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厚壳贻贝*MyD88-4*基因的生物学特性及其对沙氏弧菌的免疫应答

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摘要: 为理解髓样分化因子88(myeloid differentiation factor 88, MyD88)基因的生物学特性以及对细菌胁迫的响应, 本研究克隆了厚壳贻贝*MyD88*基因(命名为*McMyD88-4*)cDNA全长序列, 其全长3 930 bp, 开放阅读框2 607 bp, 编码868个氨基酸。其中, 13~109位的氨基酸序列为死亡结构域(death domain, DD), 347~481位的氨基酸序列为TIR(toll/interleukin-1 receptor)结构域, TIR结构域包含3个高度保守的区域Box 1、Box 2和Box 3; *McMyD88-4*蛋白的空间结构包含6个 α 螺旋(α -helix)和4个 β 折叠(β -sheet)。同源性分析显示, *McMyD88-4*蛋白序列与长牡蛎*MyD88*最相似, 其一致性和相似性分别为60%和77%; 其次, 与虾夷扇贝、海湾扇贝和菲律宾蛤仔相似度较高, 其一致性和相似性分别为40%~51%和58%~67%。系统进化树结果显示, *McMyD88-4*先与长牡蛎和扇贝聚为一支, 然后与黑腹果蝇聚为一支, 脊椎动物单独聚为一支。实时荧光定量PCR(qRT-PCR)检测发现, *McMyD88-4*基因在厚壳贻贝各组织和器官中均有表达, 其中在外套膜和鳃中的表达量最高, 而血细胞中表达量最低。厚壳贻贝经沙氏弧菌感染后, *McMyD88-4*基因表达量在免疫相关组织中急剧上升, 分别在感染后3和6 h达到峰值, 且在消化腺中的上调水平显著高于鳃和外套膜。研究表明, *McMyD88-4*在厚壳贻贝抵御外界病原体侵染过程中, 尤其是弧菌感染方面发挥重要作用。

关键词: 厚壳贻贝; 髓样分化因子88(MyD88); 基因克隆; 组织表达; 弧菌感染

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先天免疫是多细胞生物进行宿主防御的第一道防线, 当宿主的模式识别受体(pattern recognition receptors, PRRs)识别相应的病原相关分子模式(pathogen-associated molecular patterns, PAMPs)后即可被激活^[1-2]。Toll样受体(Toll-like receptors, TLRs)是目前研究最多的一种模式识别受体, 它能识别来自细菌、真菌以及病毒的PAMPs, 例如脂多糖(lipopolysaccharide, LPS)、脂蛋白(lipoprotein, LP)、肽聚糖(peptidoglycan, PGN)以及病毒性双链RNA等^[1,3-4]。髓样分化因子

88(myeloid differentiation factor 88, MyD88)是TLRs信号通路中关键接头蛋白, 在信号传递中起着重要作用^[5]。1990年, *MyD88*基因首次在小鼠(*Mus musculus*)髓样细胞中克隆得到^[6]。随着TLRs及其信号通路的深入研究, 目前, *MyD88*基因已经在人(*Homo sapiens*)^[7]、大鼠(*Rattus norvegicus*)^[8]、黑腹果蝇(*Drosophila melanogaster*)^[9]、非洲爪蟾(*Xenopus laevis*)^[10]、大黄鱼(*Larimichthys crocea*)^[11]、欧洲鳗鲡(*Anguilla anguilla*)^[12]、半滑舌鳎(*Cynoglossus semilaevis*)^[13]、栉孔扇贝(*Chlamys*

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farreri^[14]、长牡蛎(*Crassostrea gigas*)^[15]、三角帆蚌(*Hyriopsis cumingii*)^[16]、菲律宾蛤仔(*Ruditapes philippinarum*)^[17]及厚壳贻贝(*Mytilus coruscus*)^[18]等物种中被鉴定。在哺乳动物中,TLRs与各种PAMPs结合产生的信号大多是以一种依赖于MyD88的方式进行传递^[19];在黑腹果蝇中,MyD88被证明是抵抗真菌和革兰氏阳性细菌感染所需的关键因子^[9];在大黄鱼和栉孔扇贝中,同样证明MyD88在病原感染响应中起重要作用^[11, 14]。因此,MyD88被认为是激活先天免疫Toll/TLR通路下游信号的“中心环节”^[20]。

MyD88由死亡结构域(death domain, DD)和TIR(Toll/interleukin-1 receptor)结构域组成,TLR胞内的TIR结构域与MyD88的TIR结构域相互作用,将MyD88募集到TLR受体上,MyD88再通过其死亡结构域与IRAK(interleukin-1 receptor associated kinase)家族成员(如IRAK1、IRAK2、IRAK4和IRAK-M)的死亡结构域相互作用并激活IRAKs,从而导致下游一系列的信号级联反应被激活,最终激活转录因子NF- κ B,进而调控炎症细胞因子的表达^[3, 21-22]。

厚壳贻贝是我国重要的水产养殖品种之一^[23],但近年病害高发严重影响了厚壳贻贝养殖的可持续发展,并造成巨大的经济损失。以往的研究发现,厚壳贻贝TLR及其信号通路在抵抗细菌的免疫反应和调节环境压力中起着重要的作用^[24-25]。MyD88作为Toll/TLR的接头蛋白,虽已被证明在昆虫和哺乳动物的TLR信号通路中发挥关键作用,但在厚壳贻贝中的研究还较少^[18]。本实验克隆了厚壳贻贝MyD88基因cDNA全长,根据其结构与虾夷扇贝(*Patinopecten yessoensis*)、海湾扇贝(*Argopecten irradians*)系统进化关系将其命名为McMyD88-4,并对其结构特性、在各组织器官中的表达情况以及沙氏弧菌(*Vibrio chagasii*)^[26]感染后其在免疫组织(外套膜、鳃、消化腺)中的表达特性进行研究,以期深入了解McMyD88-4基因表达、免疫功能以及在TLRs信号通路的作用机制奠定分子基础。

1 材料与方法

1.1 实验动物

实验用厚壳贻贝壳长约10 cm,壳宽约5 cm,采自浙江省舟山市枸杞岛海域。实验前暂养于盐度为30的充气海水中,暂养温度为21℃,每日换水并投喂湛江等鞭金藻(*Isochrysis zhanjiangensis*)

和青岛大扁藻(*Platymonas helgolandica* var. *tsingtaoensis*)。随机取5只健康的厚壳贻贝用于取正常组织样品,取样组织包括外套膜、鳃、消化腺、足、闭壳肌、性腺和血细胞。血细胞的获取:用1 mL的无菌注射器从活体贻贝闭壳肌抽取血淋巴,放于1.5 mL离心管后立即置于冰上,800×g 4℃离心10 min,弃上清液即获得血细胞。取样后立即液氮速冻并保存于-80℃。

