

# Molecular Characterization of 14-3-3 Zeta Gene in *Musca domestica* (Diptera: Muscidae) and Its Roles in Response to Bacterial Infection

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## Abstract

The 14-3-3 gene plays important role in many biological processes, including cell survival, apoptosis, and signal transduction. However, function of the 14-3-3 homologous gene in *Musca domestica* remains unclear. Here, we identified and characterized the 14-3- $\zeta$  of *M. domestica*. We found that *Md14-3- $\zeta$*  gene was highly homologous with other close insects. The qRT-PCR analysis revealed that the *Md14-3- $\zeta$*  was highly expressed in adults, and was expressed predominantly in hemocytes and fat body. Meanwhile, the expression of *Md14-3- $\zeta$*  was up-regulated after injecting *Escherichia coli* and *Staphylococcus aureus*. Moreover, the recombinant protein r*Md14-3- $\zeta$*  strongly inhibits the growth of *E. coli* and *S. aureus*. Notably, the r*Md14-3- $\zeta$*  inhibits *E. coli* and *S. aureus* by permeating the cell membrane. Taken together, our findings suggested that *Md14-3- $\zeta$*  is involved in the immune response against bacteria through damaging the cell membrane.

**Key words:** 14-3- $\zeta$ , expression, innate immunity, *Musca domestica*

The 14-3-3 proteins were firstly discovered in the bovine brain, the proteins belong to phosphorylated-serine-binding and are highly conserved from bacteria to humans (Fu et al. 2000, Yang et al. 2019, Obsilova and Obsil 2020). All 14-3-3 proteins are acid-soluble proteins and in forms of homo or hetero dimers with a molecular mass of 28–32 kDa (Obsil and Obsilova 2011, Ulvila et al. 2011). To date,  $\beta$ ,  $\gamma$ ,  $\epsilon$ ,  $\eta$ ,  $\delta$ ,  $\lambda$ , and  $\zeta$  seven isoforms of the 14-3-3 gene were reported in mammals and fifteen isoforms were recorded in plants. Only  $\epsilon$  and  $\zeta$  isoforms of 14-3-3 genes have been found in insects (Kong et al. 2007, Shandala et al. 2011, Seong et al. 2018). Because 14-3-3 proteins have a hypervariable region at the C-terminus, more than 200 cellular proteins have been found to be associated with this protein. Thus, 14-3-3 proteins are implicated in a multitude of cellular pathways, including signal transduction, host defense, stress responses, DNA damage, and prevention of apoptosis (Fu et al. 2000, Obsil and Obsilova 2011, Shandala et al. 2011, Yang et al. 2019, Obsilova and Obsil 2020).

Numerous 14-3-3 genes have been functionally characterized in eukaryotic organisms, including fungi, plants, invertebrates, and vertebrates (Fu et al. 2000, Obsil and Obsilova 2011, Yang et al. 2019, Obsilova and Obsil 2020). Interestingly, 14-3-3 genes were found to be involved in the metamorphosis, immune response, and signal transduction in insects. For example, in *Spodoptera*

*litura* Fabricius (Lepidoptera: Noctuidae), *Bombyx mori* Linnaeus (Lepidoptera: Bombycidae), and *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae), ubiquitous expression of the 14-3-3 $\zeta$  genes in many organs during different developmental stages, and is involved in the metamorphosis and regulation of diapause processes (Kong et al. 2007, Chen et al. 2010, Feng et al. 2014). Meanwhile, the shrimp's 14-3-3 protein regulates the expression of immune-related genes, including crustin and lysozyme (Liu et al. 2016). In *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae) and *Aedes aegypti* Linnaeus (Diptera: Culicidae), the 14-3-3 $\zeta$  genes play important roles in host defense against bacteria and fungi (Trujillo-Ocampo et al. 2017, Seong et al. 2018). Moreover, the 14-3-3 $\zeta$  genes of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) not only require for neuronal differential and signal transduction, but also developmental and immune response (Shandala et al. 2011).

Housefly, *Musca domestica* (*M. domestica*), is chronically exposed to harsh environmental conditions, but seldom exhibits ill effects (Neupane et al. 2019, 2020, Hinkle and Hogsette 2021). Such observations suggest that the housefly is highly adapted to different pathogens challenges. Therefore, *M. domestica* may serve as an excellent model for investigating insect immunity (Gao et al. 2015, Zhang et al. 2019). However, to our knowledge, the 14-3-3 genes in houseflies have not been identified and characterized.

In the current study, we identified and characterized the *Md14-3-3ζ* gene from *M. domestica*, and named it *Md14-3-3ζ*. In addition, mRNA expression pattern from different developmental stages and different tissues were checked by qRT-PCR. We also analyzed expression levels following immune challenges with *Escherichia coli* and *Staphylococcus aureus*. Finally, we performed the functional analysis of the recombinant protein and found that rMd14-3-3ζ kills *E. coli* and *S. aureus* through damaging the cell membrane. Our results provide a basis for understanding the immune response mechanism of *M. domestica*.

## Materials and Methods

### Housefly Culture

Houseflies were reared at our laboratory under controlled conditions (26 ± 1°C, 65–85% RH, and a photoperiod of 14:10 (L:D) h) (Jiao et al. 2022). Larvae were reared with artificial diet (55 g bran, 150 ml milk, 3 g heat-inactivated yeast, and 0.35 g antimycotic nipagin), and adult flies were fed with sugar and milk powder, and water was supplied.

### Microorganism Culture and Immune Challenges

*E. coli* (ATCC25922) and *S. aureus* (ATCC25923) were used for immune challenges. LB medium (10 ml, 37°C) was used for the bacteria culture and harvest at OD<sub>600</sub> = 1.0. Then, both of the bacterial cells were washed with PBS (phosphate-buffered saline: 2.7 mM KCl, 138 mM NaCl, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 7.3 mM Na<sub>2</sub>HPO<sub>4</sub>, pH = 7.4) 3 times by centrifugation (2 min, 12,000 × g), the suspended cells were mixed with a small volume of PBS.

