Italy and Vigo-Spain, the other belonged to those mussels from Palavas-France. Predicted disulfide bonds of these 12 cysteine peptides suggested the presence of 2 constrained domains [34]. Recently, a partial 47 aa (no cysteine) antifungal peptide, but sharing also a weak effect on bacteria, has been isolated from a native Chilean scallop, *Argopecten purpuratus*, and used as framework to design substitutes for conventional antibiotics [2].

Occurrence of antifungal molecules and their induction have been reported in insects. In *Drosophila*, the antifungal cysteine-rich *drosomycin* [15] is strongly up regulated by fungal infection, mediated by the Toll pathway, and only slightly by the Imd pathway [26] Antibacterial *attacin* and *cecropin* genes are activated by fungi and bacteria, as well as *metchnikowin*, which is also antifungal ([20]). By contrast, strictly antibacterial *diptericin* and *drosocin* genes are mainly activated by Gram-negative bacteria through the Imd pathway [19]. Another inducible antifungal peptide, but also active against Gram-positive and Gram-negative bacteria, thanatin, has been isolated from the insect, *Podisus maculiventris* [14].

Fusarium oxysporum, a diploid filamentous fungus widely distributed in soil and water, was reported as having an extremely broad host range. Although responsible for only opportunistic infections in immune depressed humans, pathogenic activity was particularly frequent toward insects [17] and crustaceans [13,23] through the production of various toxins. *F. oxysporum* has been used in antimicrobial assays in shrimp [11] and mussels [29].

Candida albicans, a diploid fungus or yeast, is the most common human fungal pathogen. It is normally a harmless commensally organism, but it can be an opportunistic pathogen responsible for painful mucosal infections. Although not considered as an

Abbreviations: aa, amino acids; AMP, antimicrobial peptide; cds, coding sequence; EST, expressed sequence tags; MytM, mytimycin; nt, nucleotides; p.i, post-injection; q-PCR, quantitative or real-time PCR.

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important contaminant of seawater, *C. albicans* is frequently detected in the Mediterranean Sea [25]. According to the European Union Bathing Water Standards based on the presence of enterococci, 35% of the waters classified as "good" and 39% of the "excellent" contained *C. albicans*, confirming its extended repartition [12]. In an experimental assay, *C. albicans* survived at least 52 weeks in seawater, with no effect of salinity but a strong negative effect of elevated temperature with a survival limited to 5–6 weeks at 35 °C [1]. In addition, *C. albicans* was reported from all sites sampled in a rain forest freshwater stream and in near-shore coastal tropical waters of Puerto-Rico [35]. Not only in water was *C. albicans* detected, but also in oysters and mussels particularly during the colder months [4] revealing its possible transmission to humans through the consumption of seafood.

Beta-glucans are polysaccharide chains of D-glucose monomers linked by beta type glycoside bonds. They constitute the major structural components of cell wall from the yeast, *Saccharomyces cerevisiae*. Due to their capacity to enhance the innate immune system [8], they are extensively used as feed additives for cultivated crustaceans, resulting in increased survival in ponds [6]. In our study, β-glucans preparation has been used to replace intact yeast in the assays for regulating *MytM* gene expression.

In the present report, we established by q-PCR (i) the comparative expression of *MytM* gene in various tissues, (ii) the optimum dose-effect of filamentous fungus *F. oxysporum* spores, (iii) the specificity of induction by comparing the responses to *F. oxysporum* with *C. albicans*, β-glucans and bacteria injections, (iv) the response of antimicrobial peptide *mytilin* and *myticin* genes in fungal challenged mussels, and (v) measured *MytM* gene expression after a second challenge.