1.2 总RNA的提取及cDNA的合成

总RNA的提取采用RNAiso Plus(TaKaRa,日本)试剂并根据说明书进行。提取的总RNA经过Ambion Turbo DNase kit(Ambion,美国)处理去除基因组DNA的污染。分别通过NanoDrop 2000和琼脂糖凝胶电泳检测总RNA的浓度及完整性。根据SMARTer™ RACE 5'/3' Kit(Clontech,日本)试剂盒说明书分别合成5'/3' RACE cDNA第一链。

1.3 McMyD88-4基因cDNA全长克隆

根据厚壳贻贝转录组数据,用Primer Premier 5.0设计McMyD88-4基因特异性引物(表1)。3' RACE:以McMyD88-4-3' F和RACE通用引物UPM为扩增引物,通过降落PCR(touchdown PCR)反应扩增,扩增条件:94℃预变性4 min;94℃ 30 s,72℃ 2 min,5个循环;94℃ 30 s,70℃ 30 s,72℃ 2 min,5个循环;94℃ 30 s,68℃ 30 s,72℃ 2 min,25个循环;最后72℃延伸10 min。5' RACE以McMyD88-4-5' R1和RACE通用引物UPM为扩增引物,通过降落PCR反应扩增,扩增条件同上;然后以PCR产物为模板,用McMyD88-4-5' R2和RACE通用引物UPS进行巢式PCR(nested PCR),反应条件为94℃预变性4 min;94℃ 30 s,68℃ 30 s,72℃ 2 min,25个循环;72℃延伸10 min。PCR产物切胶回收后,连接到pMD19-T载体并进行转化,最终挑取阳性克隆送生工生物工程(上海)股份有限公司测序。

1.4 McMyD88-4基因序列生物信息学分析

在NCBI数据库中通过BLAST和ORF Finder在线分析序列相似性和编码区位置。使用ExpASy(<http://www.expasy.org/>)预测基因的等电点及分子量,使用DNAMAN 6.0编辑cDNA序列和氨基酸序列,使用NetNGlyc 1.0 Server(<http://www.cbs.dtu.dk/services/NetNGlyc/>)预测糖基化位点,使用SMART(<http://smart.embl-heidelberg.de/>)预测蛋白结构域,用SWISS-MODEL和PVMOL软件进行

表 1 本实验所用的引物序列

Tab. 1 Primer sequences used in this experiment

引物名称 primer name	序列 (5'-3') sequence (5'-3')	用途 application
<i>McMyD88-4-3'F</i>	TACCGCAACCAACACACGCACAT	3' RACE
<i>McMyD88-4-5'R1</i>	TCCCGATGGTTCCCGAATGCCGTAA	5' RACE
<i>McMyD88-4-5'R2</i>	CCCCATTCTCTAAACAGGCCAAACG	5' RACE巢式引物
UPM	CTAATACGACTCACTATAGGGCAAGCAGTG GTATCAACGCAGAGT	RACE通用引物
UPS	CTAATACGACTCACTATAGGGC	RACE通用引物
<i>McMyD88-4-RT-F</i>	ATGAGAGAAAAAGTGGGGGC	qRT-PCR
<i>McMyD88-4-RT-R</i>	ACGGGTCTGGGTGGTTAGAA	qRT-PCR
<i>EF-1α-F</i>	CACCACGAGTCTCTCCCTGA	qRT-PCR
<i>EF-1α-R</i>	GCTGTACCACAGACCATTCC	qRT-PCR
<i>α-tubulin-F</i>	TTGCAACCATCAAGACCAAG	qRT-PCR
<i>α-tubulin-R</i>	TGCAGACGGCTCTCTGT	qRT-PCR

3D结构预测和编辑, 使用Clustal X和GeneDoc进行死亡结构域和TIR结构域氨基酸序列的多重序列比对, 并通过PhyML 3.0软件采用最大似然法(maximum likelihood, ML)构建系统进化树。

1.5 *McMyD88-4*基因在厚壳贻贝不同组织中的表达分析

根据Transcriptor First Strand cDNA Synthesis Kit(Roche, 德国)说明书, 取500 ng总RNA进行第一链cDNA的合成, 用于实时荧光定量PCR(qRT-PCR)。选择*EF-1 α* 和 *α -tubulin*作为内参基因(表1), qRT-PCR为10 μ L反应体系: 5 μ L 2 \times FastStart Essential DNA Green Master (Roche), 上下游引物各0.3 μ L, cDNA模板1 μ L, 无菌水3.4 μ L。反应条件: 95 $^{\circ}$ C 10 min; 95 $^{\circ}$ C 10 s, 60 $^{\circ}$ C 10 s, 45个循环。分析基因熔解曲线和扩增曲线, 并对产物进行割胶回收和测序, 验证目标片段的正确性。采用绝对定量的方法检测基因的表达情况, 即采用梯度稀释法建立模板浓度与扩增循环数的标准曲线, 再以该标准曲线为基础, 通过待测样品的扩增循环数确定基因的丰度。每组样品3个生物学重复和2次技术重复, 基因表达量用平均值 \pm 标准误(mean \pm SE)表示, 用JMPTM软件对数据进行分析, $P < 0.05$ 表示有显著性差异。

1.6 厚壳贻贝*McMyD88-4*在沙氏弧菌感染后的免疫应答响应

沙氏弧菌分离于自然生物被膜表面^[23], 由

本实验室保存。将沙氏弧菌在2216E液体培养基中25 $^{\circ}$ C培养过夜, 3 500 r/min离心15 min后弃上清液, 用灭菌海水洗涤3次后将其重悬于灭菌海水, 调节实验组沙氏弧菌浓度为 10^7 CFU/mL。实验分为对照组和实验组, 每组每个取样时间点取3个重复, 对照组厚壳贻贝饲养在过滤除菌海水中, 实验组则饲养在含沙氏弧菌的海水中。分别在感染0、3、6、12、24和48 h后收集3只厚壳贻贝外套膜、鳃和消化腺, 立即用液氮速冻并保存于-80 $^{\circ}$ C。总RNA提取、cDNA的合成及qRT-PCR方法同前所述。

