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Transcriptional Evidence for the Involvement of *MyD88* in Flagellin Recognition: Genomic Identification of Rock Bream *MyD88* and Comparative Analysis

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***Abstract*—**The MyD88 is an evolutionarily conserved host-expressed adaptor protein that is essential for proper TLR/ IL1R immune-response signaling. A previously identified complete cDNA (1626 bp) of *OfMyD88* comprised an ORF of 867 bp encoding a protein of 288 amino acids (32.9 kDa). The gDNA (3761 bp) of *OfMyD88* revealed a quinquepartite genome organization composed of 5 exons (with the sizes of 310, 132, 178, 92 and 155 bp) separated by 4 introns. All the introns displayed splice signals consistent with the consensus GT/AG rule. A bipartite domain structure with two domains namely death domain (24-103) coded by 1st exon, and TIR domain (151-288) coded by last 3 exons were identified through *in silico* analysis. Moreover, homology modeling of these two domains revealed a similar quaternary folding nature between human and rock bream homologs. A comprehensive comparison of vertebrate *MyD88* genes showed that they possess a 5-exonic structure. In this structure, the last three exons were strongly conserved, and this suggests that a rigid structure has been maintained during vertebrate evolution. A cluster of TATA box-like sequences were found 0.25 kb upstream of cDNA starting position. In addition, putative 5'-flanking region of *OfMyD88* was predicted to have TFBS implicated with TLR signaling, including copies of NFκB1, APRF/ STAT3, Sp1, IRF1 and 2 and Stat1/2. Using qPCR technique, a ubiquitous mRNA expression was detected in liver and blood. Furthermore, a significantly up-regulated transcriptional expression of *OfMyD88* was detected in head kidney (12-24 h; >2-fold), spleen (6 h; 1.5-fold), liver (3 h; 1.9-fold) and intestine (24 h; ~2-fold) post-Fla challenge. These data suggest a crucial role for MyD88 in antibacterial immunity of teleosts.

***Keywords*—**MyD88, Innate immunity, Flagellin, Genomic analysis.

I. Introduction

THE innate immune system recognizes 'non-self' molecular patterns expressed on the surface of pathogens through the pattern recognition receptors (PRRs). Toll-like receptor (TLR) family members are one of the well-studied PRRs. These single membrane-spanning non-catalytic receptors recognize structurally conserved pathogen-associated molecular patterns (PAMPs) [1]-[4]. Different TLR subfamily members are involved in recognizing specific PAMPs derived from various microbial pathogens. TLR5 recognizes a substituent of bacterial flagellum called flagellin (FLA). The myeloid differentiation factor 88 (MyD88) is an evolutionarily conserved host-expressed adaptor protein required for the TLR/ IL-1R immune-response signaling [5]. It modulates the TLR signaling which culminate in the NF-κB activation and production of TNF and other inflammatory cytokines [6]. MyD88 has a bi-partite structure composed of an N-terminal death domain (DD) and C-terminal TIR domain. While the TIR domain interacts with its cognate domains located in the cytoplasmic tails of activated TLRs or IL-1R, DD mediates the interaction with the corresponding domain in IL-1R-associated kinase (IRAK) family members. Functional evidences indicated that MyD88 is a vital component of the innate immunity playing a critical role in initiating and activating the immune response, especially in the MyD88-dependent TLR/IL-1R signaling pathway [6], [7].

MyD88 has been reported from many species including several teleosts such as zebrafish [8], Japanese flounder [9], large yellow croaker [10] and rainbow trout [11]. Our previous documentation of *MyD88* from rock bream (*OfMyD88*) was limited to its cDNA identification and mRNA expression in healthy fish, and animals challenged with LPS and *Edwardsville tarda*[12]. However, the gene structure and genomic evolution of *MyD88*, and its involvement in flagellin induced TLR5 signaling in fish was not studied. The current study was designed to evaluate its exon-intron structure, and to examine its transcription upon flagellin challenge.

II. Materials and Methods

A. Identification of cDNA and gDNA

Full-length cDNA of *MyD88* was identified from a previously characterized transcriptome library [13], and characterized (HM035064) [12]. A bacterial artificial chromosome (BAC) library was screened using gene-specific oligos (F1, 5'-AATCCTGACGCAGGTGGAGAGAAA-3' and R1, 5'-AGCTGTCAACCTCTGGAACCTGAA-3') designed for the above cDNA, as described previously [14], and gDNA of *MyD88* was retrieved from the positive BAC clone located, by GS-FLX sequencing.