For the immune challenges experiment, *M. domestica* adults (*n* = 6 per group) were microinjected with 5 μL suspensions of *E. coli* 1 × 10<sup>5</sup> colony forming units (CFU), and *S. aureus* 1 × 10<sup>5</sup> CFU using a microsyringe. Control groups (*n* = 6 per group) were injected with 5 μL of PBS. The samples were then collected at 0, 3, 6, 12, 24, 36, and 48 h for RNA extraction.

### RNA Extraction and Reverse Transcription

Housefly samples were collected at six stages, including eggs, first instar larvae, second instar larvae, third instar larvae, pupae, and adults (*n* = 6/group). The hemocytes, integument, fat body, midgut, Malpighian tubules, trachea, and salivary glands were collected from adult houseflies (*n* = 6/group). High quality RNA extraction was performed by using a Trizol Reagent Total RNA Kit. Then reverse transcribed cDNA was synthesized by using M-MLV Reverse Transcriptase (Invitrogen) from 2 μg of total RNA (Jiao et al. 2022).

### Cloning and Sequence Analysis of *Md14-3-3ζ* Gene

*Md14-3-3ζ* gene was selected from the cDNA library of *M. domestica* (GenBank accession number XP-005182436). A pair of specific primers, *Md14-3-3F* and *Md14-3-3R* was used for PCR (Table 1; Gene-1). PCR reaction was performed with predenaturation at 94°C for 4 min, followed with 35 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 3 min, as well as a final extension at 72°C for 10 min. The products of PCR were cloned into the pMd18-T and then transformed into *E. coli* DH5α. Finally, the anti-Ampicillin agar plate was used for detecting positive recombinants. Three positive clones were amplified by PCR and then sequenced.

The sequences of *Md14-3-3ζ* were searched using BLAST in Gen Bank, and ORF was detected by using ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The sequence alignments were performed by the National Center for Biotechnology Information

(<https://blast.ncbi.nlm.nih.gov>). The signal peptide was predicted by SignalP program (<http://www.cbs.dtu.dk/services/SignalP/>). The phylogenetic tree was constructed by MEGA 7.0.

### Expression of *Md14-3-3ζ* in Different Developmental Stages and Tissues

The mRNA expression of *Md14-3-3ζ* from different tissues (*n* = 6 per group), different developmental stages (*n* = 6 per group), and immune challenges experiment (*n* = 6 per group at each time point) were tested by qRT-PCR with qRT-PCR System (ABI 7500, Applied Biosystems). The reaction solution of the qRT-PCR was containing 7.5 μL of TransStart Top Green qRT-PCR Super Mix (final concentration at 2 μM; TransGen), 0.3 μL of primer (final concentration at 40 μM; Table 1, Gene 2), 50 ng of cDNA, and 3.9 μL of RNase-free water. The reaction procedures were 40 cycles of 95°C for 5 s and 60°C for 30 s and 72°C for 30 s. RPS18 (ID: 101887462) was used as a reference gene. The relative mRNA expression of *Md14-3-3ζ* was determined by the 2<sup>-ΔCT</sup> method (Jiao et al. 2022).

### Recombinant Expression and Purification of rMd14-3-3ζ

To amplify the mature peptide of *Md14-3-3ζ*, the gene specific primer was designed (Table 1, Gene 3). The DNA fragment was transformed into an expression vector pET-28a (Novagen), and recombinant plasmids were transformed into competent *E. coli* BL21 (DE3) cells. No insert fragment of pET-28a vector as the control, and insert fragment of pET-28a vector were incubated in LB medium and shaken (300 g) at 37°C until the OD<sub>600</sub> = 0.5. Then, 0.5 mM of isopropyl β-D-thiogalactoside was mixed and shaken for 4 h. After that, bacterial cells were harvested by washing with PBS and then sonicated on ice. The rMd14-3-3ζ was then purified using the High-Affinity Ni-NTA Resin. Finally, the proteins were stored at -80°C until use (Li et al. 2015).

### Western Blotting Analysis of rMd14-3-3ζ

The size of purified rMd14-3-3ζ protein was confirmed by western blotting analysis. Briefly, the purified rMd14-3-3ζ protein was quantified by using a BCA Protein. After this, 12% SDS-PAGE was used for loading and separating the purified protein (25 μg), which was transferred to a nitrocellulose membrane. Then, the membrane was incubated with mouse anti-His mono-clonal antibody at 4°C for 12 h. Finally, the membrane was incubated with goat anti-mouse immunoglobulin G at 26°C for 2 h (Li et al. 2015).

**Table 1.** Primer sequences used in gene cloning (Gene-1), qRT-PCR (Gene-2), and recombinant expression (Gene-3)

Primer	Primer sequence
Md14-3-3ζ -F-1	5'-TTCCTCCATTGAGCAGA-3'
Md14-3-3ζ -R-1	5'-CTTCATAACAGATTTCCCTA-3'
RPS18-F-2	5'-AAGGGTGTGGGTCGCCGTT-3'
RPS18-R-2	5'-GCAATGGGTTGGAGATGAT-3'
Md14-3-3ζ -F-2	5'-TTCCTCCATTGAGCAGA-3'
Md14-3-3ζ -R-2	5'-CTTCATAACAGATTTCCCTA-3'
Md14-3-3ζ -F-3	5'-CGCGAATTCATGTGCACAGTC GATAAAGAGG-3'
Md14-3-3ζ -R-3	5'-CGCAAGCTTTTAAAAAGGAAA CAATTTAGTTTGGG-3'

### Antimicrobial Activity Assay

The antimicrobial activity assay was performed by LB agar plates and the 96-well sterilized microplates. First, 6 and 10 µg of rMd14-3-3ζ was mixed with *E. coli* and *S. aureus* (50 CFU), respectively. Then, the solution was spread on LB agar plates and incubated at 37°C. The inhibitory effect of rMd14-3-3ζ on the growth of *S. aureus* and *E. coli* were observed after 12 h. Ampicillin (3 µg/ml) was used as a positive control, and PBS was used as a negative control for the assay. Meanwhile, 100 µL bacterial cultures were allocated to 96-well sterilized microplates, and 10 µg of rMd14-3-3ζ were mixed into each well. Finally, the microplates were incubated in an orbital shaker-incubator (37°C, 120 g). The absorbance was measured after the microplates were incubated for 0, 2, 4, 6, 8, 10, 12, and 24 h. Experiments were done in duplication and repeated at least three times for each assay.