2. Material and methods

2.1. Mussels and fungi

Adult mussels, *Mytilus galloprovincialis* (6–7 cm shell length), were purchased from the marine farm Les Compagnons de Maguelone (Palavas-France) out of the reproduction period. They were acclimated for 24–48 h in the laboratory airlift system of oxygenated re-circulating seawater at 20 °C before challenge. Filamentous fungus, *F. oxysporum,* and yeast, *C. albicans,* were a gift from Prof. Alain Clérivet (IUT Département Génie Biologique, Montpellier-France). They were allowed to grow on 1.5% agar PDA (Potatoes Dextrose Agar, Sigma–Aldrich) in Petri dishes incubated at room temperature in the dark. After 2–4 days, the dishes were washed with phosphate buffered solution isotonic to seawater (PBS-NaCl: 2 mM KH_2HPO_4, 10 mM Na_2HPO_4, 3 mM KCl, 600 mM NaCl in distilled water, pH 7.4). The collected liquid was filtered through cotton wool and the spore number adjusted to 2×10^5/ml with PBS-NaCl. Beta-glucans containing β-(1→3) and β-(1→6) components, were from baker's yeast *S. cerevisiae* (Sigma). They were diluted in PBS-NaCl as a stock solution of 330 μg/ml.

2.2. In vivo challenges, RNA extraction and reverse transcription

Routine challenges with fungi consisted of one injection of 2×10^4 spores in 100 μl PBS-NaCl into the posterior adductor muscle. Challenge with β-glucans consisted of one injection of 100 μl of PBS-NaCl containing 33 μg of β-glucans into the posterior adductor muscle. After injection, mussels were returned to seawater at 20 °C. cDNAs from mussels challenged with bacteria were from Li et al [22]. Briefly, mussels from Palavas-France were challenged by one injection of 10^7 *Vibrio splendidus* LGP32, *Vibrio anguillarum* (Gram-) or *Micrococcus lysodeikticus* (Gram+) into the posterior adductor muscle and returned to seawater at 20 °C.

Hemolymph was collected from the posterior adductor muscle with a 1 ml syringe containing 0.2 ml of the anti-coagulant modified Alsever's solution buffer, before challenge (UC) and 1, 3, 6, 9, 12, 24, 48 and 72 h post-injection (p.i.). Hemolymph from 10 mussels per time point was pooled and hemocytes pelleted by 15 min centrifugation at 800 g, 4 °C. To analyze constitutive expression in tissues, hemolymph was collected, and gills, hepatopancreas, mantle, muscle and foot were dissected from 5 unchallenged mussels and pooled. For dose-effect responses, 10 mussels per dose were injected with 100 μl of PBS-NaCl containing 0, 10^2, 10^3, 10^4 and 10^5 spores of *F. oxysporum* and hemolymph collected at 9 h p.i. For second challenge assays, 10 mussels per end point were injected with 10^4 spores of *F. oxysporum*, and then re-injected 24 h later with 10^4 spores of *F. oxysporum*. Hemolymph was collected as above and all experiments have been repeated 4 times.

Total RNA was extracted using the Trizol Reagent protocol (Invitrogen). Quantity of RNA was evaluated by spectrophotometer using NanoDrop ND-1000 (NanoDrop Technologies). Quality was assayed by electrophoresis using Agilent RNA Nano LabChip (Agilent Technologies). First strand cDNA synthesis was performed on 1 μg of total RNA using random primers (Promega) and murine leukemia virus reverse transcriptase (Promega), and kept in nuclease-free water at −20 °C until use.

2.3. Quantitative PCR (q-PCR)

Primers for house keeping gene 28S rRNA, mytilin and myticin mRNA quantifications were from previous report [5]. Mytimycin primers were F2 ($^{5\prime}$-GTGTTGTCATTGGCATGGCG-$^{3\prime}$) and R2 ($^{5\prime}$-TCGTCCATGATTGACCAATG-$^{3\prime}$) giving a unique amplicon of 367 nt with a melting temperature of 82.34 ± 0.08 °C. q-PCR was performed using the SYBR Green chemistry on a LightCycler 480 × 384 well-plate (Roche Diagnostics) as previously reported [21] with 1 μM of each primer and 65 °C of hybridization temperature.

Gene expression levels were quantified by the Livak ($2^{-\Delta\Delta CT}$) method using the ratio of equivalent target amount (ETA) for the considered gene on ETA for *28S rRNA*. Internal reference of 28S expression was measured in each time point as recommended [10]. Normalization of the ratios was calculated considering each ratio equal to 1 in unchallenged mussels and expressed as x-fold the ratio for unchallenged mussels. Results were expressed as the arithmetic mean of 3–4 different experiments ± SD. Statistical analyses for low numbers of assays, used the Mann–Whitney test ($P < 0.05$).