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B. Molecular and Genomic Characterization

The gDNA and cDNA sequences of *OfMyD88* were aligned and gene-structure was determined. Putative 5'-flanking region was obtained by genome walk approach (R2, 5'-GCTTCAGGCGTCCACAGGTTAC-3') and subjected to prediction of transcription factor binding sites (TFBS) using *in silico* tools. Molecular models of DD and TIR domain were generated based on human MyD88 (3mopC and 2z5vA) by homology modeling approach. Protein sequences of MyD88 homologs were compared using ClustalW and MatGAT matrix analyzer.

C. Animals, Challenge and Tissue Collection

Details of animal rearing, tissue collection and processing have been described elsewhere [14]. For the FLA challenge, healthy *O. fasciatus* fish (~96g) were acclimatized to laboratory conditions in our Fish Vaccine Development Center, Jeju National University (Republic of Korea). 100µL of PBS containing FLA (Invivogen) at 2.4µg $fish^{-1}$ was injected by intraperitoneal (i.p.) injection (n=20). An untreated control group (n=10) was established as negative control. A group (n=20) which was injected with 100 µL of PBS only was considered as the control. Different tissues including head kidney, spleen, intestine, liver, were collected from four fish (n=4) at time points of 3, 6, 12, and 24h post-injection.

D. Transcriptional Analysis

Total RNA extraction, cDNA synthesis and transcriptional analysis were performed as described earlier [14]. Quantitative real-time PCR (qPCR) was conducted to investigate the *OfMyD88* mRNA levels. Briefly, the cDNA synthesized from RNA samples of FLA- or PBS-injected and unchallenged animals was used as template in standard SYBR Green qPCR (TaKaRa), using *OfMyD88* (F1 and R1) and *β-actin* (internal control; F2, 5'-TCATCACCATCGGCAATGAGAGGT-3' and R3, 5'-TGATGCTGTTGTAGGTGGTCTCGT-3') gene-specific oligos. Data was analyzed by Livak-comparative Ct ($2^{-\Delta\Delta Ct}$) method.

III. Results and Discussion

A putative coding sequence of 867 bp encoding a peptide of 288 residues (~33 kDa) and untranslated regions (UTRs) at 5' (122 bp) and 3' (637 bp) terminals of coding sequence were identified. Domain analysis revealed the presence of a death domain (DD; 24-103) and a TIR domain (151-288) in OfMyD88 protein (Fig. 1).

The DD of OfMyD88 was composed of six helices and TIR was composed of five helices and four major sheets. The homology modeling analysis revealed a similar folding pattern between MyD88 domains of human and rock bream homologs (Fig. 2). In addition, pairwise homology analysis revealed that teleost homologs share a tight conservation among them, and OfMyD88 shares a higher overall homology with its Mandarin fish homolog (Table II). This comparison further showed that relative homology in TIR domain is higher than that of DD.

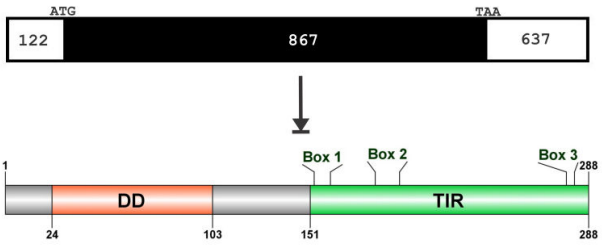

Fig. 1 The schematic diagram of rock bream MyD88 (OfMyD88) transcript and comprehensive domain architecture of OfMyD88 protein. OfMyD88 is composed of DD and TIR domain. In TIR domain, three conserved motifs are found.

TABLE I
Properties of Rock Bream MYD88 (*OFMYD88*) at Different Molecular Levels

	Characteristics	OfMyD88
Nucleotide (cDNA)	Length of cDNA	1626
	5' UTR	122
	CDS (bp)	867
	3' UTR	637
	Polyadenylation signal	^{1608}AATAAA^{1613}
Protein	Peptide (aa)	288
	Death domain	24-103
	TIR domain	151-288
	N-glycosylation site	1 (^{19}NMSV)
Genome	Length of gDNA (bp)	3761
	Number of exons	5
	Number of introns	4
	TFBS in putative promoter[a]	Sp1, NF-κB1, STAT1/2, IRF1/2 and APRF (STAT3)
Transcripts	Dominant mRNA expression (qPCR)	Liver

[a]Predicted using AliBaba2.1, JASPAR and PROMO servers.

