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Research Article

Molecular characterization and expression of the antimicrobial peptide defensin from the housefly (*Musca domestica*)

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Abstract. A 430-bp cDNA encoding the insect antimicrobial peptide defensin was cloned from the housefly, and designated *Musca domestica* defensin (*Mdde*). The open reading frame of the cDNA encoded a 92-amino acid peptide with an N-terminal signal sequence followed by a propeptide that is processed by cleavage to a 40-amino acid mature peptide. Northern analysis and *in situ* hybridization identified the corresponding mRNA in the fat body of bacterially challenged houseflies and in the epidermis of the body wall of naive and challenged houseflies. The Gram-negative bacterium (*Escherichia coli*) is a strong inducer of the gene. By RT-PCR, *Mdde* mRNA was also detected in naive and challenged insects. These findings suggest that the defensin gene is constitutively expressed in the epidermis of the housefly body wall. The predicted mature form of Mdde was expressed as a recombinant peptide in *E. coli* and *Pichia pastoris*. The recombinant Mdde expressed in *Pichia* was active against Gram-positive and some Gram-negative bacteria.

Keywords. Housefly (*Musca domestica*), antimicrobial peptide, defensin, expression pattern, recombinant expression.

Introduction

The innate immune system is the only pathogen-defense system in invertebrates [1]. It targets a variety of invaders ranging from viruses and unicellular organisms to multicellular fungi, worms, and other metazoa. The immune response is activated by pattern recognition receptors that recognize conserved microbe-specific molecular structures (usually referred to as pathogen-associated molecular patterns). Following recognition, a second important step is modulation by a serine protease proteolytic cascade. The cascade not only confirms the signal, but also trigger responses, such as melanization. The signal then activates multiple and complex signal transduction pathways that ultimately regulate the transcription of target genes encoding effector molecules [1]. Antimicrobial peptides (AMPs) are one of the most important effector molecules of the innate immune system. In the fruit fly *Drosophila*, for example, cationic peptides are the major form of defense against infection [2].

The major AMPs/proteins already found in insects include cecropins, insect defensins, and peptides with an over-representation of some amino acids (proline rich, histidine rich, *etc*.) [3–5]. The insect immune genes and their related molecules have been thoroughly studied in some model insects, such as *Drosophila* [2] and *Anopheles gambiae* [1]. Insect defensins were first identified from *Sarcophaga peregrina* by Matsuyama and Natori [6] and from *Phormia terranovae* by Lambert et al. [7]. Since then, over 70 different invertebrate defensins have been isolated from arthropods belonging to diverse taxa such as insects, crustaceans, ticks, spiders, scorpions,

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and mollusks [5]. The insect defensin family is the most widespread group of AMPs that exist in insects and other invertebrates. They are cysteine-rich peptides and work primarily against Gram-positive bacteria.

Several dipteran insects are vectors of parasites and pathogens that cause major human infectious diseases. The housefly (*Musca domestica)* is considered to be a mechanical vector of many kinds of pathogens, such as bacteria, protozoa, viruses, and worms. Despite the importance of the housefly as a vector of disease, very little is known about its immune system. Previous work from this laboratory purified an AMP of 10 kDa. The peptide is active against certain Gram-positive and Gram-negative bacteria [8]. In our previous study, we cloned a new gene for cecropin AMPs from the adult housefly, detected its expression in larvae and adults, and expressed it in *Escherichia coli* [9]. In the present study, we examined and characterized the defensin AMPs from the housefly.

Materials and methods

Animals and materials

Houseflies, *Musca domestica* Linnaeus (Insecta, Diptera), were raised in the laboratory. A 3′-Full RACE core set kit, Ex-*Taq* polymerase, T4 DNA ligase, and restriction enzymes were obtained from TaKaRa Biotechnology (Dalian, China). The Super SMART™ PCR cDNA Synthesis kit and Clonetech PCR-Select cDNA Subtraction kit were from BD Biosciences Clontech (Palo Alto, CA). The cDNA synthesis kit was from Sangon Company (Shanghai, China). Quickprep™ Micro mRNA Isolation kit are purchased from Amersham (Uppsala, Sweden). The DIG RNA Labeling Kit was from Roche Applied Science (Basel, Switzerland). Glutathione-Sepharose 4B, thrombin, and pGEX-4T1 were purchased from Amersham Pharmacia. Plasmid pPIC9K and *P. pastoris* (KM71) were purchased from Invitrogen (Carlsbad, CA). All other chemicals were of analytical grade for biochemical use.