2 结果

2.1 *McMyD88-4*基因全长及序列分析

*McMyD88-4*基因cDNA全长3 930 bp, 包括188 bp的5'非编码区(untranslated region, UTR)、2 607 bp的ORF编码区和1 135 bp的3' UTR(图1)。该基因共编码868个氨基酸, 预测其蛋白质分子量为97.07 ku, 等电点为5.32。SMART预测厚壳贻贝*MyD88-4*的结构域, 结果显示, 第13~109位的氨基酸为死亡结构域, 第347~481位的氨基酸为TIR结构域(图1), 死亡结构域和TIR结构域是*MyD88*家族所共有的典型特征。

2.2 *McMyD88-4*结构特征

通过SMART在线预测厚壳贻贝*McMyD88-4*的二级结构(图2-a), 与已报道的厚壳贻贝

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1   atcatgcttgatttcaacttaacgttaaccctgctacagtgacggaaatattcattttaaatctatacaaaatggctcttcca
85  ataatagtgacataggaaatagttatataagataaactgattanaangttcttgagataangataangtctataatitttcaa
169 gccatataatattgtgacgtcATGGGGGAAGGAGTGAACCTTTGGAAGAATAAATGACTTACCAATAAGAATACTTAGCCCTT
1   M G E G V N L S E E Y N D L P I R I L S P

253 CAACTAGACGGTTGATGTCAATTATCATAGATCCACCAGCAGTTATACCGTCTCACATGGTGTCTAGGTGACATGCTGGAC
22  S T R R L M S I H L D P P A V I P S S H G V L G D M S G

337 TAGCAGAAGTACTGGGTTGAGTTTGTGCATATTCGATATTTGAAACTAAGTATAGCAGAAGCTTGGCTGTAGGAGGAAT
50  L A E L A G F E F C H I R Y F E T R Y S R T F A C L E E

421 GGGATTACGGCATTGGGAACCATCGGAATCTACTGAAGAAATTTGGTTATCATGGAAGGTTGACTGTCTCTGAAATTA
78  W G L R H S G T I G N L L K N L V I M E R F D C L S E I

505 AAGAATCCGATTGAGAGATACAAAATCACATCGAAAGGACACGAAATAGTGAGATGGTAAACATTGGGAATATTG
106 K E S V L R D I K N H I E R T T R N S E M V L N I G N I

589 ATAATGAAACAATCTGGTTCAAGTACCGCATGCAAACTGTAAACATGGTAAACACAGATCTCAAAGTTGTTGGTAAACA
134 D N E N N L G S S T A C K T V T S L N T D L K V V G E N

673 ACTTTTCAGATAACTGTTTACCATCGATAAACACAGACAAAAAGTTATTGGTAAATAACTTCCGATAACTGTTTACCGT
162 N F S D N C L P S I N T D K K V I G E N N F S D N C L P

757 CGTTAAACATAGATAAAATGTTTTGGAGAAAACAACTTGTCCGACAACTGTTTACAAGAAGTGAAGGCTATAGGACGTCT
190 S L N I D K N V F G E N N L S D N C L Q E D E G Y R T C

841 CGTTAAATGATGAAGAACTTCGATCCTTAATAATGGAACGAAAAAGGAGGAGTGAATCTTCAACAAGTGGATAGTGTGAGA
218 S L N D E E L R S L I N G N E N E A V N L Q Q V D S V E

925 GTACAATAAGTAGAATTAGCAGAGAGAACACATCTATTACAGATCACAATAGGAATGAGACAACAACTTGCATATTAACA
246 S T I S R I S R E N T S I T D H N R N B T T K L A N I N

1 009 ATACAGGAGATATAGAGATAAACATTGCTGTGACGTTGAAAAATAAAAAAGCGGGACAGTTGAAAAATAGCAAAATCAAAAAGT
274 N T G D I E I N I R C A V E N K K R G T V E N S K I K K

1 093 TAGATAAGGTTGAAGACACAGAGAAATCATATGTTGATGATGGAGTTGTTATCTGGTACCGGACCCAGTCAGTCCAGTTT
302 L D K V E D T E R I I C S D D G V R Y L V P R P V S P V

1 177 TTGAAGACGATGGATTCTACTACAGACGATAGGGAAGTTTTCTAAAAAGATAATCTGCTGCATTGTGTGCTGATGAAA
330 F E D D G F L T T D D R E G F L K R Y S A C I C C A D E

1 261 ATATAAATCTGCCAATGAAATCATGAAATACAAAGACGAAAGCTGATTCTTTTCCACAGGAAGCATGCTAAGTG
358 N I N L A N E I I M K Y K D Q K A D F F L P Q E A L L S

1 345 GAAAAACGAAATTTGAAACCTTAGCGGAAGTCATAGAAACAGGTTGACAGCAGACTATAGTATTCTCTAAACATTACC
386 G K Y E F E T L A E V I E T R C D S R L I V I L S K H Y

1 429 TTACCAGTCTGCTGTGATTTGCAACACAGTTGTCAAGACACTGATCCAGATGCTAAACGCAAGAAATATTCCCGTAC
414 L T S P A C V F A T Q F V K T L D P D A K R R K I I P V

1 513 TCCTAGATGAAGATGATCTTATCCCGTGTCTTACGTGGCATTAGCTCAATTAAGCCCGGGCGCTAAAGTTGGACATGGAT
442 L L D E D V S Y P R V L R G I S S I K A R R L K F G H G

1 597 TTTGAAATTTACTGAGTTCGCTGCTAGCTCTAAAAAAGTTATAAATGATGAAGAAAAATTAACAATGCCATAAACACAACA
470 F W N L L S S S L R L R K V I N D E E N L N N A H K H N

1 681 TGAACAGTTTCGACAAAACCGATCAATTTGTTACACAACAGAGTGTGCAACTCAACAGATGAAAAAGACTGAAAAATA
498 M N S F D K T D Q F C L H K Q S D A T Q Q M E K T E R N

1 765 ATTCATTCCAACAACAATGGATCATTAAATTTACGGAAAAAATGCTGTGATGCTGACTAATGATTTCCATCGGATAAAT
526 N S F Q Q Q M D H L N S T E K M S C D L T N D F P S D K

1 849 CTGCCATGACGGTTGCTGTGTGATCTACCAAGTGGCTCAGGAATATGCAAAAAGAACAATATGCGAGTCTGGCAGGG
554 S A M S V A C C D L P S G A S G I C Q K E Q Y A S L A T

1 933 GTAAGAAAGACAAGAAAAATCCGGACTTAAACTCTCGACGGATTATGAAAAGTTAAATATCATTGACCGGAAGCCAAAAT
582 G K K D K K N P G L K T L R R I M K S L N I I D R K P K

