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Toll-like receptors and MyD88 adaptors in *Mytilus*: Complete cds and gene expression levels

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ABSTRACT

TLR- and MyD88-related sequences have been previously investigated in Mytibase and then in new transcript reads obtained by Illumina technology from the mussel, *Mytilus galloprovincialis*. Based on full cds and domain organizations of virtual translations, we identified 23 Toll-like receptors (TLRs) and 3 MyD88 adaptors. *Mg*TLRs can be arranged in 4 clusters according to extra-cellular LRR domain content. *Mg*TLR-b, -i and -k were the only ones containing a multiple cysteine cluster (mccTLR), a domain composition also found in *Drosophila* Toll-1 and 18-wheeler. The 3 MyD88 we identified in *M. galloprovincialis* were also retrieved from *Mytilus edulis*, as well as *Mg*TLR-b and -i. All *Mg*TLRs were constitutively expressed in digestive gland whereas only 4 of them were also present in hemocytes. On the opposite, the 3 *Mg*MyD88s were constitutively expressed in all the tissues. *In vivo* challenge of *M. galloprovincialis* with bacteria caused the up regulation of only *Mg*TLR-i, but of all the 3 *Mg*MyD88s. Highest response was induced by Gram-negative *Vibrio anguillarum* at 9 h p.i. Injection of filamentous fungus, *Fusarium oxysporum*, resulted in up regulation of *Mg*TLR-i and *Mg*MyD88-c at 9 h p.i. Such similar pattern of responses suggested *Mg*MyD88-c represents the intra cytoplasm partner of *Mg*TLR-i. Their interaction constituted the first cellular event revealing the existence of a Toll-signaling pathway in Lophotrochozoa.

1. Introduction

Leucine rich repeat (LRR) domains are common structures of numerous molecules among which are Toll and Toll-like receptors (TLRs), an evolutionarily very successful family (reviewed by Leulier and Lemaitre (2008)). Across metazoans, these structurally related receptors are involved in immunity by being either the equivalent of cytokine receptors, for instance Toll in *Drosophila* recognized Spätzle (Hoffmann, 2003), or of pathogen-recognition receptors recognizing pathogen-associated-molecular-patterns (PAMPs) such as TLRs. Following binding of the ligand to the extra cellular segment, the intra cellular conserved Toll-interleukin-1-receptor (TIR) domain interacts with the cytosolic adaptor protein myeloid-differentiation-factor-88 (MyD88) (Muzio et al., 1997). The resulting signaling cascade ends with the translocation of the transcription nuclear-factor-κB (NF-κB) inside the nucleus, where it activates the expression of immune-related genes (Cornwell and Kirkpatrick, 2001). Partially depending on the cell type and stimulated cell surface receptors, MyD88 and interleukin 1-associated kinase (IRAK-4) can activate various transcription factors, which mediated a general and protective immune response (von Bernuth et al., 2012).

Initially, the name Toll was given to the product of 1 locus responsible for embryonic lethal mutants of *Drosophila melanogaster* (Nusslein-Volhard and Wieschaus, 1980). Later, Toll was indicated to mediate the fruit fly resistance to fungal infection (Lemaitre et al., 1996) and to control the expression of antimicrobial-peptide (AMP) genes (Tauszig et al., 2000). Both TLR and Toll are glycoproteins consisting of an assembly of multiple extra cellular LRR domains capped by characteristic N- and C-terminal sequences, but only TLRs detect PAMPs. Extra cellular region, including LRR domains, are largely variable whereas intra cellular region, including TIR domains, appears to be evolutionary conserved (Kanzok et al., 2004). Not only circulating immune cells express TLRs (reviewed by Szatmary (2012)), but also some cells from digestive epithelium in mammals (reviewed by Takahashi (2010)) and in insects (Wu et al., 2010), fibroblasts, endothelial and glial

Abbreviations: aa, amino acids; AMP, antimicrobial peptide; bp, base pair; cds, coding sequence; EF1-α, elongation factor 1-alpha; EST, expressed sequence tags; LRR, leucine rich repeat; mccTLR, multiple cysteine clusters in TLR; MyD88, myeloid-differentiation-factor-88; NF-κB, nuclear factor kappa B; nt, nucleotide; ORF, open reading frame; PAMPs, pathogen-associated molecular patterns; p.i., post-injection; sccTLR, single cysteine cluster within TLR; TIR, Toll-interleukin 1 receptor; TLR, Toll-like receptor; TM, transmembrane; SSW, sterile seawater.