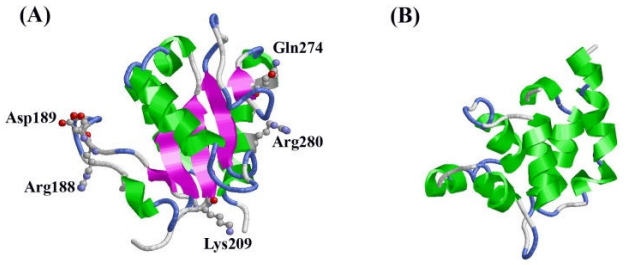

Fig. 2 Homology modeling of rock bream MyD88 (OfMyD88) (A) TIR domain and (B) DD. Conserved residues in TIR domain are shown

These *in silico* data confirmed that OfMyD88 essentially features the common properties of other MyD88s, in particular, those identified from teleosts. Similar to TLRs, the key adaptor proteins in TLR signaling pathways, including MyD88, have been conserved among different vertebrate classes [15], [16]. Hence, teleost MyD88 could be assumed to have similar biological roles as demonstrated in mammals.

TABLE II
HOMOLOGY INDICES OF THE ROCK BREAM MYD88 (OFMYD88) WITH THE OTHER HOMOLOGS

Species	Identifier	Acc. No.	Length	Degree of identity [I%]			Degree of similarity [S%]		
			(aa)	Whole	DD	TIR	Whole	DD	TIR
Mandarin fish	ScMyD88	ADM25313	288	91.3	86.3	96.4	95.5	92.5	99.3
Large yellow croaker	LcMyD88	ACL14361	287	86.2	82.5	94.9	93.4	91.3	98.6
Zebrafish	DrMyD88	AAZ16494	284	70.9	55.0	87.7	81.3	71.3	93.5
Frog	XlMyD88A	NP_001081001	283	59.5	50.0	74.6	74.0	67.5	84.8
Chicken	GgMyD88	A5HNF6	299	61.2	49.4	78.3	75.3	69.1	88.4
Mouse	MmMyD88	NP_034981	296	61.6	50.0	77.5	78.0	75.0	88.4
Human	HsMyD88	AAP36509	297	60.4	47.5	76.3	73.7	67.5	86.3

Degree of identity [I%] and similarity [S%] was calculated by the MatGAT program using BLOSUM62 scoring matrix maintaining first gap penalty and extending gap penalty levels at 12 and 1, respectively. MotifScan was employed in determining the domain span in each species.

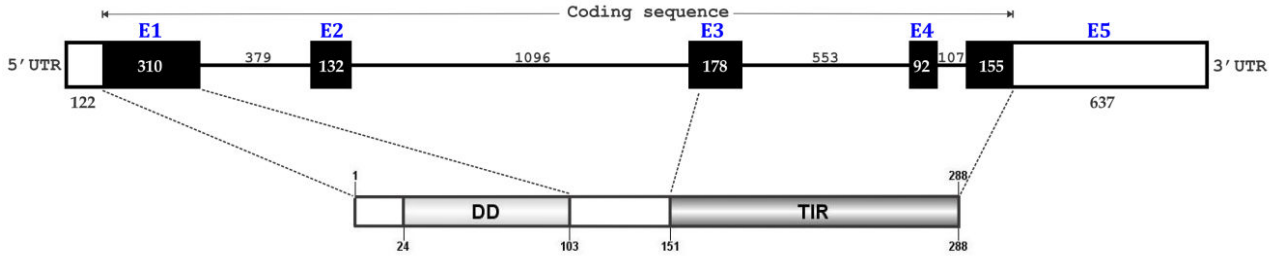

Fig. 3 Genomic organization of rock bream *MyD88* (*OfMyD88*).Gene-structure and domain coding regions of *OfMyD88*. Exons and introns are shown by box (white, UTR and black, CDS) and lines, respectively

Previously reported *OfMyD88* sequence and the gDNA sequence (3761 bp) of current study varied by a single bp in the CDS causing a single amino acid variation. The *OfMyD88* exhibited a quinquepartite genome organization composed of five exons split by four introns. All the introns displayed splice signals consistent with the consensus GT/AG rule. While the DD was encoded by first exon, the TIR domain was encoded by last three exons (Fig. 3).

Comparison of vertebrate *MyD88* genes led us to propose a common quinquepartite gene structure for *MyD88*. The last three exons coding the TIR domain were equal in size (181, 92 and 155 bp) in all the vertebrate classes, except the third exon of teleost *MyD88* which slightly varied (178 bp), revealing the rigid conservation of its TIR domain and its functional importance. This is in agreement with the amino acid homology data. In contrast, first two exons broadly varied in their sizes in different classes (Table III).