2 017 TACAATACAAAAACGTTCTCATTCGACAGGAGGCTATACCGCAACCAACACCGCACATGTAGAGCCGAGATCACATTCTA
610 L Q Y T K R S H S T G E A I P Q P T H A H V E P R S H S

2 101 CTAATGTTCTCCCAATGGATACCTATTCTCTGATGAAGTGGCCATACATGTTCTCACTACCTATTTTAAACATGTCATA
638 T N V P P T M D T Y F S D E L A H T C S S P I F N I C H

2 185 CAGAATCAGAGCTAACACTTTCAAGTATAGCGATACATTGCGGCTCAAGATAGTACCAACAATTTGTCAGCTGTGAGTACA
666 T E S E L T L S S D S D T L S A Q D S T N N L S S C E Y

2 269 GTGATATAAAAAATCTTCAATGATTGCAAAATTTGCATAAGAGAGTAATGCTAATTTCTAGTCAATCAAGTGGTC
694 S D I K N N L S N D L Q N L H K K S N A N S S S Q S S G

2 353 AAAATATTCTAGCCCAATATAGAACCGCTATCACCTAAGGATTTTCAACTGAAACGAACAATCTTGATTCAACATCAAGAC
722 Q N I L S P N I E P L S P K D L S T E T N N L D S T S R

2 437 AAACAGCACGTGATGATAATGTAATACCAGATACCGATCTACGTCAATGATACCAGGACTTATTGTCGGAAGTCAAGTT
750 Q T A R D D N V I P D T G S T S Y D T T S L L S E V T S

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(图1 Fig. 1)

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2 521 CAGATAACAATAGCATTGTTTCGAATTCAGAAAGGAATCGTTAGACAATTGCAGTTATGCGTCTTCCTTCGACAAATGAAAT
778 S D N N S I V S N S R K E S L D N C S Y A S S F R Q M K
2 605 TTGGGAACGACAGTCGACAGACCTCTTTCGAGAGTAATAGGTCAGCTTCGTTTGACGGGAATAGTATGACGCTCAATCGAAGACA
806 F G N D S R Q T S F E S N R S A S F D G N S M T S I E D
2 689 ACATGGCTGCTGATAAACAAACAGGATTTTTTCATGTGAACAAATTGTCAGTAGTGACAAAGGAACTAGAAGGAAATCAGGAG
834 N M A A D K Q T G F F S C E Q I V S S D K G T R R K S G
2 773 TCGTGTTCGAAATATGCTGTAAaataatgatgtgtgcaagaagatgtgttctctttgatcgaanaataacagaccattgaaa
862 V V F E I M L *
2 857 aataacggnaatagctatgatgataaaaaaataat taantggcattatgtttataaaacatatcgttatagtcnaaantatg
2 941 taatcttgttgtgctgcaataat taaatgttaagtgtgaaatgcttgcctgcacaaatgttatcaatcaccattgta
3 025 tcatagtaatctttgaattatttcattttggtaccgaacaaatggatataaatgaatgccnagcaacgacttaaacctttaa
3 109 acacattgcaatattctcagctgtgcaat tagatttgtttacattatcagtcacatcaccaccggatacttgtgcagcaac
3 193 gttagtcatcttagat tggtaattgctgtagcagttcaatgtcattcaactgaccaagt gnaacgctagaataatcaacgag
3 277 acttagtgaatgcacagacttagatcagataagttctctttttgtcttatagaacgctaatgagagaaaangtggg
3 361 ggcacataaattgagatagcaacaacgacacaaagaaccacaaagatacttgaggtaacataaagctctactaaaatgtaaaa
3 445 tatcctcacaatatatgtgaaattc taaccaccgaccgtacaaccgggtagasaanaatgggactggaataaanaacctc
3 529 aatcaacaataatgttcatcaatccaanaagttgtgatttaactgatgctgacaccttacacagttcttgatttggataggt
3 613 ataganaatgtagtgggat tagactattatctgacaaatcaccctctcttataatcttatacaatlgattaactaacgtta
3 697 catcgaagaacttgcataaaatcagataggattggacaaataaacat taaaaacaccagaaccagctacagctataaagaacttc
3 781 ttgaacttctcttagttgatataatgtctttagtactagtaattacattttgtatttgatgcaaaactgatagaacaac
3 865 ccgatttgagatcttgtaatacaattaaaagatatatcgtaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

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图 1 厚壳贻贝 *McMyD88-4* 基因全长序列及氨基酸序列

小写字母分别代表 5' 和 3' 非编码区序列, 大写字母代表编码区序列; 上面为核苷酸序列, 下面为编码的氨基酸序列; 起始密码子 (ATG)、终止密码子 (TAA)、终止信号 (aataaa) 已加粗; 死亡结构域和 TIR 结构域用灰色阴影表示; 糖基化位点用加粗斜体表示

Fig. 1 cDNA and amino acid sequence of *McMyD88-4* gene from *M. coruscus*

Lowercase letters represent 5' and 3' untranslated region sequences respectively, and capital letters represent coding region sequences, with nucleotide sequences above and encoded amino acid sequences below, the initiation codon (ATG), the stop codon (TAA) and termination signal (aataaa) are characterized in bold, the death domain and TIR domain are shaded, glycosylation sites are indicated in bold italics