3. Results

3.1. Expression pattern in tissues

As measured by q-PCR, the presence of MytM mRNA in gills, hepatopancreas, muscle and foot appeared marginal (Fig. 1). Clearly hemocytes had the highest content of MytM mRNA, a level significantly different from all other tissues ($p < 0.001$). Only in the mantle was there a noticeable expression, about 6 times higher than in gills, but significantly lower when compared to hemocytes ($p = 0.003$). Consequently, further quantification of *MytM* gene expression will be restricted to hemocytes.

3.2. Dose-effect response of F. oxysporum

Increasing quantities of spores were injected into mussels and the expression of *MytM* gene measured at 9h p.i. (Fig. 2). In PBS-injected mussels, as well as in mussels injected with lower spore concentrations (10^2 and 10^3), no significant changes in expression were observed. In contrast, injecting higher doses resulted in

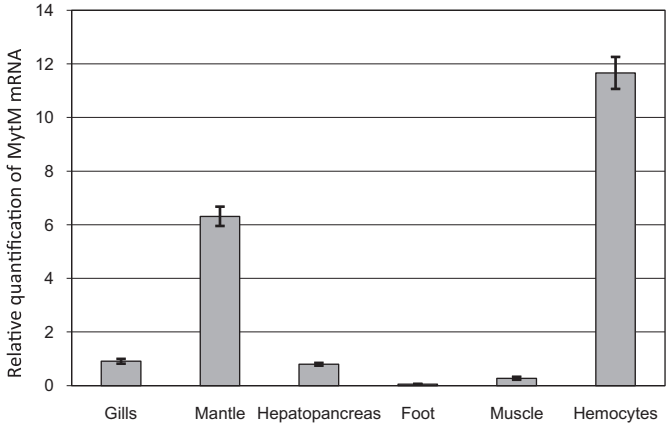

Fig. 1. Quantification of MytM mRNA in various tissues expressed as the mean of $2^{-\Delta\Delta cp}$ for 3 to 4 different samples related to 28S mRNA ± SD (bars). Note the largest expression in hemocytes, a value significantly different from mantle ($p = 0.003$) and from gills, hepatopancreas, muscle and foot ($p < 0.001$).

significantly elevated expression. Such increase was 12.6 ± 2.9 folds that of those unchallenged ($p = 0.0004$) with 10^4 spores and was still significantly higher with 10^5 spores (2.1 ± 0.02 folds, $p = 0.02$).

3.3. Specificity of regulation

Kinetics and specificity of the induction of *MytM* gene expression were investigated in pools of mussels injected with bacteria previously obtained for other experiments ([22]). In mussels injected with different bacteria (*V. splendidus*, *V. anguillarum* and *Micrococcus lysodeikticus*), no significant changes in expression were observed (Fig. 3). Similarly, injection of the yeast, *C. albicans,* did not increase the expression of *MytM* gene. By contrast, injection of spores from the filamentous fungus, *F. oxysporum,* resulted in a rapid and significant up-regulation of *MytM* gene expression peaking suddenly at 9 h p.i., with a stimulation index of 25.7 ± 2.1 folds the expression in unchallenged ($p < 0.001$). Later, the intensity of *MytM* gene expression decreased gradually, but was still

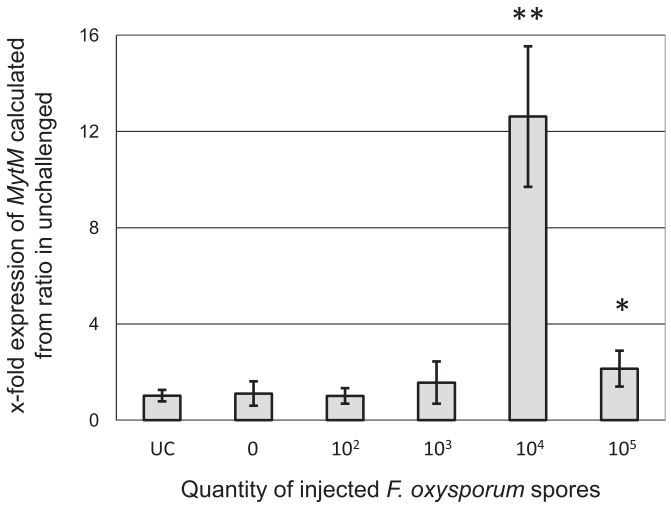

Fig. 2. Dose-effect response of *MytM* gene expression following one injection of 100 µl of PBS-NaCl (0) alone, or containing various quantities of *F. oxysporum* spores. Data are from 3 to 4 pools of 10 mussels each per injected quantity, measured by q-PCR and expressed as multiplicand factors related to expression in unchallenged (UC) ± SD (bars). Note the strongest up-regulation observed with 10^4 spores. *: $p < 0.05$; **: $p < 0.001$.