Infection experiment

E. coli 8099 and *Staphylococcus aureus* ATCC 6538 was used for the infection. The bacteria were cultured in 10 ml LB medium at 37 °C to an OD_{600} of 1 and washed twice with 0.9% NaCl by centrifugation at 12 000 *g* for 2 min. The washed cells were thoroughly resuspended and mixed in the small volume of saline (about 1/10 of original volume).

We used housefly larvae (2nd instar) and adult (aged 2–4 days) for the infection experiment. Septic injury was performed by pricking the larva body or adult thorax of the anaesthetized flies with a sterile needle previously dipped into a concentrated mixture of the bacteria. The flies were then cultured in an air-conditioned room (25–28 °C) and collected at specific times after infection. A sterile needle was used for injury of control larva and adults.

cDNA cloning

Extraction of total RNA. Total RNAs were isolated by acid guanidinium thiocyanate-phenol-chloroform extraction [10] from larva or adult houseflies and dissolved in water treated with DEPC. The purity of the RNA was assessed by measuring absorbance at 260 and 280 nm. Those with an absorbance ratio greater than 1.8 at 260 to 280 nm were used for further experiments.

Suppression subtractive hybridization (SSH). Poly(A+) RNA was extracted from larvae 12 h post injury infection using the QuickPrep micro mRNA Purification Kit (Amersham Biosciences). cDNA was synthesized using the Super SMART-PCR cDNA Synthesis Kit, according to the manufacturer's instructions. SSH was performed to generate a subtracted cDNA library between the bacteria-challenged housefly larvae (tester) and the naive housefly larvae (driver) using the PCR-Select cDNA Subtraction Kit, according to the manufacturer's protocol. Briefly, double-stranded cDNA was synthesized from RNA harvested from tester and driver. After purification, the cDNA was cut into short fragments by *Rsa*I. The cDNA from the tester was separated into two equal parts, and adapters 1 and 2R were each added to one of the two parts. The tester was then hybridized with driver. After the first hybridization, the two products (adaptor 1 and adaptor 2R) were mixed together and immediately hybridized again with an excess of fresh denatured driver cDNAs. Two rounds of hybridization generated a normalized population of tester-specific cDNAs with different adaptors on each end. PCR amplification was performed twice with PCR primer 1 (5′-CTAATACGACTCACTATAGGGC-3′) and nested PCR primer 1 and primer 2R (5′-TCGAGCGGCCGCCCGGGCAGGT-3′/5′-AGCGTGGTCGCGGCCGAGGT-3′) specific to the two adaptors, respectively. The two rounds of PCR resulted in suppression of sequences common to the two cDNA populations and exponential amplification of the differentially expressed sequences.

The subtracted target cDNAs were ligated with the pGEM-T Easy vector using T4 DNA ligase and transformed into *E. coli* DH5α competent cells. The transformed bacteria were plated onto LB agar plates containing ampicillin, X-gal, and IPTG, followed by overnight incubation at 37 °C. Randomly selected white clones were sequenced. The sequences were searched in GenBank with BLASTx.

Cloning of the full-length Md-defensin cDNA. From the subtracted library, four clones were identified as the

defensin fragments after sequencing and BLAST analysis. They were named '*Md*-defensin' (*Mdde*). The 3′ end of *Mdde* was amplified using the cDNA library (λgt11) of the housefly [11] with the specific primer *Mdde* F (5′-GCCACGTGCGATTTGTTGAGC-3′) and the reverse primer λgt R (5′-CTTATGAGTATTTCTTCCAGGGTA-3′) from the λgt 11. The 5′ end of *Mdde* was also amplified from the library by PCR. The primers used for PCR were the λgt11 forward primer (5′-GGTGGCGACGACTCCT-GGAGCCCG-3′) and a specific reverse primer of *Mdde* R (5′-ACGCAGACAGCCTTGCCATTG-3′).