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cells. In vertebrate, TLRs do not control AMP production but various cytokines and other immune pathways instead.

The myeloid-differentiation primary response gene 88 (MyD88) was originally discovered and cloned in mice (Lord et al., 1990). This protein is a universal intra cytoplasm adaptor containing a single death domain (Death) associated to a single downstream TIR domain, and used by TLRs to activate the transcription factor NF-κB through a complex cascade (Muzio et al., 1997).

As filter feeders, marine mollusks are exposed to multiple microorganisms present in the seawater. Most of them are beneficial, but some represent potential pathogens. Compared to other bivalves, the severe mortalities reported for mussels were not linked to any microorganisms (Myrand and Gaudreault, 1995), suggesting mussel anti infectious defense system is very efficient. Invertebrates rely on the sole innate immunity, and AMPs are the major effectors of their immune system. *M. galloprovincialis* AMPs have been extensively studied, (reviewed by Li et al. (2011)), including the regulation of their gene expression following experimental infections. However, we do not know how diverse are the Mollusks (Lophotrochozoa) TLRs, and how universal is the *Drosophila* (Ecdysozoa) Toll molecular architecture. Few sequences of mollusk TLR and MyD88 are available in databases but some wrong annotations limit our knowledge.

The aim of the present study was (i) to identify as much TLRs and MyD88s as possible in the Mediterranean mussel, *M. galloprovincialis*, (ii) to establish their complete nucleotide sequences, (iii) to look for phylogenic relationships, (iv) to measure their constitutive tissue-specific expression, and (v) to investigate their involvement in innate immune response by quantifying a gene expression following *in vivo* challenges with bacteria and fungus.

2. Material and methods

2.1. Mussel transcriptome mining and bioinformatic analysis

We used the 18,788 ESTs of mixed tissues previously obtained by Sanger sequencing (Venier et al., 2009) and the 286.4 millions of reads we obtained by paired-end (2 × 100 bp) Illumina Hiseq2000 RNA-sequencing of digestive gland (97 million reads) and gills (189.4 million reads, unpublished) from North Adriatic Sea mussels. Overall *de novo* assembly has been performed using Trinity (Grabherr et al., 2011) and the CLC Genomic Workbench v.5.1 (CLCbio, Aarhus, Denmark). First, the contigs generated by the CLC assembler have been translated into the six possible reading frames and translations have been scanned with HMMer 3 (Eddy, 2011) for the detection of TIR domain homology (IPR000157). MyD88 was identified by the contemporary presence of a TIR domain and of a Death domain (IPR000488). Fragmented transcripts of interest have been identified by comparison with the contigs generated by Trinity and, whenever possible, they were elongated by manual assembly. The process has been reiterated until 3′ and 5′ ends were reached. Uniform mapping of paired-end reads on complete sequences assessed correct reconstruction of full-length transcripts. Only transcripts encoding proteins containing a C-terminal TIR domain, a trans-membrane region and LRR N-terminal domains have been considered as putative TLRs.

Leucine-rich repeat (LRR), LRR-carboxy terminal (LRR-CT), LRR-amino terminal (LRR-NT) and TIR domains have been retrieved using SMART (http://smart.embl.de) and SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui), completed by manual alignments using already reported TLR sequences. Signal peptide region has been also predicted by SignalP-3.0 (http://www.cbs.dtu.dk/services/SignalP/) and trans-membrane domain detected with TMHMM v.2.0 (http://www.cbs.dtu.dk/services/TMHMM). Evolutionary relationships have been calculated by using the neighbor-joining algorithm (CLC Genomic Workbench v.6.6.2) based on total aa sequences (http://www.clcbio.com/products/clc-genomics-workbench/). Trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenic relationships.