Furthermore, inspection of *OfMyD88 g*DNA indicated a putative cluster of TATA box-like sequence quite far away from the TIS. Several potential TFBS were predicted to be located in this 5'-flanking region including sites for Sp1, NF-κB1, STAT1/2, IRF1/2 and APRF (STAT3), all of which have been demonstrated to impact the transcriptional expression of *MyD88* [17]. This suggests that *OfMyD88* is also might be regulated by various transcription factors.

The levels of *OfMyD88* were quantified by qPCR technique and found that it is ubiquitously expressed with higher levels in liver and other immune relevant tissues. Both LPS and *E. tarda* up-regulated its expression in blood, liver and head kidney [12]. In the current study, involvement of *OfMyD88* in TLR5 mediated signaling was tested. Data revealed a significantly induced expression of *OfMyD88* in head kidney (12-24 h; >2-fold), spleen (6 h; 1.5-fold), liver (3 h; 1.9-fold) and intestine (24 h; ~2-fold), in response to FLA-challenge.

However, FLA-mediated induced, highest expression occurred in a tissue-specific manner as shown in Fig. 4. Our results were in consistent with the report in Indian major carp, mrigal [18], in which FLA induced the expression of *MyD88* (along with *TLR5* and *TRAF6*) in multiple tissues examined.

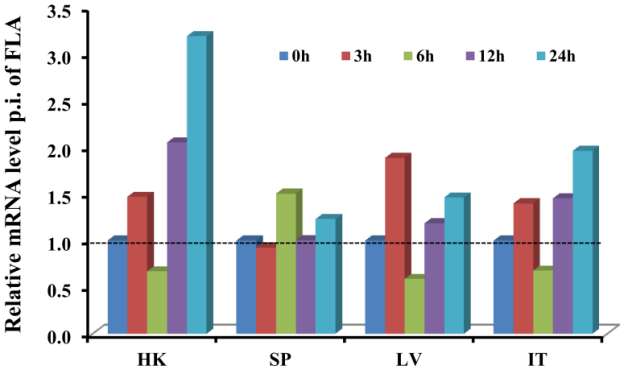

Fig. 4 *OfMyD88* expression analysis after FLA-challenge. Transcriptional expression was analyzed in head kidney (HK), spleen (SP), liver (LV), and intestine (IT) post-FLA challenge using qPCR. Relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method relative to PBS-injected controls and normalized with the same, using the *β-actin* as the reference gene.

The up-regulated expression of *MyD88* in *E. tarda* treated animals [12] might have occurred due to the FLA-mediated transcriptional induction, at least in part. Although these lines of evidence suggested an involvement of *MyD88* in TLR(5) signaling, the significance of increased *MyD88* mRNA and its

association with TLR5-mediated responses, such as cytokine production needs further experiments. In addition, functional aspects of teleost MyD88 remains to be elucidated.

TABLE III
COMPREHENSIVE GENE-STRUCTURAL COMPARISON OF VERTEBRATE *MYD88*S

Lineage	Species	Size	E1*		I1	E2	I2	E3	I3	E4	I4	E5*		Accession no.
		(bp)	5'	CDS								CDS	3'	
Mammalia	*H. sapiens*	4545	184	367	835	135	389	181	188	92	283	155	1736	ENST00000396334
	M. musculus	2707	82	328	921	135	389	181	160	92	243	155	21	ENSMUST00000035092
Aves	*G. gallus*	12685	-	340	3600	132	1958	181	836	92	1888	155	3503	ENSGALT00000044477
Reptilia	*C. pictabellii*	17469	5	346	7275	120	1430	181	1525	92	2408	155	3932	XM_005297139
Amphibia	*X. tropicalis*	5537	147	316	1267	111	1026	181	772	92	927	152	546	NM_001016837
Pisces	*D. rerio*	3941	136	304	785	123	80	181	414	92	1319	155	352	NC_007135
	P. olivaceus	3120	-	310	974	123	808	178	398	92	82	155	-	AB221347
	T. rubripes	5471	-	310	3896	132	389	178	226	92	93	155	-	NM_001113195
	O. fasciatus	3761	122	310	379	132	1096	178	553	92	107	155	637	This study

E, exon; I, intron; the first (E1) and last (E5) exons (*) have been divided into two parts (5'/3'-UTRs and CDS).

IV. CONCLUSION

Current study evaluated the genomic evolution of *MyD88* and its involvement in FLA-mediated TLR5 signaling. Our results suggest that *MyD88* is an evolutionarily conserved molecule in terms of gene-structure and protein domain architecture; and therefore, mammals and teleost *MyD88* may share similar functional roles. FLA stimulated *OfMyD88* expression at transcriptional level in different tissues of challenged fish indicated its important role in FLA-mediated TLR5 signaling.

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