7.3 ± 0.2 folds at 48h p.i. ($p = 0.006$). The highest stimulation observed at 9h was statistically significantly different from that observed at 12h (11.6 ± 1.0, $p < 0.001$). Injection of β-glucans induced a discrete increase of *MytM* gene expression only after 48h (4.2 ± 1.4) but significantly different from expression in unchallenged ($p = 0.01$).

Specificity of the *MytM* gene response was investigated measuring the kinetics of expression of the antimicrobial peptide (AMP) genes, *mytilin* and *myticin,* in the fungi injected mussels (Fig. 4). Maximum stimulation index was 3.1 ± 0.4 (*myticin* induced by *F. oxysporum* at 48 h), the only observed significantly different up-regulation ($p = 0.002$). All other indices were below 2.4, and insignificantly different from unchallenged, regardless of stimulation or the time p.i.

3.4. Response to a second challenge

Mussels were re-injected with *F. oxysporum* 24 h after the first, and *MytM* gene expression measured between 1 and 72 h after this second challenge (Fig. 5). Significant up-regulation was observed at 9 h after the first injection (14.0 ± 1.4, $p < 0.001$ compared to unchallenged). This confirmed that the new batch of mussels reacted as it had been previously observed (Fig. 3). Consequently, and based on Fig. 3 data, we considered that expression at 24h returned to baseline. Second challenge performed 24 h after the first one resulted in up-regulation measured at 9h (3.5 ± 1.7, $p = 0.03$ compared to unchallenged) and lasted at least 72 h (4.0 ± 0.8, $p < 0.001$ compared to unchallenged). Meanwhile, the kinetics observed following first and second challenge were different. Compared to Fig. 3, no peaking occurred at 9 h and the average stimulation index of only 3.5 was maintained until 72 h, when the first stimulation gradually decreased and returned to baseline at 24 and 72 h. In addition, the stimulation index recorded 9 h after the second challenge was statistically significantly different ($p = 0.001$) from the stimulation index measured after the first challenge.

4. Discussion

Used for decades, extracts from fungal cell walls containing β-glucans have been found to trigger various immune defense mechanisms in plants, invertebrates and even vertebrates. Effect of β-glucans on immune mechanisms of invertebrates was reviewed in 2004 involving insects, crayfish, shrimp and earthworms [38]. However, the role of β-glucans on AMP induction was poorly investigated. Only cecropin, one of the *Drosophila* AMPs, is induced by β-glucans in hemocytes *in vitro* ([33]). In the mussel *M. galloprovincialis*, β-glucans exert no effect on antibacterial activity, but increased the *in vitro* production of nitric oxide and the release of free oxygen radicals by hemocytes [24]. By contrast, the same authors reported that hemolymph collected from *Ruditapes decussatus* injected with β-glucans, inhibited the growth of *V. splendidus*, *Vibrio alginolyticus* and *E. coli.* Unfortunately, the active moiety was not identified.

In a previous report we analyzed the diversity of MytM mRNA purified from hemocytes [34]. In the present work, we quantified such mRNA in several tissues. Clearly hemocytes had the highest content, i.e. more than 10 times higher than in gills. Considered as an immune molecule, is it not surprising that *MytM* gene is expressed mainly in hemocytes, which represent the most active immune cells of mussels. The mantle also contained noticeable *MytM* gene expression, but half the expression in circulating hemocytes. Detectable expression at least in the mantle and gills probably resulted from hemocyte infiltration as we reported for defensin, mytilin, and myticin mRNAs [30].