### Minimum Inhibitory Concentration (MIC) Assay

The MIC of rMd14-3-3ζ was assessed by 96-well microtiter assay, and resazurin was used as an indicator (Elshikh et al. 2016). Briefly, different amount of rMd14-3-3ζ (250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.95, 0.97, 0.48, 0.24, 0.12, 0.06, 0.03, and 0.015 µg) were added into each well, and then mixed with 100 µL of nutrient broth and 10 µL of resazurin indicator solution. After that, 10 µL of bacterial cell suspension ( $5 \times 10^2$  CFU/ml) was mixed in each well, 37°C incubated for 24 h, and observed the color change. PBS was used as the control. The wells that remained blue were scored above the MIC value.

### Bacterial Cell Membrane Permeabilization Assay

Bacterial cell membrane permeabilization was examined by releasing cytoplasmic β-galactosidase from *E. coli* and *S. aureus* in the culture

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1   ATGTCGACAGTCGATAAAGAGGAATTGGTCCAAAAGGCAAAATGGCCGAACATCCGAA.
1   M S T V D K E E L V Q K A K L A E Q S E .
61  AGATACGATGACATGGCTCAAGCTATGAAATCAGTCACAGAAACCGGTGTCGAACGTCA.
21  R Y D D M A Q A M K S V T E T G V E L S .
121 AATGAAGAAAGAAATTTGCTATCAGTTGCTTACAAAAATGTTGTTGGTGGCCGCAGATCA.
41  N E E R N L L S V A Y K N V V G A R R S .
181 TCATGGAGAGTAATTTCCCTCCATTGAGCAGAAAACCGAAGCCTCCGCCAGAAAACAACAA.
61  S W R V I S S I E Q K T E A S A R K Q Q .
241 CTCGCCCGGAGTACAGAGAACCGTAGAGAAGGAACCTAGGGAAATCTGTTATGAAGTT.
81  L A R E Y R E R V E K E L R E I C Y E V .
301 TTGGGTCTTCTTGACAAATACCTTATTCCATAAGCTAGCAATCCAGAAAGTAAGGTTTTC.
101 L G L L D K Y L I P K A S N P E S K V F .
361 TATCTGAAAATGAAGGGAGATTATTACAGGTATTTAGCTGAAGTAGCCACAGGAGATGCC.
121 Y L K M K G D Y Y R Y L A E V A T G D A .
421 CGCAACACCGTTGTTGACGACTCCAAACCGCATAACAGGATGCATTGAAATTAGCAAG.
141 R N T V V D D S Q T A Y Q D A F E I S K .
481 GGTAAAATGCAACCAACACATCCCATCAGATTGGGTCTTGCACTTAATTTCTCAGTCTTC.
161 G K M Q P T H P I R L G L A L N F S V F .
541 TACTATGAGATTCTGAATTCTCCAGACAAAGCTTGCCAATTGGCCAAACAGGCATTTCGAT.
181 Y Y E I L N S P D K A C Q L A K Q A F D .
601 GATGCGATAGCTGAACTGGATACTCTCAACGAGGATTCGTACAAGGACTCAACTCATC.
201 D A I A E L D T L N E D S Y K D S T L I .
661 ATGCAATTGTTGAGAGATAACCTTACCCTCTGGACATCGGACACCCAGGGTGGCGAT.
221 M Q L L R D N L T L W T S D T Q G D G D .
721 GAACCACAAGAAGTGGTGACAAC TAACACCAAAC TAAATTGTTCCTTTTTAA.
241 E P Q E V V T T N T Q T K L F P F * .

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**Fig. 1.** Nucleotide and deduced amino acid sequence of the *Md14-3-3Zeta*. The start codon (ATG) and termination codon (TAA) was shown in the black frame. The putative transmembrane regions (172-180aa) were shown in double underlined. In 6-238aa were the structural domains of the *Md14-3-3Zeta*. The two potential conserved sites (44-54aa, 214-233aa) were shown in single underlined.

medium (Liu et al. 2004). First, bacterial cultures grown in LB medium were harvested. Then, it was washed and suspended with PBS. The bacterial cell suspension was adjusted at  $OD_{430} = 1.2$ . Each well of standard microtiter plate contains 1,000  $\mu\text{L}$  of bacterial suspension ( $5 \times 10^2$  CFU/ml), and then 100  $\mu\text{L}$  ONPG (o-Nitrophenyl- $\beta$ -D-galactoside, ONPG; 30 mM) were mixed into the well. Finally, 0.144 mg of rMd14-3-3 $\zeta$  was mixed. Plates were incubated at 37°C for 12 h and absorbance was monitored at 430 nm.

### Statistical Analysis

SPSS 17.0 were used for statistical analyses. The Levene test and Shapiro–Wilk test were used for checked the homogeneity and normality of variances, respectively. Then, the differences between control and treatment were analyzed by using Student's *t*-test ( $P \leq 0.05$ ). The differences among groups were analyzed by using one-way analysis of variance (ANOVA) and the Tukey ( $P \leq 0.05$ ). All dates were presented as the mean  $\pm$  SE.