Sequence and phylogenetic analysis

Database searches were performed with Blastx (http//www.ncbi.nlm.nih.gov/). Translation of the cDNA and characteristic prediction of deduced peptide were performed with ExPASy (http://www.expasy.org/). Signal sequence and domain prediction were undertaken with SMART (Simple Modular Architecture Research Tool) (http://smart.embl-heidelberg.de/). Alignment and phylogenetic analysis were performed with MEGA 3.0 [12]. The sequence data were transformed into a distance matrix (p-distance). One thousand bootstraps were performed for the NJ trees to check for repeatability of the results.

Gene expression analysis

RT-PCR. The expression of *Mdde* in naive larvae and bacteria-challenged larvae was investigated by RT-PCR. RNA (5 μg) from larvae at different times following the challenge was reverse transcribed into cDNA as the template for PCR by the RevertAid™ First Strand cDNA Synthesis Kit (MBI). The specific forward primer (*Mdde* F 5′-ATGGCTACTTGCGATTTGTTGAGC-3′) and the reverse primer (*Mdde* R 5′-**G**AACACCTCAGTTACG-GCAAAC-3′) were used in the PCR reactions. The procedure for the PCR reaction includes 2 min at 94 °C, 25 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 10 min. PCRs were standardized using β-actin specific primers (actin F 5′-CAC-GCCATCCTGCGTCTGGA-3′ and actin R: 5′-CCA-CATCTGCTGGAAGGTGG-3′). The amplified products were analyzed by agarose gel electrophoresis.

Northern blot analysis. The recombinant *Mdde*-pGEM-T-Easy plasmid, with the positively oriented insert of the cDNA, was linearized by digestion with *Nde*I and *Apa*I separately and then transcribed *in vitro* with T7 or SP6 RNA polymerase (DIG RNA Labeling Kit, Roche) into antisense and sense digoxigenin (DIG)-RNA probes, respectively. Northern blot analysis was performed to detect the gene expression at different induction times. Total RNA was isolated from larvae and adult houseflies by the method mentioned above. Approximately 10 μg RNA was denatured and electrophoresed on a formaldehyde-containing agarose gel. After electrophoresis, the RNA was transferred to a nylon membrane. The target mRNA was hybridized with DIG-labeled antisense RNA probes (100 ng/ml) in 50% formamide, at 68 °C. After a stringency wash at 68 °C, anti-DIG-phosphatase antibody was used to detect the probe. 5-Bromo-4-chloro-3-indolyl phosphatase and nitroblue tetrazolium chloride were used to visualize the signals.

***In situ* hybridization**. The larvae were fixed by incubation for 24 h at room temperature in a PBS solution containing 4% paraformaldehyde and, after dehydration, were embedded in paraffin. Paraffin-embedded tissue was sectioned at a thickness of 7 μm and rehydrated in DEPC-treated water. Prehybridization and hybridization were performed according to the procedures from the supplier of the DIG RNA labeling kit (SP6/T7). The final concentration of antisense RNA probes labeled by DIG used in the experiment was 400 ng/ml. The negative control used the same amount of the sense RNA probe labeled by DIG. Tissue sections of larva houseflies were probed with DIG-labeled antisense probes and detected with alkaline phosphatase conjugated with anti-DIG antibodies.

Recombinant expression

Expression, purification and antiserum preparation of recombinant Mdde in *E. coli*. The DNA fragment encoding mature *Mdde* was obtained by PCR amplification with the following primers designed from the cloned *Mdde* sequence: forward primer *Mdde* pGEX F 5′-GCGCGAATTCGCTACTTGCGATTTGTTGAGC-3′ containing an *Eco*RI site (underlined) and reverse primer *Mdde* pGEX R 5′-GCGCCTCGAGAACACCTCAGT-TACGGCAAAC-3′ containing an *Xho*I site (underlined). The amplified fragment was digested with *Eco*RI and *Xho*I, and subcloned into a pGEX-4T-1 expression vector digested with the same enzymes. The constructed plasmid was denoted as *Mdde*-pGEX and transformed into competent cells of *E. coli* BL21 for fusion expression.