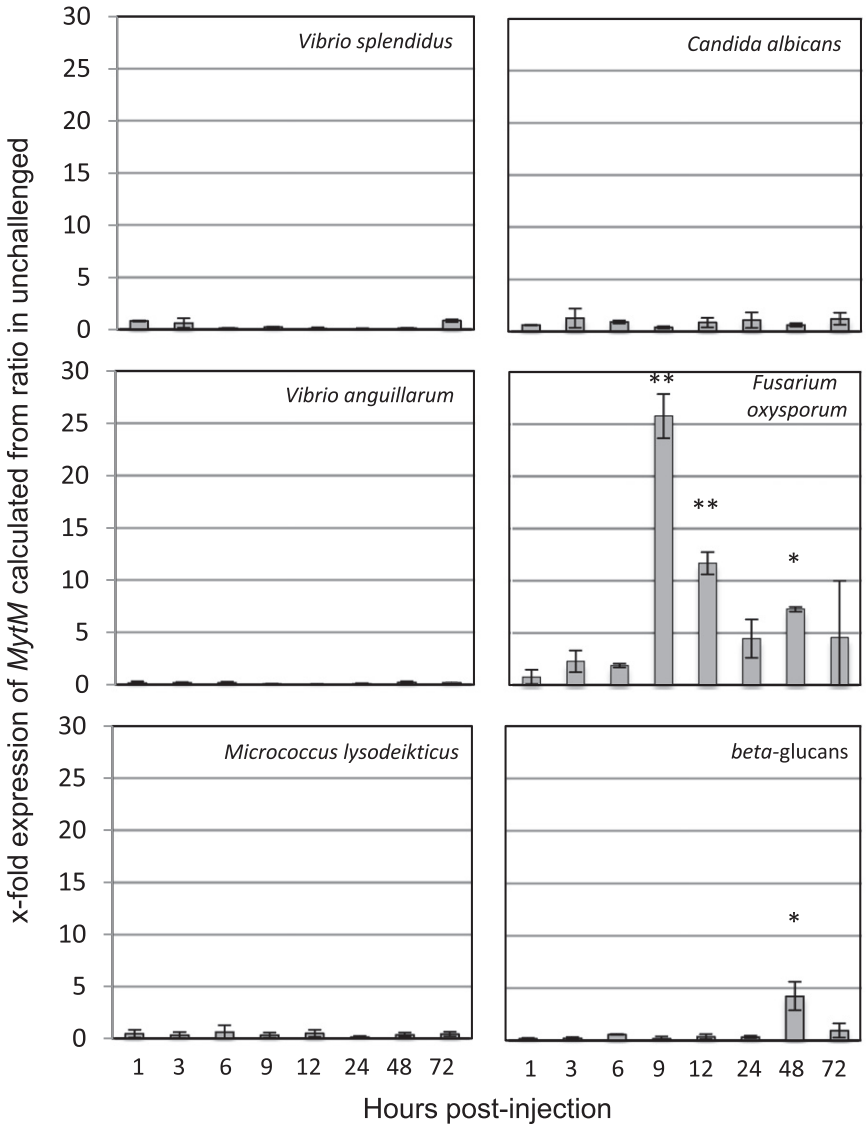

Fig. 3. Kinetics of *MytM* gene expression in response to one injection of Gram-negative (*V. splendidus, V. anguillarum*), and Gram-positive (*M. lysodeikticus*) bacteria compared to one injection of yeast (*C. albicans*), filamentous fungus (*F. oxysporum*) and fungal cell wall extract (*beta*-glucans). Data are from 3 to 4 pools of 10 mussels each per end point, measured by q-PCR and expressed as multiplicand factors related to expression in unchallenged ± SD (bars). *: $p < 0.05$; **: $p < 0.001$. Note the strongest up-regulation observed with *F. oxysporum*.