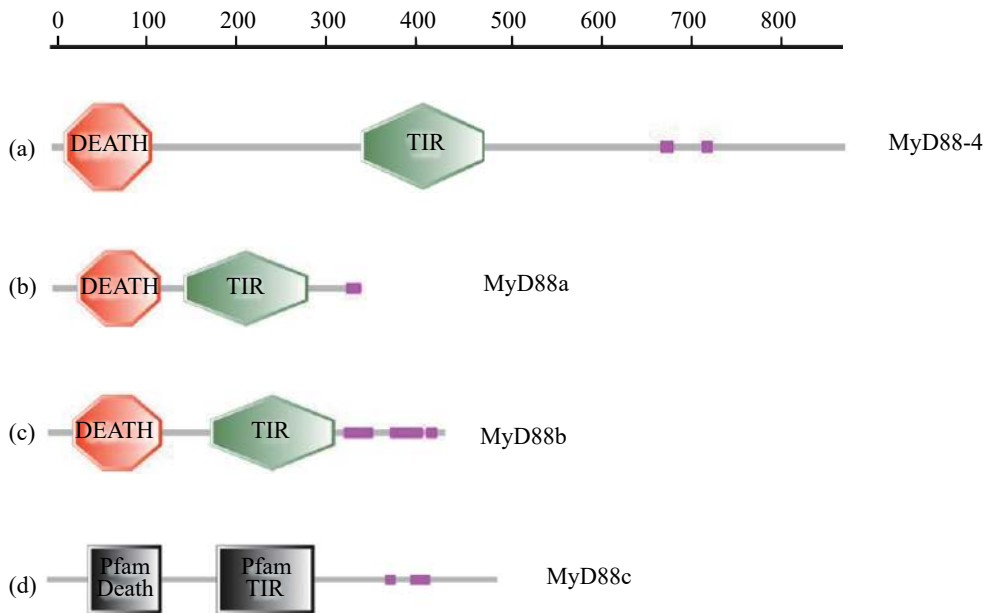


图 2 厚壳贻贝 *MyD88-4*(a) 与已知的厚壳贻贝 *MyD88a*(b)、*MyD88b*(c) 和 *MyD88c*(d) 结构域比较

Death 和 TIR 分别表示死亡结构域和 TIR 结构域

Fig. 2 Comparison among the protein secondary structures of *MyD88-4* (a), *MyD88a* (b), *MyD88b* (c) and *MyD88c* (d) from *M. coruscus*

Death and TIR represent the death domain and TIR domain respectively

McMyD88a(图 2-b)、*McMyD88b*(图 2-c) 和 *McMyD88c*(图 2-d) 相比, 它们均由死亡结构域和 TIR 结构域组成, 但在氨基酸总长及结构域的相

对位置不尽相同(图 2)。使用 ExPASy 在线分析的 SOPMA 功能对厚壳贻贝 *McMyD88-4* 氨基酸序列的二级结构进行分析, 其中 α 螺旋占全结构的

35.60%，延伸序列占14.98%， β 折叠占8.87%，不规则卷曲占40.55%。使用SWISS-MODEL构建的厚壳贻贝McMyD88-4蛋白3D结构(图3-a)，共有6个 α 螺旋和4个 β 折叠；其三维空间结构与已知蛋白序列的其他3个McMyD88亚型相比，在 α 螺旋、 β 折叠和不规则卷曲部位均有不同(图3-a~d)，但大部分空间结构均能相互吻合(图3-e~g)。

2.3 厚壳贻贝McMyD88-4与其他物种MyD88氨基酸序列多重比对及系统进化分析

将厚壳贻贝McMyD88-4氨基酸序列的功能域序列与其他物种进行多重序列比对，发现不同物种(包括脊椎动物和无脊椎动物)的死亡结构域和TIR结构域保守性都较高，其中TIR结构域包含3个高度保守的区域：Box 1、Box 2和Box

3(图4)。厚壳贻贝McMyD88-4蛋白序列与长牡蛎CgMyD88最相似，其一致性和相似性分别为60%和77%；其次，与虾夷扇贝、海湾扇贝和菲律宾蛤仔相似度较高，其一致性和相似性分别为40%~51%和58%~67%，而与厚壳贻贝其他3个亚型的MyD88相似度较低(表2)。基于MyD88氨基酸序列的系统进化树表明，厚壳贻贝McMyD88-4先与长牡蛎MyD88以及扇贝MyD88-4聚为一支，然后与黑腹果蝇聚为一支；厚壳贻贝McMyD88a、McMyD88b、McMyD88c分别与紫贻贝(*Mytilus galloprovincialis*)MgMyD88a、MgMyD88b、MgMyD88c聚为一支；脊椎动物MyD88则单独聚为一支(图5)。

2.4 McMyD88-4基因在厚壳贻贝不同组织中的表达情况

经qRT-PCR检测发现，McMyD88-4基因在厚壳贻贝各组织和器官中均有表达(图6)。其中，在外套膜和鳃中的表达量最高($P < 0.05$)，而在消化腺、足、闭壳肌、血细胞等器官和组织中的表达量都较低(图6)。

2.5 沙氏弧菌感染对厚壳贻贝免疫组织McMyD88-4基因表达量的影响

厚壳贻贝经沙氏弧菌感染后，外套膜McMyD88-4基因表达水平在3 h时已开始显著上升($P < 0.05$)，并在6 h时表达量达到最高($P < 0.05$)，随后开始下降(图7-a)。在鳃中，McMyD88-4基因表达量在感染后3 h即上升到最高水平($P < 0.05$)，然后开始下降，在12 h恢复到正常水平，随后48 h时又呈现显著上升(图7-b)。在消化腺中，McMyD88-4基因表达水平也在3 h达到最高($P < 0.05$)，这种高表达水平一直持续到感染后6 h，然后开始降到正常水平，并且在消化腺中的上调水平明显高于鳃和外套膜(图7-c)。

3 讨论

同多数无脊椎动物一样，软体动物缺乏特异性免疫反应以及免疫记忆，因此只能依赖细胞和体液成分介导的先天免疫来抵御病原体^[27]。近年来，许多模式识别受体和免疫效应器已在软体动物中得到报道，它们作为先天免疫系统的重要组成部分，在环境变化和病原胁迫下的免疫应答中发挥着重要作用^[28-29]。研究已表明，

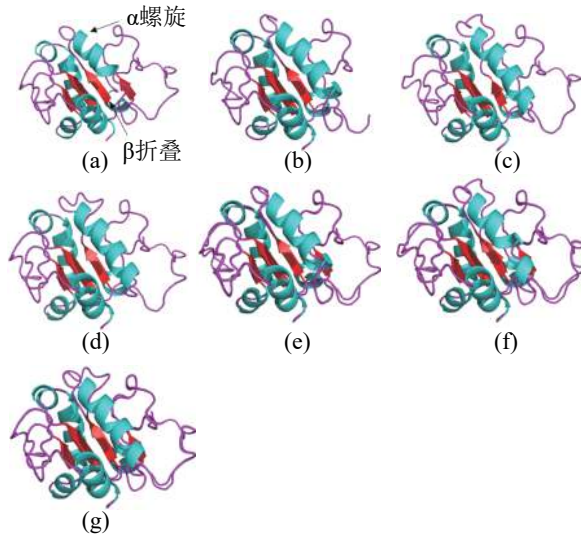


图3 厚壳贻贝McMyD88-4与已知的其他3个亚型的空间结构比较

(a)厚壳贻贝McMyD88-4空间结构模拟；(b)厚壳贻贝McMyD88a空间结构模拟；(c)厚壳贻贝McMyD88b空间结构模拟；(d)厚壳贻贝McMyD88c空间结构模拟；(e)厚壳贻贝McMyD88-4与McMyD88a空间结构重合模拟；(f)厚壳贻贝McMyD88-4与McMyD88b空间结构重合模拟；(g)厚壳贻贝McMyD88-4与McMyD88c空间结构重合模拟