## Results

### Md14-3-3 $\zeta$ Gene Sequence Analysis

The gene sequence of 14-3-3 $\zeta$  was obtained from the cDNA library of *M. domestica* (GenBank accession number XP-005182436) and named it Md14-3-3 $\zeta$ . The open reading frame (ORF) is 771 bp in length and encodes 257 amino acids. Signal peptide was not predicted in Md14-3-3 $\zeta$ . The domain prediction revealed that amino acids 6-238 were Md14-3-3 $\zeta$  structural domains with a transmembrane domain (170–180 aa), two conserved sites (44–54 aa, and 214–233 aa; Fig. 1). The predicted molecular mass and isoelectric point of Md14-3-3 $\zeta$  were 29.35 kDa and 4.87, respectively.

### Phylogenetic Analysis of Md14-3-3 $\zeta$

Multiple sequence alignment revealed that Md14-3-3 $\zeta$  amino acid sequences were significant similarity with 14-3-3 $\zeta$  of other close insects (up to 86%), in which, 14-3-3 $\zeta$  of housefly showed 95%

and 99% homology with *D. melanogaster* and *Stomoxys calcitrans* Linnaeus (Diptera: Muscidae), respectively. Moreover, phylogenetic analysis revealed that 14-3-3 $\zeta$  sequences of seventeen insects were divided into eight groups. The 14-3-3 $\zeta$  in these insects was considerably conserved, and the Md14-3-3 $\zeta$  was clustered with *D. melanogaster* and *S. calcitrans* (Fig. 2).

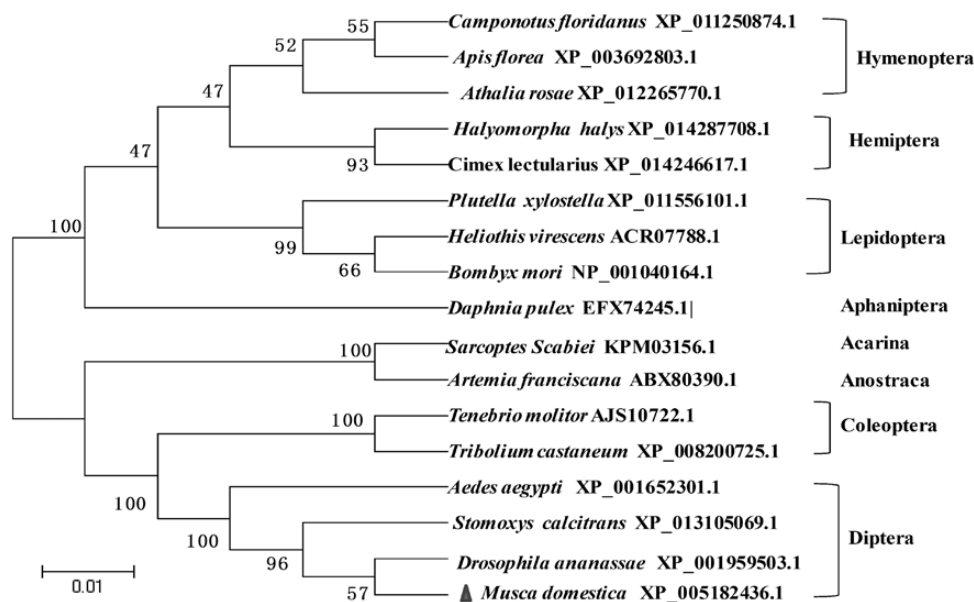
### Expression Profiles of Md14-3-3 $\zeta$ Gene

In order to investigate Md14-3-3 $\zeta$  gene in the context of housefly immunity, we first checked its expression pattern during all the developmental stages of the housefly. Results showed that Md14-3-3 $\zeta$  gene was constitutively expressed in all the six developmental stages of the housefly, with higher expression levels in adults, which was 157-fold greater than that of eggs with lowest expression ratio ( $F_{5,12} = 111.287$ ,  $P < 0.001$ ; Fig. 3A).

Next, we checked the expression pattern of Md14-3-3 $\zeta$  in different tissues, including immune tissues (hemocytes, integument, and fat body), digestive tissues (midgut), excretory tissues (Malpighian tubules), respiratory tissues (trachea), and reproductive tissues (salivary glands). We found that Md14-3-3 $\zeta$  was highly expressed in the immune tissues compared with other tissues ( $F_{6,14} = 21.621$ ,  $P < 0.001$ ; Fig. 3B). These results imply that Md14-3-3 $\zeta$  may play a key role in housefly immunity.

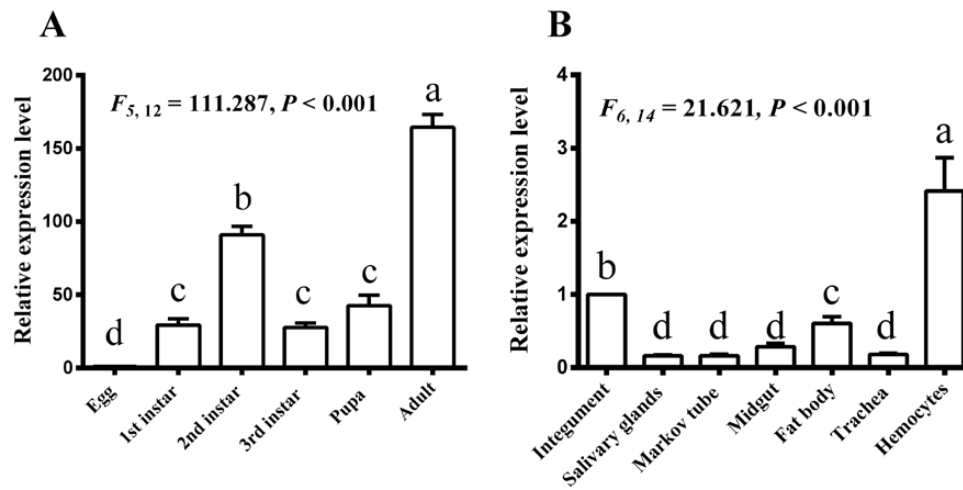
### Expression Profile of Md14-3-3 $\zeta$ Responding to Bacteria Challenge

In order to investigate the putative function of Md14-3-3 $\zeta$  in innate immunity, *M. domestica* adult was challenged with *E. coli* and *S. aureus*. The relative mRNA level of Md14-3-3 $\zeta$  was increased and peaked (14.7-fold) at 3 h after *E. coli* injecting ( $F_{5,12} = 43.889$ ,  $P < 0.001$ ; Fig. 4A). Similarly, the relative mRNA level of Md14-3-3 $\zeta$  was increased and peaked (11.8-fold) at 3 h after *S. aureus* injecting ( $F_{5,12} = 150.061$ ,  $P < 0.001$ ; Fig. 4B). These results indicate that Md14-3-3 $\zeta$  is involved in the immune response against *E. coli* and *S. aureus*.