Mussel defensin, mytilin and myticin shared antibacterial and antifungal properties. In fact, the so-called antifungal activity has been tested only against filamentous fungi: *Neurospora crassa* for *M. edulis* [7] and *F. oxysporum* for *M. galloprovincialis* [31]. Challenge with Gram-positive *M. lysodeikticus* resulted in general down regulations of AMP gene expression [5]. Gram-negative challenge effect was more complex with rapid up-regulation (from 1 to 24 h) of *defensin* by *V. splendidus*, to late up regulation (from 1 to 3 days) of *mytilin* by *V. anguillarum*. In another study, the 3 AMPs were reported as down regulated by all challenging bacteria whatever geographic origin of the mussels [22], confirming previous observations done from 6 to 72h [28]. Assays through microarray devoted to *M. galloprovincialis* immunome revealed a general down-regulation of AMP genes following challenges with *V. splendidus*, and particularly *MytM* with relative expression of −3.02 compared to unchallenged at 48h [37]. In the present report, only the filamentous fungus, *F. oxysporum*, strongly stimulated expression of *MytM* gene. Stimulation appeared suddenly 9 h after injection, suggesting that a delay is required for recognition and signal transduction. Later, over expression gradually decreased, but was still significantly different from those unchallenged after 48 h. In contrast to induction of AMP gene expressions observed in *Drosophila* [16], no significant up-regulation of *MytM* gene was observed with the yeast, *C. albicans*, revealing that the two fungi induced different gene responses.

Injection of β-glucans, representing soluble extracts of the fungus cell wall, did not modify *MytM* gene expression, excepted a limited up-regulation observed at 48 h. Significant different responses to spores compared to soluble extracts suggested that stimulation requires solid particles. In fact, internalization by hemocytes is achieved by different mechanisms according to the size of the foreign body: pinocytosis for soluble particles, phagocytosis for solid ones. Therefore, it is hypothesized that triggering of *MytM* gene resulted from phagocytosis, a receptor-mediated endocytosis, requiring specific recognition. In addition, mussel innate immune system clearly discriminates between filamentous

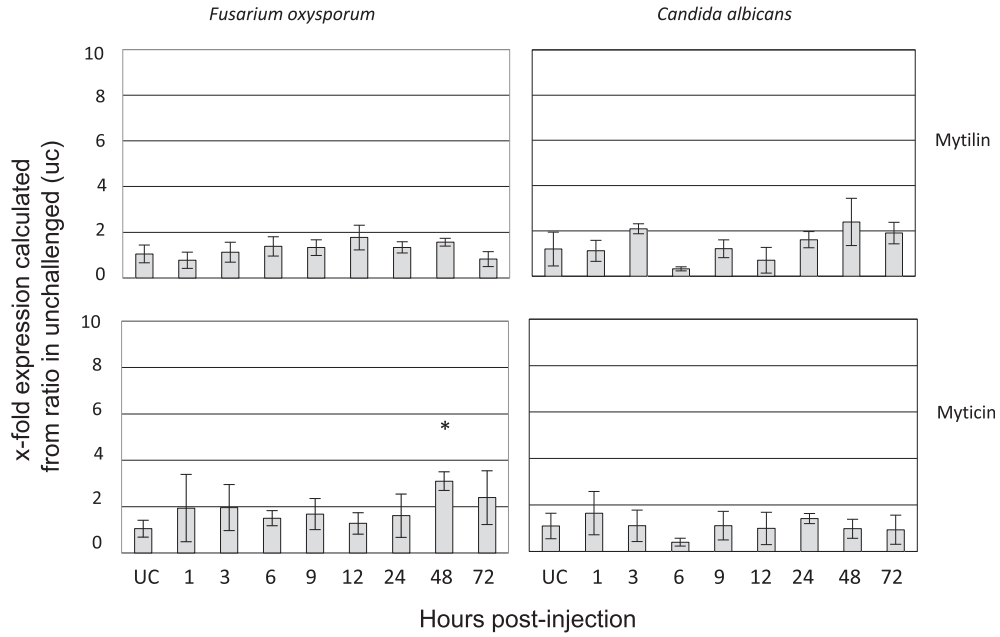

Fig. 4. Kinetics of expression of the AMP *mytilin* and *myticin* genes in response to one injection of the filamentous fungus (*F. oxysporum*) and of the yeast (*C. albicans*). Data are from 3 to 4 pools of 10 mussels each per end point, measured by q-PCR and expressed as multiplicand factors related to expression in unchallenged ± SD (bars). *: $p < 0.05$. Note the absence of stimulation higher than 3.1, the only statistically significantly different from unchallenged.

and yeast spores, the latest not modifying expression. As they are of similar sizes (6–9 μm for oval-spherical microconidia of *F. oxysporum* versus 2–7 × 3–8 μm for ovoïd blastoconidia for *C. albicans*), the different behaviors may result from differences in cell wall composition.