2.2. Mussel samples and PCR

Adult wild Mediterranean mussels, *M. galloprovincialis*, have been collected in Palavas–Prévost (France) lagoon. *In vivo* challenges with bacteria were from (Li et al., 2010) and consisted in one injection of 10^7 bacteria (*Vibrio splendidus*, *V. anguillarum*, *Micrococcus luteus*). *In vivo* challenge with fungus was from (Sonthi et al., 2012) and consisted in one injection of 2×10^4 spores of filamentous fungus, *Fusarium oxysporum*. Briefly, 10 mussels per end point received one injection of 100 μl into the posterior adductor muscle. Controls were both the unchallenged mussels and mussels injected with 100 μl of sterile seawater (SSW). Complete sets of challenges and sampling have been performed 4 times. Hemolymphs have been collected into anti-coagulant modified Alsever's solution buffer from the posterior adductor muscle and pooled. Hemocytes have been pelleted by centrifugation and RNA extracted by Trizol Reagent (Invitrogen). Good conservation of RNA kept at −80 °C for several years has been checked by spectrophotometer using NanoDrop ND-1000 (NanoDrop Technologies) for quantity and by electrophoresis using Agilent RNA 6000 Nano LabChip (Agilent Technologies) for quality. To evaluate constitutive and tissue-specific expression, total RNA from hemocytes and from dissected foot, digestive gland, muscle, gills and mantle, was extracted by Trizol Reagent (Invitrogen) from 1 pool of 3 mussels and purified by precipitation with sodium acetate 0.3 M. First strand cDNA has been synthesized from 1 μg of total RNA using 0.5 μg of hexaprimers (Promega), 2 mM dNTPs (Promega) and 200 U of murine leukemia virus reverse transcriptase (M-MLV RT, Promega) in 25 μl final volume. Reverse transcriptase products have been diluted 1/10 in nuclease-free water for end-point PCR and 1/20 for qPCR, and kept at −20 °C until use.

Primers to be used in PCR and qPCR have been designed using the LightCycler Probe Design 2.0 software V.1.0.R.36 (Roche) and completed by hand optimization regarding specificity and efficacy. Among the numerous possibilities addressing *Mg*TLRs, 9 pairs have been retained (Table 1). Following numerous assays, 3 pairs for *Mg*MyD88s and one pair for EF1-α, have been also optimized. All the resulting amplicons have been sequenced (LGC Genomics, Berlin, Germany) to control their expected identity.

End-point PCR mix contained 0.5 μl of template, 0.4 μM of each specific primer, 0.8 mM of dNTPs and 0.625 U of Gotaq polymerase (Promega) in 25 μl final volume. PCR program started with initial denaturation at 95 °C for 2 min, followed by 35 cycles including 30 s at 95 °C, 30 s annealing at 62 °C (EF1-α), 56 °C (*Mg*TLT-h) or 60 °C (all the other genes), 30 s elongation at 72 °C, and 5 min final elongation at 72 °C. Results have been visualized by electrophoresis migration on 2% agarose gel.

qPCR has been performed on a LightCycler 480 (Roche) in 384-wells plates. PCR mix contained 2 μl of template, 0.4 μM (*Mg*TLR-n/k), 0.6 μM (*Mg*TLR-b/i/h/s and *Mg*MyD88-a/c) or 0.8 μM (*Mg*TLR-a/r/q, *Mg*MyD88-b and EF1-α) of each specific primer, 3 μl of Lightcycler 480 SYBR Green I Master (Roche), adjusted to 6 μl with nuclease-free water. Dispensation was done by JANUS automated workstation (Perkin Elmer). PCR program started with initial heating at 95 °C for 10 min, followed by 45 cycles including 10 s at 95 °C, 10 s annealing at 62 °C (EF1-α), 56 °C (*Mg*TLR-h) or 60 °C (all others) and a final step of 10 s at 72 °C. Melting temperatures were measured by returning to 65 °C in 30 s and gradual heating to 95 °C. Complete sets of 4 biological replicates have been measured twice. Negative control wells containing water in place of