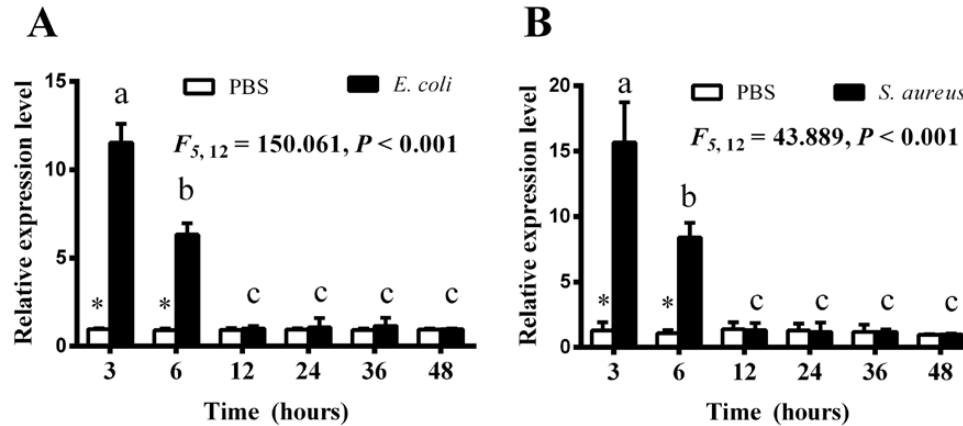


**Fig. 2.** Phylogenetic relationships of 14-3-3 $\zeta$  in different insect species using the neighbor-joining method with a bootstrap value of 1,000. The phylogenetic trees were constructed using MEGA 7.0 software with the neighbor-joining method. A bootstrap analysis of 1,000 replicates was used, and bootstrap values are shown in the cladograms.





**Fig. 3.** Expression of *Md14-3-3Zeta* in different developmental stages and tissues. (A) *Md14-3-3Zeta* gene expression in different developmental stages. (B) *Md14-3-3Zeta* gene expression in different tissues of *M. domestica* adult. Relative expression levels were calculated using the  $2^{-\Delta\Delta Ct}$  method. Data are presented as the mean  $\pm$  SE. The same letters indicate no significant difference (ANOVA and Tukey test  $P > 0.05$ ) among groups.



**Fig. 4.** *Md14-3-3Zeta* mRNA expression profile after injection with *E. coli* (A) and *S. aureus* (B) at 3, 6, 12, 24, 36, and 48 h. PBS was used as a control, and the *Musca domestica* adult was collected for qRT-PCR analysis after injecting *E. coli* and *S. aureus*. Relative expression levels were calculated using the  $2^{-\Delta\Delta Ct}$  method. Data are presented as the mean  $\pm$  SE. The same letters indicate no significant difference (ANOVA and Tukey test  $P > 0.05$ ) among groups at different time points. The asterisks indicate significant differences (Student's *t*-test  $P \leq 0.05$ ) between control and treatment.

### The rMd14-3-3 $\zeta$ Inhibits the Growth of Bacteria

To investigate Md14-3-3 $\zeta$  protein function in vitro, the recombinant protein of Md14-3-3 $\zeta$  was expressed in *E. coli*. PAGE and western blotting with anti-HIS indicated rMd14-3-3 $\zeta$  is around 30 kDa protein (Fig. 5A and B). Compared with those of the control, the rMd14-3-3 $\zeta$  strongly inhibited the growth of *E. coli* and *S. aureus* in LB agar plates (Fig. 6A). The growth curve of *E. coli* and *S. aureus* was maintained from 0 to 24 h (Fig. 6B and C). Furthermore, the MIC of rMd14-3-3 $\zeta$  inhibiting *E. coli* and *S. aureus* was 0.16 mg/ml and 0.25 mg/ml, respectively. These results confirmed that Md14-3-3 $\zeta$  is involved in the immune response against *E. coli* and *S. aureus*.

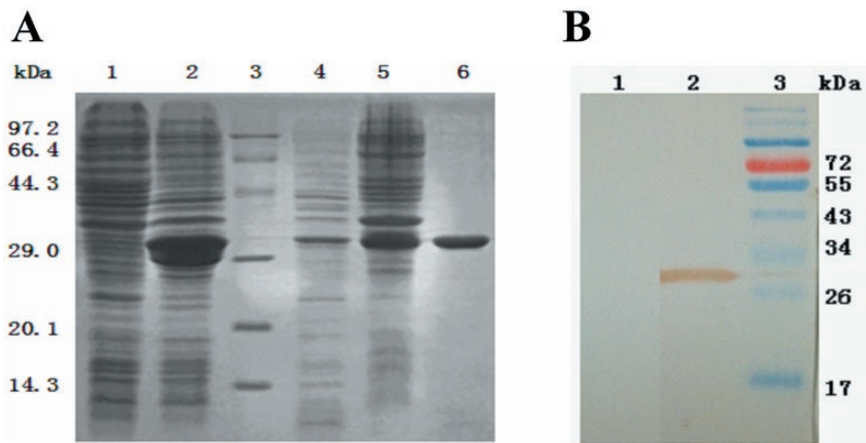
### The rMd14-3-3 $\zeta$ Permeates the Cell Membrane of Bacteria

The release of cytoplasmic  $\beta$ -galactosidase that absorb at 430 nm is an indication of cell membrane damage. When the suspensions of *E. coli* and *S. aureus* were treated with rMd14-3-3 $\zeta$ , the OD<sub>430</sub> increased rapidly from 30 to 120 min, while the control was maintained at all-time points (Fig. 7A and B). These results indicate that rMd14-3-3 $\zeta$  inhibits *E. coli* and *S. aureus* by permeating the cell membrane.