Glutathione S-transferase (GST)–Mdde fusion protein was expressed in *E. coli* BL-21 and induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 4 h at 37 °C. The expression efficiency of different transforms was assessed by analysis of the target protein band in SDS-PAGE using LabWorks software in UVP Gel Imagining-Formation System (Ultra-Violet Products, Cambridge, UK). The transform with high expression efficiency was chosen for large scale expression. Bacterial pellets were collected, resuspended in 5% of the original volume of PBS with 1% Triton X-100 and lysed by probe sonication.

Fusion protein was purified from the supernatant by one-step affinity chromatography on glutathione-Sepharose 4B, which was run according to the manufacturer's instructions for the GST purification column (Pharmacia). The purified fusion protein was digested with thrombin at 25 °C and then applied to Sephadex G-100 for the separation of Mdde. The purified fusion protein GST-Mdde was used as an antigen for making rabbit polyclonal antibodies by the traditional method.

Expression and purification of recombinant Mdde in *Pichia pastoris*. The DNA fragment encoding mature *Md*-defensin-1 was amplified from the *mdde*-pGEM-T easy vector. The PCR products were digested with *Eco*RI and *Not*I and ligated into the *Eco*RI/*Not*I-digested pPIC9K vector. This plasmid (*Mdde*-pPIC9K) was transformed into *E. coli* DH5α. The transformants were confirmed by PCR screening and DNA sequencing.

The PEG method of transformation, *in vivo* screening of multiple inserts, and expression of the recombinant of *P. pastoris* KM71 followed the manufacturer's instructions (Multi-Copy *Pichia* Expression Kit, Invitrogen). The recombinant (r) Mdde was purified using CM-Sepharose CL-6B from the culture medium and analyzed by SDS-PAGE. Fractions containing rMdde were collected, desalinated by dialysis, lyophilized, and kept as dry powder.

Tricine SDS-PAGE and Western blot. The rMdde was analyzed by 15% tricine SDS-PAGE, according to the method of Schägger and von Jagow [13]. After electrophoresis, the peptides were transferred to nitrocellulose membranes using semi-dry electrophoretic transfer. The Western blot was performed as described by Sambrook et al. [14]. The rabbit anti-GST-Mdde polyclonal antibody (1 : 500) was used as the primary antibody, and peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody.

N-terminal sequencing analysis. Purified rMdde was boiled in SDS-PAGE sample buffer. The supernatant was run on 15% tricine SDS–PAGE gels and transferred to PVDF membranes (Perkin-Elmer, Foster City, CA) by semi-dry electrophoretic transfer. Protein bands were stained with Ponceau 3R, excised and sequenced directly by the Edman method on a Procise Sequencer (Perkin-Elmer).

MALDI-TOF analysis. rMdde was analyzed by MALDI-TOF mass spectrometry using a Voyager™ Linear-DE/K Mass Spectrometer (PerSeptive Biosystems, Biospectrometry Workstation, Framington, MA). The samples were mixed with the matrix solution of 3-5-dimethoxy-4-hydroxycinnamic acid (10 mg/ml) containing 30% acetonitrile and 0.01% TFA. The spectra were obtained in the linear positive ion mode and externally calibrated with standard peptides.

Bioassays

Microorganisms. The following seven Gram-positive bacteria were tested in this study: *Staphylococcus aureus* ATCC6538, *Bacillus megaterium*, *Bacillus cereus*, *Bacillus subtilis* ATCC 9372, *Bacillus thuringiensis israelensis*, *Bacillus thuringiensis kurstaki* and *Micrococcus luteus* ATCC4698. The Gram-negative bacteria tested were *E. coli* and *Klebsiella pneumoniae.* In addition, the following fungi (plant pathogens) were tested: *Pythium aphanidermatum*, *Colletotrichum lagenarium*, *Piricularia oryzae.* Among the bacteria, *S aureus* ATCC6538, *Bacillus t. israelensis* and *B. subtilis* ATCC 9372 are animal pathogens and others are used as marker bacteria.