Fig. 3 Comparison of three-dimensional structures between McMyD88-4 and other three subtypes

(a) the predicted three-dimensional structure of McMyD88-4; (b) the three-dimensional structure of McMyD88a; (c) the three-dimensional structure of McMyD88b; (d) the three-dimensional structure of McMyD88c; (e) the predicted three-dimensional structure of McMyD88-4 overlapped with McMyD88a; (f) the predicted three-dimensional structure of McMyD88-4 overlapped with McMyD88b; (g) the predicted three-dimensional structure of McMyD88-4 overlapped with McMyD88c

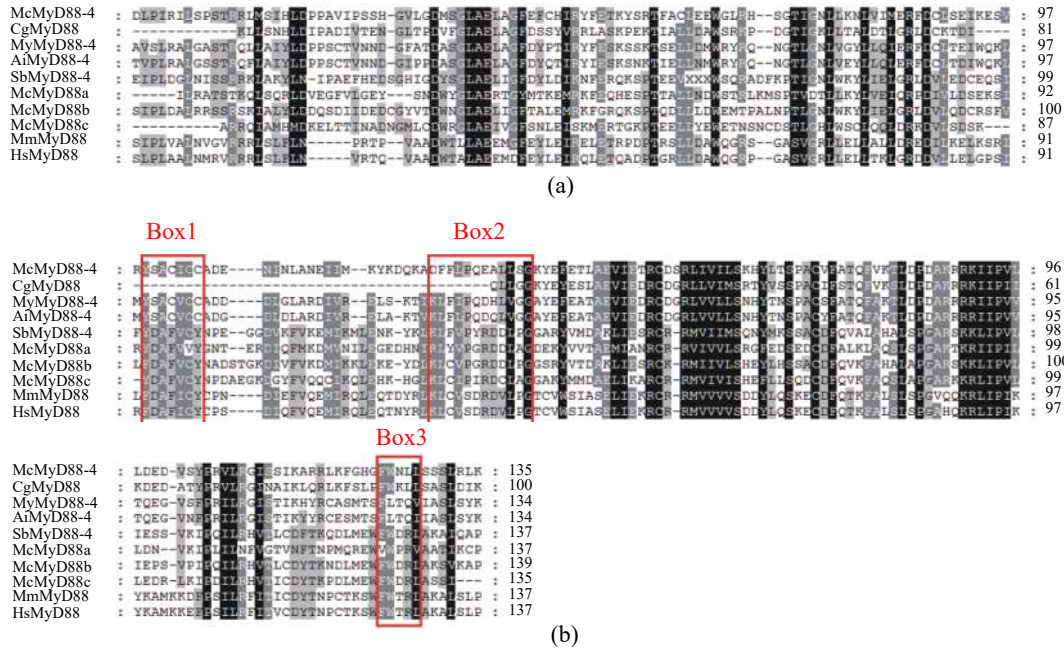


图 4 MyD88死亡结构域(a)和TIR结构域(b)氨基酸多重序列比对

用于多重序列比对其他物种的氨基酸序列分别是长牡蛎(EKC32208.1), 蛎夷扇贝(AKN04689.1), 海湾扇贝(AVP74320.1), 魁蚶(ALZ42090.1), 厚壳贻贝(AYA22345.1, AYA22346.1, AYA22347.1), 小鼠(NP_034981.1), 人(Q99836.1); Box1、Box2和Box3表示TIR区域的3个保守基序

Fig. 4 Multiple alignment of the amino acid sequences of DD (a) and TIR domains (b) of MyD88

The amino acid sequences used for multiple alignment includes *C. gigas* (EKC32208.1), *P. yessoensis* (AKN04689.1), *A. irradians* (AVP74320.1), *Scapharca broughtonii* (ALZ42090.1), *M. coruscus* (AYA22345.1, AYA22346.1, AYA22347.1), *M. musculus* (NP_034981.1), *H. sapiens* (Q99836.1). Box 1, Box 2 and Box 3 indicate the three conserved motifs of the TIR domain

表 2 厚壳贻贝和其他物种MyD88蛋白序列一致性和相似性比较

Tab. 2 Identity and similarity of MyD88 protein sequence between *M. coruscus* and other species

基因名 gene name	物种名 species	GenBank登录号 accession no.	氨基酸数 amino acid number	氨基酸一致性/ 相似性/% amino acid identity/similarity	死亡结构域一致性/ 相似性/% DD domain identity/similarity	TIR结构域一致性/ 相似性/% TIR domain identity/similarity
<i>McMyD88-4</i>	厚壳贻贝 <i>M. coruscus</i>		868	100/100	100/100	100/100
<i>CgMyD88</i>	长牡蛎 <i>C. gigas</i>	EKC32208.1	644	60/77	43/62	70/87
<i>PyMyD88-4</i>	蛎夷扇贝 <i>P. yessoensis</i>	AKN04689.1	590	51/67	49/71	55/70
<i>AiMyD88-4</i>	海湾扇贝 <i>A. irradians</i>	AVP74320.1	580	43/60	46/71	55/71
<i>RpMyD88</i>	菲律宾蛤仔 <i>R. philippinarum</i>	AEF32114.1	471	40/58	42/60	31/52
<i>SbMyD88-4</i>	魁蚶 <i>S. broughtonii</i>	ALZ42090.1	441	37/60	37/57	29/54
<i>McMyD88b</i>	厚壳贻贝 <i>M. coruscus</i>	AYA22346.1	439	32/53	32/53	35/57
<i>McMyD88c</i>	厚壳贻贝 <i>M. coruscus</i>	AYA22347.1	482	31/60	34/50	31/60
<i>HsMyD88</i>	人 <i>H. sapiens</i>	Q99836.1	296	29/47	32/47	28/48
<i>MmMyD88</i>	小鼠 <i>M. musculus</i>	NP_034981.1	296	28/47	33/53	28/49
<i>McMyD88a</i>	厚壳贻贝 <i>M. coruscus</i>	AYA22345.1	339	26/49	32/51	29/55