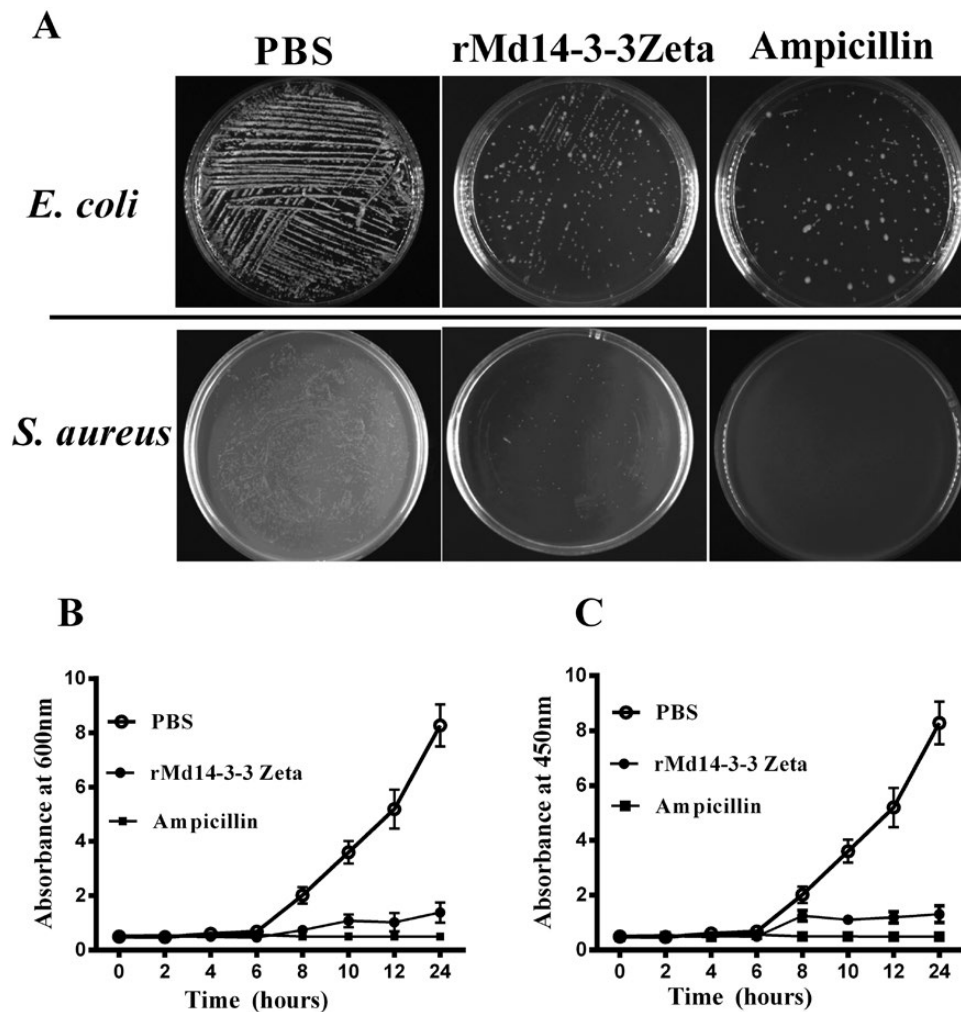
### Discussion

As a chaperone protein, the 14-3-3 protein is involved in numerous biological functions in vertebrates and invertebrates, including development, immune response, and metabolism (Fu et al. 2000, Obsil and Obsilova 2011, Shandala et al. 2011, Trujillo-Ocampo et al. 2017, Obsilova and Obsil 2020). However, the protein function in the housefly remains unexplored. In recent years, the housefly has become a good model for investigating insect immunity (Gao et al. 2015, Andoh et al. 2018, Neupane et al. 2020, Hinkle and Hogsette 2021). In this study, the 14-3-3 $\zeta$  gene of housefly was identified and characterized.

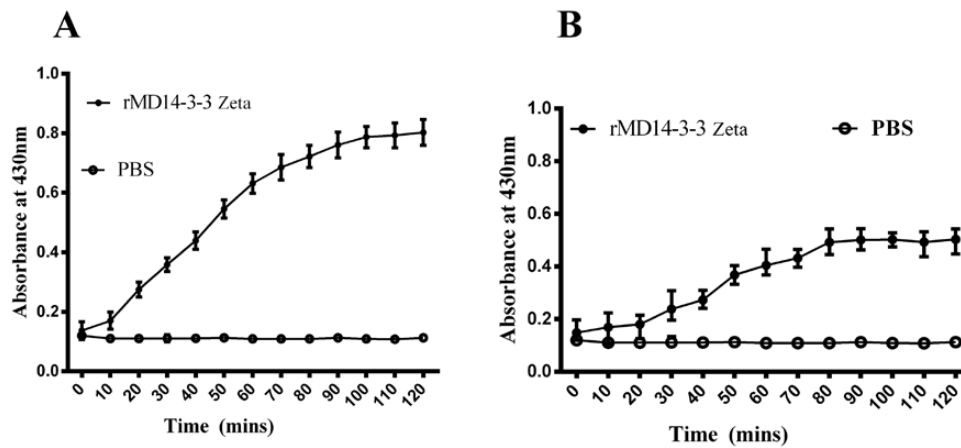
The 14-3-3 $\zeta$  protein is highly conserved from yeast to humans, it has been identified and found the protein contained the 14-3-3 homology domain and showed high sequence identity to their respective orthologues in eukaryotes (Fu et al. 2000, Obsil and Obsilova 2011, Shandala et al. 2011, Liu et al. 2016, Obsilova and Obsil 2020). We also found that Md14-3-3 $\zeta$  holds a typically 14-3-3 structural domain, a transmembrane domain, and two conserved sites, as well as lacks the signal peptide. These features were consistent with the characteristics of 14-3-3 protein. Moreover, the phylogenetic tree