Antimicrobial tests. The minimal growth inhibition concentration (MIC) was determined by liquid growth inhibition assays [15, 16]. MIC is expressed as the lowest final concentration of the peptide at which no microbe growth is observed [17, 18]. The purified rMdde was quantified by the Bradford method [19] and made into a stock solution. The stock solution of rMdde was serially diluted twofold, each with 0.01% acetic acid and 0.2% bovine serum albumin (BSA). Aliquots (10 µl) from each dilution sample were incubated on a 96-well polypropylene microtiter plate. Each well was inoculated with 100 µl of a suspension of a mid-logarithmic phase culture of bacteria in Poor Broth medium (1% tryptone, 0.5% NaCl, pH 7.5), to a final concentration of 2×10^5–7×10^5 CFU/ml, or with 80 µl fungal spores (final concentration: 10^4 spores/ml). Antifungal assays were performed in Potato Dextrose Broth supplemented with tetracycline (10 µg/ml final concentration) in the stationary culture for about 48 h at 25 °C. Bacteria cultures were grown for 24 h with gentle shaking at 30 °C. Bacterial and fungal growth were evaluated by measuring the absorbance of cultures at 600 nm using a microplate reader.

Results

cDNA cloning of the *Mdde*

From the subtracted library, four clones (177 and 300 bp) were identified as the defensin fragments after sequencing and BLAST analysis. They were named '*Md*-defensin' (*Mdde* in short). Using the two specific primers *Mdde*F2 and *Mdde*R2 that were designed according to the fragments, the 3′ end and the 5′ end fragments of the defensin cDNA from the housefly, respectively, were cloned. A 430-bp full-length cDNA was determined by splicing the three overlapping cDNA fragments (GenBank no. AY260152). Finally, sequences of these

cDNA fragments were confirmed by sequencing the full-length *Mdde* cDNA with 5′ and 3′ end primers (SF 5′-ATGAAATATTTTACAATGTTC-3′and SR 5′-ACCTCAGTTACGGCAAACACA-3′). Sequence analysis revealed that the open reading frame of the cDNA encoded a 92-amino acid peptide (Fig. 1). The first 23 amino acids represent a putative signal sequence, followed by a propeptide (24–52) and a mature peptide (53–92).

BLASTX searches of the NCBI databases revealed that the sequence identities with other known insect defensins are between 51% and 73%. The mature peptide, with a predicted molecular mass of 4.0 kDa and p*I* of 8.69, has one negatively charged amino acid and four positively charged ones. The putative housefly defensin is characterized by six invariant cysteine residues forming three disulfide bonds.

The alignment of defensin mature peptide sequences from several insects, Arachnida (spiders), Mollusca and fungus indicated the similarity and conservation of these peptides among different animals (Fig. 2a). Phylogenetic analysis shows that all defensins were divided two groups. Group I includes the defensins from different insects (Diptera, Hymenoptera, Coleoptera, Hemiptera), spiders (*Demacentor and Haemaphysalis*), mollusca (*Haliotis*) and fungus (*Aspergillus fumigatus*); all defensins from Diptera insects except only one from Lepidoptera insect form the group II. The *Mdde* belongs to group I and shared the greatest identities with those from *Protophormia terranovae* and *Sarcophaga peregrine* (Fig. 2b).