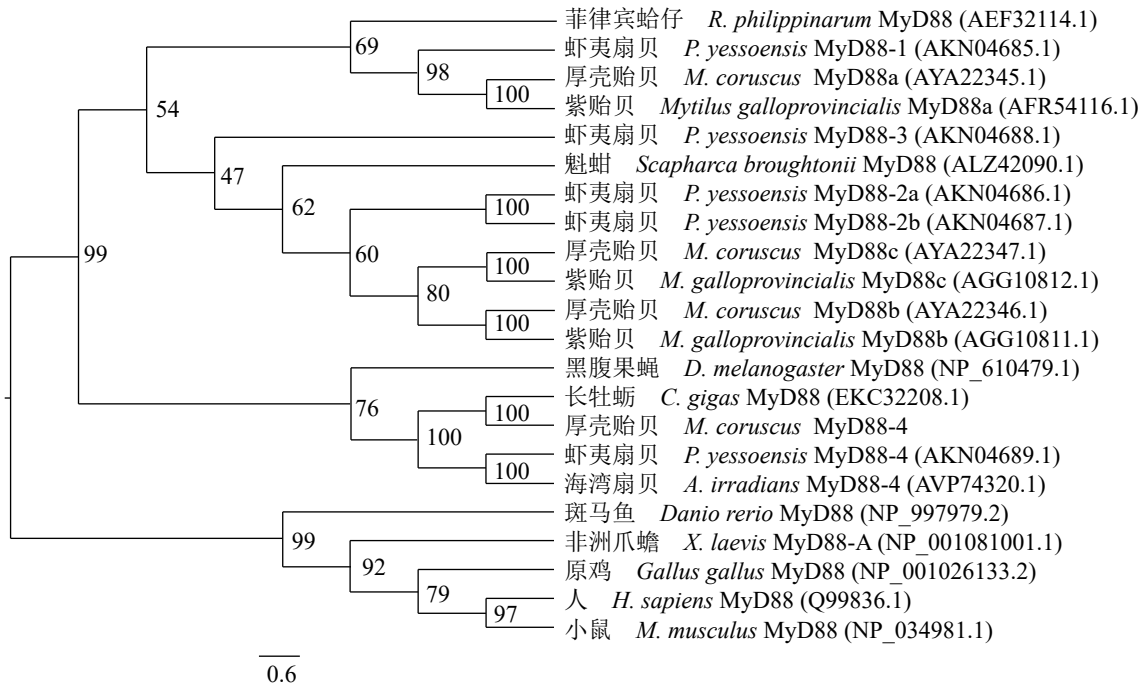


图5 厚壳贻贝和其他物种MyD88氨基酸序列系统进化树

Fig. 5 Phylogenetic tree of the MyD88 amino acid sequences between *M. coruscus* and other species

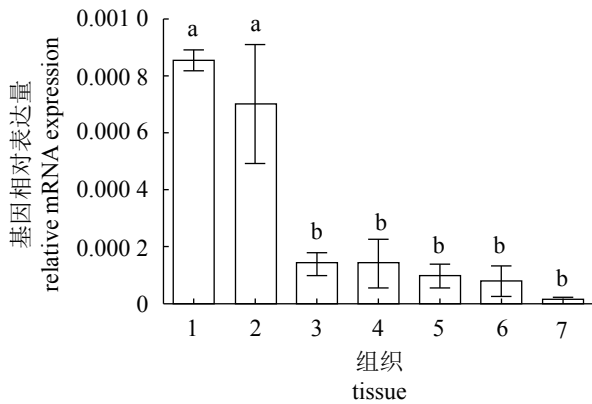


图6 McMyD88-4基因在厚壳贻贝各组织中的表达

1. 外套膜, 2. 鳃, 3. 足, 4. 消化腺, 5. 闭壳肌, 6. 性腺, 7. 血细胞; 不同字母表示差异显著($P < 0.05$); 下同

Fig. 6 mRNA expression of *McMyD88-4* in different tissues of *M. coruscus*

1. mantle, 2. gill, 3. foot, 4. digestive gland, 5. adductor muscle, 6. gonad, 7. haemocytes, bars with different letters were significantly different ($P < 0.05$), the same below

在栉孔扇贝、菲律宾蛤仔和长牡蛎中, MyD88在其依赖的信号通路(如TLR信号通路)中发挥着重要作用^[14, 17, 30-31]。而在厚壳贻贝中, 关于MyD88的研究甚少, 其免疫相关功能尚不清楚。因此, MyD88作为TLR通路中的关键因子, 研究McMyD88在厚壳贻贝免疫应答中的作用具有重

要意义。

本研究克隆了厚壳贻贝*McMyD88-4*的cDNA全长, 编码868个氨基酸。其氨基酸序列包含MyD88蛋白家族典型的死亡结构域和TIR结构域, 同时TIR结构域包含3个保守区域: Box 1、Box 2和Box 3。与已报道的厚壳贻贝MyD88a、MyD88b和MyD88c^[18]相比, 本研究在氨基酸序列总长及死亡结构域、TIR结构域的相对位置上有一定区别; 同时, 三维空间结构上, 其 α 螺旋、 β 折叠和不规则卷曲部位均有不同, 但大部分空间结构均能相互吻合。已有研究表明, 含死亡结构域的蛋白能介导蛋白质之间的相互作用, 在细胞凋亡和炎症反应相关过程中起关键作用^[32]。而TIR结构域则在激活TLR/IL-1R超家族介导的先天免疫应答过程中十分重要^[33]。在脊椎动物中, TIR结构域中的3个保守区在蛋白质相互作用和信号转导中也起着重要作用^[34-36]。因此, 从结构上可以推测, McMyD88-4在厚壳贻贝先天免疫防御系统的TLR信号通路中起重要作用。系统进化树结果表明, 厚壳贻贝McMyD88-4先与长牡蛎以及扇贝聚为一支, 然后与黑腹果蝇聚为一支, 暗示它们可能起源于共同的祖先。此外, 厚壳贻贝McMyD88-4与其他软体动物整体均聚为一支, 证明McMyD88-4属于MyD88家族。

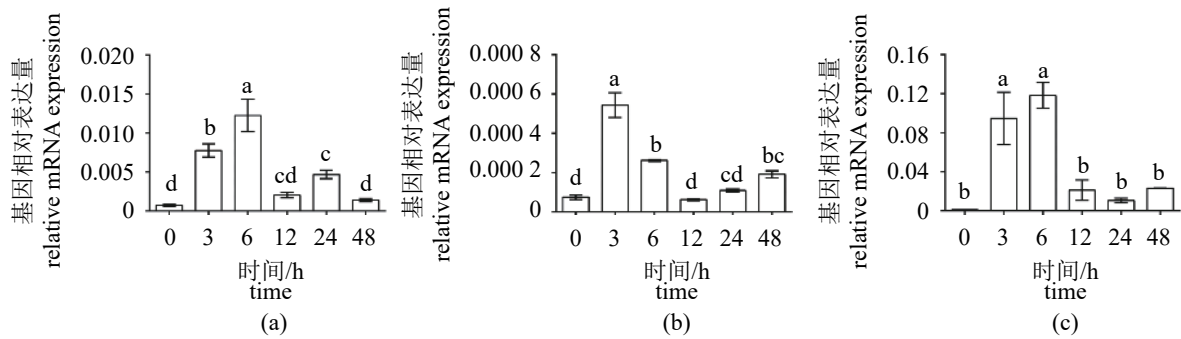


图 7 沙氏弧菌感染后 *McMyD88-4* 在厚壳贻贝外套膜(a)、鳃(b)和消化腺(c)中表达水平的变化

Fig. 7 mRNA expression of *McMyD88-4* in mantle (a), gill (b) and digestive gland (c) of *M. coruscus* after being challenged with *V. chagasii*