**Fig. 5.** Analysis of recombinant rMd14-3-3 $\zeta$  using SDS-PAGE and western blotting. (A) The detection of Md14-3-3 $\zeta$  recombinant protein by SDS-PAGE analysis. Lane 1, pET-28a (-) Md14-3-3 $\zeta$  not induced. Lane 2, pET-28a (+) Md14-3-3 $\zeta$  induced. Lane 3, Marker. Lane 4, supernatant after crushing. Lane 5, sedimentation after crushing. Lane 6, purified protein of rMd14-3-3 $\zeta$ . (B) Western blotting analysis using rabbit serum anti-His monoclonal antibody as the first antibody. Lane 1, control serum. Lane 2, purified protein of rMd14-3-3 $\zeta$ . Lane 3, marker.



**Fig. 6.** Antibacterial activity of the recombinant rMd14-3-3 $\zeta$  protein. (A) No inhibition of growth was observed by PBS at 12 h, which were used as negative controls and Ampicillin was used as a positive control. (B) The growth of *E. coli* was measured at OD 600 nm every 2 h. (C) The growth of *S. aureus* was measured at OD 450 nm every 2 h. Data are presented as the mean  $\pm$  SE. Experiments were run in triplicate for each assay.



**Fig. 7.** The rMd14-3-3 $\zeta$  permeates the membrane of *E. coli* (A) and *S. aureus* (B). Bacterial cell membrane permeabilization was measured by the release of cytoplasmic  $\beta$ -galactosidase from bacteria. PBS was used as the control. The absorbance was measured at OD 430 nm every 10 min. Data are presented as the mean  $\pm$  SE. Experiments were run in triplicate for each assay.

has shown that 14-3-3 $\zeta$  was clustered with that of *Diptera* species. Our results indicated that Md14-3-3 $\zeta$  shared a high level of conservation with other closed insects, and had a closer evolutionary relationship with the *Diptera*.

Previous studies showed that the 14-3-3 $\zeta$  gene has different expression patterns in many insects depending on specific roles (Kong et al. 2007, Obsil and Obsilova 2011, Trujillo-Ocampo et al. 2017). For instance, the 14-3-3 $\zeta$  gene of *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae) was expressed in all tested tissues and developmental stages, and no significant differences were found in the tested tissues, thereby suggesting that the gene acted as a house-keeping gene (Bian et al. 2015). Moreover, the 14-3-3 $\zeta$  gene of *S. litura*, *B. mori*, and *H. armigera* was identified and found relatively high expression level in prepupa, suggesting that the genes were related to larval metamorphosis and diapause processes (Kong et al. 2007, Chen et al. 2010, Feng et al. 2014). Meanwhile, 14-3-3 $\zeta$  gene of *T. molitor* and *S. litura* was mainly expressed in immune tissues—hemocytes and fat body, suggesting that the genes were related to insect immunity (Feng et al. 2014, Seong et al. 2018). This is consistent with our results that Md14-3-3 $\zeta$  was predominantly expressed in the hemocytes, integument, and fat body. We also found that expression of the Md14-3-3 $\zeta$  gene could be induced by *E. coli* and *S. aureus*. Because the innate immune responses of insects could be induced by pathogens, and integument was the first checkpoint component, while the hemocytes and fat body were regarded as key tissues related to insect immunity (Kong et al. 2007, Andoh et al. 2018, Zhang et al. 2019).

Importantly, RNAi and recombinant protein-based strategies have mainly performed functional analysis of the genes in many studies. Based on RNAi, the previous study found that 14-3-3 $\zeta$  is required for microbial resistance and phagocytosis in *D. melanogaster* (Shandala et al. 2011). Meanwhile, knocking down 14-3-3 $\zeta$  of *S. litura* increased insects' sensitivity to *Nomuraea rileyi* and resulted in higher mortality (Feng et al. 2014). Similarly, silencing of the 14-3-3 $\zeta$  in *A. aegypti* and found it involved in phagocytosis of Gram-positive and Gram-negative bacteria (Trujillo-Ocampo et al. 2017). However, the recombinant protein rTm14-3-3 $\zeta$  could inhibit the growth of *E. coli* and *C. albicans* (Seong et al. 2018). Our results showed that the recombinant protein rMd14-3-3 $\zeta$  inhibits the growth of *E. coli* and *S. aureus*. Moreover, our findings showed that rMd14-3-3 $\zeta$  could permeate the cell membrane of the bacteria, thereby inhibiting the growth of *E. coli* and *S. aureus*.

## Conclusions

In conclusion, we have identified and characterized the 14-3-3 $\zeta$  gene from housefly. The gene is predominantly expressed in the immune tissues, and the expression of the gene could be induced by *E. coli* and *S. aureus*. The recombinant protein rMd14-3-3 $\zeta$  inhibits the growth of bacteria by permeating the cell membrane. Our results provide novel information for investigating the immune response mechanism of the housefly.

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## Author Contributions

Z.J. and Y.Y. conceived research and writing; J.P.: statistical analyses; J.X.: review; X.S. carried out some of experiments; G.G. designed the study.