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-72                                                        CTCAAACACTAC
-60  AGCTCGTCTGTTTGTAACCATCCAACCCAACCAGCCATCAATCAAACAAATCCAATAAAA
  1  ATGAAATATTTTACAATGTTCGCTTTTTTCTTTGTGGCCGTCTGTTACATCAGCCAGTCG
       M  K  Y  F  T  M  F  A  F  F  F  V  A  V  C  Y  I  S  Q  S   -33
 60  AGTGCCTCACCTGCCCCCAAAGAAGAGGCAAATTTCGTCCATGGAGCTGACGCCCTTAAG
       S  A  S  P  A  P  K  E  E  A  N  F  V  H  G  A  D  A  L  K   -13
120  CAGTTGGAGCCTGAGTTACATGGCCGTTATAAGAGAGCTACTTGCGATTTGTTGAGCGGT
       Q  L  E  P  E  L  H  G  R  Y  K  R  A  T  C  D  L  L  S  G     8
180  ACTGGTGTTGGCCACTCGGCCTGCGCTGCCCATTGTCTGTTGCGCGGCAACCGTGGAGGC
       T  G  V  G  H  S  A  C  A  A  H  C  L  L  R  G  N  R  G  G    28
240  TACTGCAATGGCAAAGGTGTTTGTGTTTGCCGTAACTGAGGTGTTGTTTTTTGTTATTGT
       Y  C  N  G  K  G  V  C  V  C  R  N  *                        40
300  AGTTTATAAGGAAGTTAACAAGATGTATTATTGTATGTAATAAATTAAAAAAAAAAAA
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Figure 1. cDNA and deduced amino acid sequence of defensin from *Musca domestica* (*Mdde*). The sequence of the predicted mature peptide is underlined. The Poly(A) signals (AATAAA) are double-underlined. The asterisk (*) indicates the stop codon (TGA) followed by the powerful stop codon (TAA) at the same ORF. The nucleotide sequence is numbered on the left, and the amino acid sequence is numbered on the right.