目前, *MyD88* 基因的组织表达模式在许多物种中都有报道, 发现大多在免疫相关组织器官中有高表达^[16-17, 37]。本研究中, *McMyD88-4* 基因在厚壳贻贝各组织和器官中均有表达, 在外套膜和鳃中表达量最高。推测这与外套膜和鳃通过感知水流或滤食不断与外界环境接触, 是宿主防御的前线。以往研究发现厚壳贻贝 *McMyD88b* 基因在鳃中有高表达, *McMyD88a* 和 *McMyD88c* 基因在血细胞中高表达^[18], 这种表达模式与其他贝类极为相似, 如海水贝类菲律宾蛤仔中, *RpMyD88* 在鳃中表达量最高^[17]; 虾夷扇贝中 *PpMyD88-3* 和 *PpMyD88-2b* 表达量分别在鳃和血细胞中最高^[37]。淡水贝类三角帆蚌中, *HcMyD88-1* 和 *HcMyD88-2* 基因都在肝胰腺中表达量最高^[16]。由此猜测, *MyD88* 基因可能与这些组织器官的先天免疫应答密切相关。

为进一步了解厚壳贻贝 *McMyD88-4* 基因在先天免疫应答中的作用, 本实验检测了经沙氏弧菌感染后厚壳贻贝 *McMyD88-4* 基因应答响应。经沙氏弧菌感染后, 厚壳贻贝外套膜、鳃和消化腺中 *McMyD88-4* 基因均有快速而明显的上调, 在 3 或 6 h 达到峰值, 且在消化腺中的上调水平明显高于鳃和外套膜。这暗示厚壳贻贝消化腺对于沙氏弧菌的免疫反应可能比鳃和外套膜更敏感。与此同时, Liu 等^[38] 的研究也发现, 紫贻贝消化腺中免疫基因对于哈维氏弧菌 (*V. harveyi*) 的反应比鳃中更敏感。此外, 研究表明, 溶藻弧菌 (*V. alginolyticus*) 亦能引起厚壳贻贝血细胞中 *McMyD88* 各亚型的显著上调^[18]。三角帆蚌中, 鳃弧菌 (*V. anguillarum*) 感染能引起肝胰腺 *HcMyD88-1* 和 *HcMyD88-2* 表达量上调^[16]。此外, 鱼类中, 副溶血弧菌 (*V. parahaemolyticus*) 和鳃弧菌分别能

使大黄鱼血细胞和脾脏、半滑舌鳎脾脏、肝脏和头肾等组织器官中 *MyD88* 基因显著上调^[11, 13]; 而在凡纳滨对虾 (*Litopenaeus vannamei*) 中, 副溶血弧菌能使血细胞中的 *LvMyD88* 表达上调, 但幅度较小^[39]。除弧菌感染外, 研究者们发现在栉孔扇贝^[14]、欧洲鳗鲡^[12]、大西洋鲑 (*Salmo salar*)^[40] 中, 病原相关分子模式 (如 LPS、poly I:C、PNG 和 SAV3) 刺激后, 其 *MyD88* 的表达水平也显著升高。上述研究表明, *MyD88* 在机体先天免疫系统抵御外界病原体方面起着至关重要的作用。然而, 其中具体的分子机制还有待进一步的研究。

本实验获得了厚壳贻贝 *McMyD88-4* 基因的 cDNA 全长, 并对其结构和表达特性进行了研究。厚壳贻贝 *McMyD88-4* 具有死亡结构域和 TIR 结构域, 其在外套膜和鳃中表达量最高; 且在沙氏弧菌感染后, *McMyD88-4* 基因在厚壳贻贝外套膜、鳃和消化腺中的表达水平显著升高, 且在消化腺中的上调水平明显高于鳃和外套膜。以上表明, *McMyD88-4* 基因在厚壳贻贝抵御外界病原微生物方面发挥了重要作用, 同时为厚壳贻贝生态健康养殖产业发展提供了理论依据。

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Identification of *MyD88-4* in *Mytilus coruscus* and expression changes in response to *Vibrio chagasii* challenge

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Abstract: Myeloid differentiation factor 88 (MyD88) is a crucial adaptor protein in the Toll-like receptor (TLR) signaling pathway and plays an important role in signal transmission. In this study, the full-length cDNA sequence of a *MyD88* gene from *Mytilus coruscus* (*McMyD88-4*) was cloned. Its cDNA sequence is 3 930 bp, with a 2 607 bp open reading frame encoding a protein of 868 amino acids. Amino acids at position of 13-109 are the dead domain, and amino acids at position of 347-481 are the Toll/interleukin-1 receptor (TIR) domain, which contains three highly conserved regions Box 1, Box 2 and Box3. The three-dimensional structure of *McMyD88-4* contains six α -helix and four β -sheets. According to the comparison with known MyD88 amino acids sequence, the putative protein of *McMyD88-4* was most similar to that of *Crassostrea gigas*, with the identity and similarity of 60% and 77% respectively. Then it was more similar to *Patinopecten yessoensis*, *Argopecten irradians*, *Ruditapes philippinarum*, with 40%-51% of identity and 58%-67% of similarity. The result of the phylogenetic tree showed that *McMyD88-4* of *M. coruscus* firstly clustered with *C. gigas* and scallops, and then with *Drosophila melanogaster*, while vertebrates formed a separate cluster. qRT-PCR revealed that *McMyD88-4* was expressed in all tissues and organs examined. The highest expression was found in the mantle and gill, while the lowest expression was found in haemocytes. After infection by *Vibrio chagasii*, the expression of *McMyD88-4* was up-regulated sharply in immune-related tissues, and reached the peak at 3 h and 6 h, and the up-regulation level in digestive gland was significantly higher than those in gill and mantle. The present results showed that *McMyD88-4* plays an important role during the immune response of *M. coruscus* against external pathogens, especially *Vibrio* infections.

Key words: *Mytilus coruscus*; myeloid differentiation factor 88 (MyD88); gene cloning; tissue expression; *Vibrio* infection

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