## References Cited

- Andoh, M., T. Ueno, and K. Kawasaki. 2018. Tissue-dependent induction of antimicrobial peptide genes after body wall injury in house fly (*Musca domestica*) larvae. *Drug Discov. Ther.* 12: 355–362.
- Bian, D.-D., B.-M. Ge, C.-L. Zhou, Q.-N. Liu, and B.-P. Tang. 2015. Molecular characterization of a 14-3-3 zeta gene from *Plodia interpunctella*: a potential marker for phylogenetic inference. *Biochem. Syst. Ecol.* 60: 171–176.
- Chen, L., W. Ma, X. Wang, C. Niu, and C. Lei. 2010. Analysis of pupal head proteome and its alteration in diapausing pupae of *Helicoverpa armigera*. *J. Insect Physiol.* 56: 247–252.
- Elshikh, M., S. Ahmed, S. Funston, P. Dunlop, M. McGaw, R. Marchant, and I. M. Banat. 2016. Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants. *Biotechnol. Lett.* 38: 1015–1019.
- Feng, E., H. Chen, Y. Li, W. Jiang, Z. Wang, and Y. Yin. 2014. Gene cloning, expression, and function analysis of SpL14-3-3 $\zeta$  in *Spodoptera litura* and its response to the entomopathogenic fungus *Nomuraea rileyi*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 173: 49–56.
- Fu, H., R. R. Subramanian, and S. C. Masters. 2000. 14-3-3 proteins: structure, function, and regulation. *Annu. Rev. Pharmacol. Toxicol.* 40: 617–647.
- Gao, Y., T. Tang, J. Gu, L. Sun, X. Gao, X. Ma, X. Wang, F. Liu, and J. Wang. 2015. Downregulation of the *Musca domestica* peptidoglycan recognition protein SC (PGRP-SC) leads to overexpression of antimicrobial peptides and tardy pupation. *Mol. Immunol.* 67: 465–474.

- Hinkle, N. C., and J. A. Hogsette. 2021. A review of alternative controls for house flies. *Insects*. 12: 1042.
- Jiao, Z., P. Su, Y. Li, W. Zhao, L. Yang, C. Sun, J. Xiu, X. Shang, and G. Guo. 2022. Identification and function analysis of chitinase 2 gene in housefly, *Musca domestica*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 259: 10.
- Kong, L., Z. Lv, J. Chen, Z. Nie, D. Wang, H. Shen, X. Wang, X. Wu, and Y. Zhang. 2007. Expression analysis and tissue distribution of two 14-3-3 proteins in silkworm (*Bombyx mori*). *Biochim. Biophys. Acta*. 12: 23.
- Li, Y. P., M. Xiao, L. Li, C. X. Song, J. L. Wang, and X. S. Liu. 2015. Molecular characterization of a peptidoglycan recognition protein from the cotton bollworm, *Helicoverpa armigera* and its role in the prophenoloxidase activation pathway. *Mol. Immunol.* 65: 123–132.
- Liu, H., Y. Du, X. Wang, and L. Sun. 2004. Chitosan kills bacteria through cell membrane damage. *Int. J. Food Microbiol.* 95: 147–155.
- Liu, N., X. W. Wang, J. J. Sun, L. Wang, H. W. Zhang, X. F. Zhao, and J. X. Wang. 2016. Akirin interacts with Bap60 and 14-3-3 proteins to regulate the expression of antimicrobial peptides in the kuruma shrimp (*Marsupenaeus japonicus*). *Dev. Comp. Immunol.* 55: 80–89.
- Neupane, S., D. Nayduch, and L. Zurek. 2019. House flies (*Musca domestica*) pose a risk of carriage and transmission of bacterial pathogens associated with bovine respiratory disease (BRD). *Insects*. 10: 358.
- Neupane, S., K. White, J. L. Thomson, L. Zurek, and D. Nayduch. 2020. Environmental and sex effects on bacterial carriage by adult house flies (*Musca domestica* L.). *Insects*. 11: 401.
- Obsil, T., and V. Obsilova. 2011. Structural basis of 14-3-3 protein functions. *Semin. Cell Dev. Biol.* 22: 663–672.
- Obsilova, V., and T. Obsil. 2020. The 14-3-3 proteins as important allosteric regulators of protein kinases. *Int. J. Mol. Sci.* 21: 8824.
- Seong, J. H., Y. H. Jo, G. W. Seo, S. Park, K. B. Park, J. H. Cho, H. J. Ko, C. E. Kim, B. B. Patnaik, S. A. Jun, et al. 2018. Molecular cloning and effects of *Tm14-3-3ζ*-silencing on larval survivability against *E. coli* and *C. albicans* in *Tenebrio molitor*. *Genes*. 9: 330.
- Shandala, T., J. M. Woodcock, Y. Ng, L. Biggs, E. M. Skoulakis, D. A. Brooks, and A. F. Lopez. 2011. *Drosophila* 14-3-3ε has a crucial role in anti-microbial peptide secretion and innate immunity. *J. Cell. Sci.* 124: 2165–2174.
- Trujillo-Ocampo, A., F. E. Cázares-Raga, R. M. Del Angel, F. Medina-Ramírez, L. Santos-Argumedo, M. H. Rodríguez, and F. C. Hernández-Hernández. 2017. Participation of 14-3-3ε and 14-3-3ζ proteins in the phagocytosis, component of cellular immune response, in *Aedes* mosquito cell lines. *Parasit. Vectors*. 10:362.
- Ulvila, J., L. M. Vanha-aho, A. Kleino, M. Vähä-Mäkilä, M. Vuoksio, S. Eskelinen, D. Hultmark, C. Kocks, M. Hallman, M. Parikka, et al. 2011. Cofilin regulator 14-3-3zeta is an evolutionarily conserved protein required for phagocytosis and microbial resistance. *J. Leukoc. Biol.* 89: 649–659.
- Yang, Z., C. Wang, Y. Xue, X. Liu, S. Chen, C. Song, Y. Yang, and Y. Guo. 2019. Calcium-activated 14-3-3 proteins as a molecular switch in salt stress tolerance. *Nat. Commun.* 10:1191.
- Zhang, Y., F. Wang, Q. Feng, H. Wang, T. Tang, D. Huang, and F. Liu. 2019. Involvement of trehalose-6-phosphate synthase in innate immunity of *Musca domestica*. *Dev. Comp. Immunol.* 91: 85–92.