We demonstrated herein that injection of bacteria did not stimulate expression of the *MytM* gene. Whatever the Gram coloration, bacterial cell wall composition is different from the fungal. As MytM has been reported as strictly antifungal [7], one can hypothesize that its gene expression will depend on the recognition of specific fungal cell wall component(s), if presented in a solid form, and might use a different signal transduction pathway, as demonstrated for *drosomycin* [27]. Either *mytilin* or *myticin* genes were not up regulated by fungal challenge. Joined to the fact that bacterial challenges do not trigger *MytM* gene expression, this observation strongly suggests the existence of different recognition mechanisms or signal transduction pathways in mussels. This suggestion based on functional observations is reinforced by previous report on the putative presence of several MyD88 transcripts, including a Toll/IL-1 receptor domain, in the Zhikong scallop, *Chlamys farreri* [32], in the Manila clam, *Ruditapes philippinarum* [18] and in *M. galloprovincialis* hemocytes [37]. Existence of different signal transduction pathways is well documented in insects where the Toll pathway is engaged in antifungal defense, whereas the Imd pathway triggers different array of antimicrobial peptides [9]. Existence of these two pathways remains to be established in mollusks.

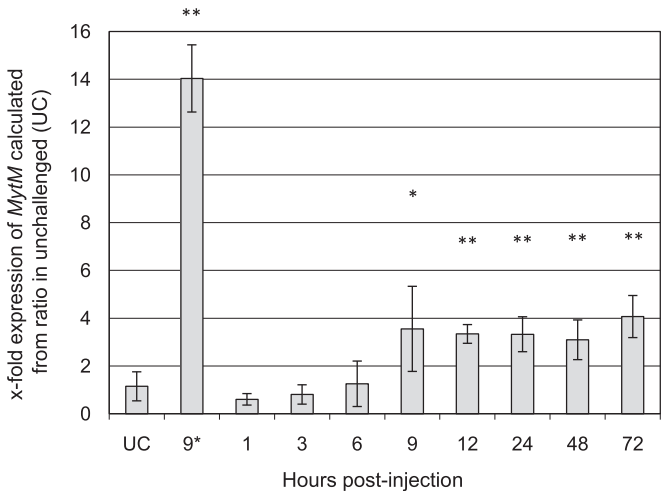

Fig. 5. Kinetics of *MytM* gene expression in response to a second injection of *F. oxysporum* performed 24h after the first challenge. Data are from 4 pools of 10 mussels each, measured in quadriplicate by q-PCR and expressed as multiplicand factors related to expression in unchallenged (UC) ± SD (bars). 9*: expression measured 9 h after the first challenge; 1 to 72: expressions measured after the second challenge; *: $p < 0.05$; **$p < 0.001$. Note the significant but lower expression 9h after the second challenge compared to the first challenge.

Immune memory in invertebrates is a controversial concept. We suggested that one way to resolve the debate might be to quantify the expression of particular genes or immune effectors following a first and a second challenge [3]. Surprisingly, reports of such studies are rare and based on unusual host-parasite associations, and no one regarding bivalves. With the actual technology to precisely quantify the expression of thousands of genes, it becomes possible to investigate the memory concept in invertebrates. Regarding mussels, we reported herein the expression of the sole *MytM* gene following a second challenge performed at a time the first response was lowered: no more rapid or intense response was noticed. By contrast, the second response to *F. oxysporum* challenge was significantly lower than the first suggesting a less efficient response more than a better protection and arguing against memory. It must be underlined that, to avoid additional stress, re-challenged mussels were not the ones sampled to control the stimulation at 9 h. We speculated that all the mussels up regulated at 9 h, returning to background of expression at 24 h, before the second challenge. Although individual variability exists among mussels, we hoped to exclude this bias by measuring 4 pools of 10 mussels each, as previously validated [5].

In conclusion, different responses according to different challenges suggested the existence of two signal transduction pathways, one activated by bacteria and yeast, the other triggered by filamentous fungi. Evidences of these two pathways might be obtained by screening Mytibase [36] looking for intermediate molecules similar to the ones known in insects.

Acknowledgments

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