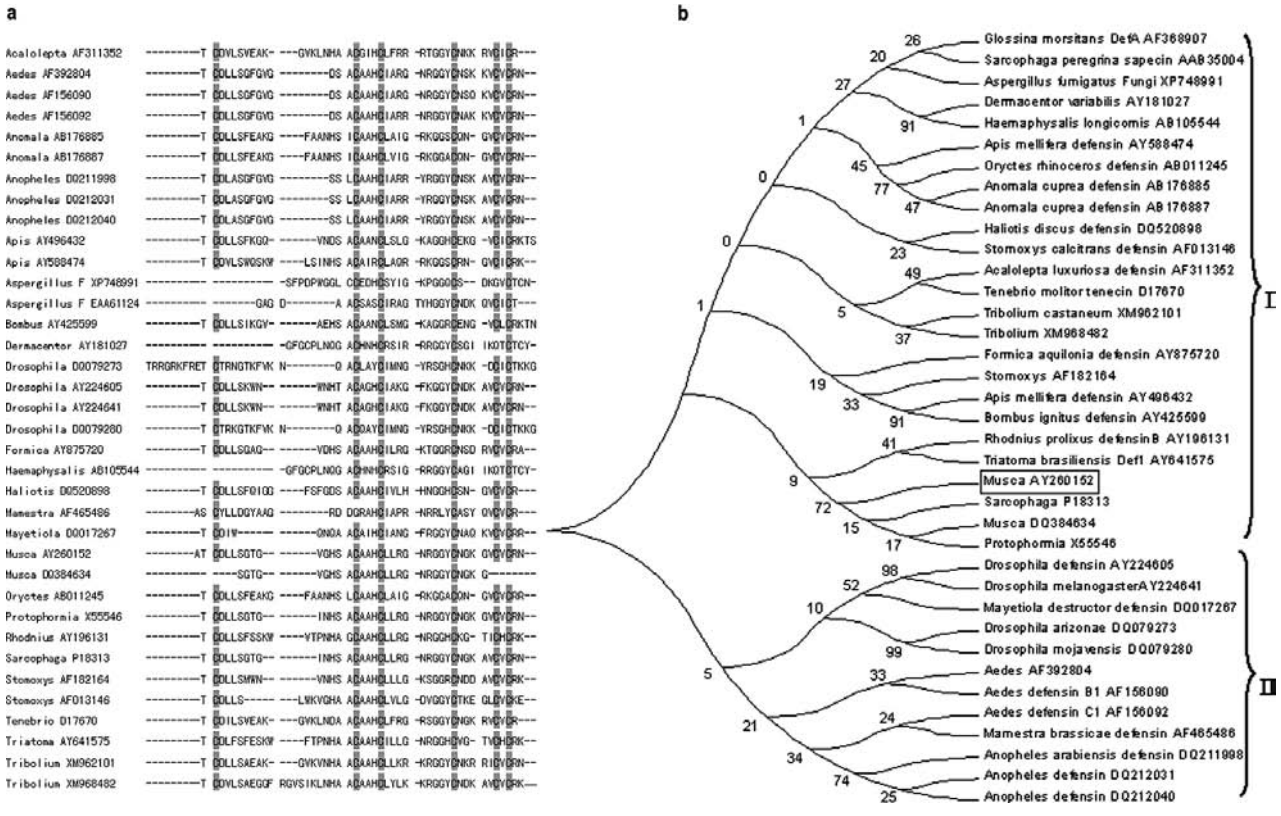

Figure 2. (*a*) Comparative alignment of amino acids of pre prodefensins from insects. The sequences were selected from GenBank. Dashes indicate maximal similarity. (*b*) Phylogenetic analysis of defensin sequences s from different animals and fungus. The NJ tree was obtained using MEGA with complete deletions of gaps. One thousand bootstraps were performed for the NJ trees to check for the repeatability of the results. *Mdde* (Musca AY260152) in group I was boxed.

Mdde gene expression after bacterial challenge in larvae and adults

The expression patterns of *Mdde* in the larvae and adults of the housefly after bacteria challenge were examined by Northern blot analysis. As shown in Figure 3a, the *Mdde* probe detected a ~0.4-kb transcript in larvae from 10 to 48 h after the infection challenge and in adults from 10 to 24 h after the challenge but not at 48 h. The *Mdde* gene expression was not detectable in naive adults and larvae by Northern analysis. To confirm and extend these observations, RT-PCR was performed using *Mdde*-specific primers (pGEX F and pGEX R) and total RNA from the larvae. As shown in Figure 3b, a prominent 150-bp product corresponding to the expected size for mature *Mdde* was amplified from larvae 5–48 h after the challenge. A faint product of the *Mdde* was also observed in naive larvae. The expression data indicated that the *Mdde* transcripts were constitutively expressed at low levels in naive larvae and up-regulated after the larvae were challenged with bacteria.

We compared the induction level of *Mdde* gene in larvae after challenge by Gram-positive and –negative bacteria. As illustrated in Figure 3c, the level of induction of the *Mdde* gene in immune-challenged larvae varied strikingly with the microorganism tested. Gram-negative bacterium (*E. coli*) was the potent inducer of the defensin gene. The expression level was increased 3 h post challenge and reached the higher level from 36 to 48 h post challenge. In contrast, Gram-positive bacterium (*S. aureus*) did not induce expression above the level of a simple injury. The only difference between the Gram-positive bacterium challenge and the injury was the expression time of the gene, it lasted a little longer time for the challenge of *S. aureus*.

To localize the expression of *Mdde* mRNA in the different tissues, *in situ* hybridization was performed on sections of the housefly larvae. As shown in Figure 4, *Mdde* mRNA was detected in the fat body of the challenged larvae and the epidermal tissue of both the challenged and naive housefly larvae. No signal was detected in the midgut and tracheae tissues. Also, no signal was detected when the sections were exposed to the sense *Mdde* RNA probe. These results indicated that the expression of the *Mdde* gene in the epidermis was constitutive expression, and that in fat body was inducible expression.

Purification and antiserum preparation of rMdde

rMdde was expressed in *E. coli* as a GST-fusion peptide and represented 33% of the total cell proteins. It was purified via GST affinity chromatography (Fig. 5a). The GST was removed from rMdde by cleaving the fusion peptide with thrombin. rMdde was further purified by cation-exchange chromatography (Fig. 5b). The antimicrobial activity of purified rMdde was assessed in

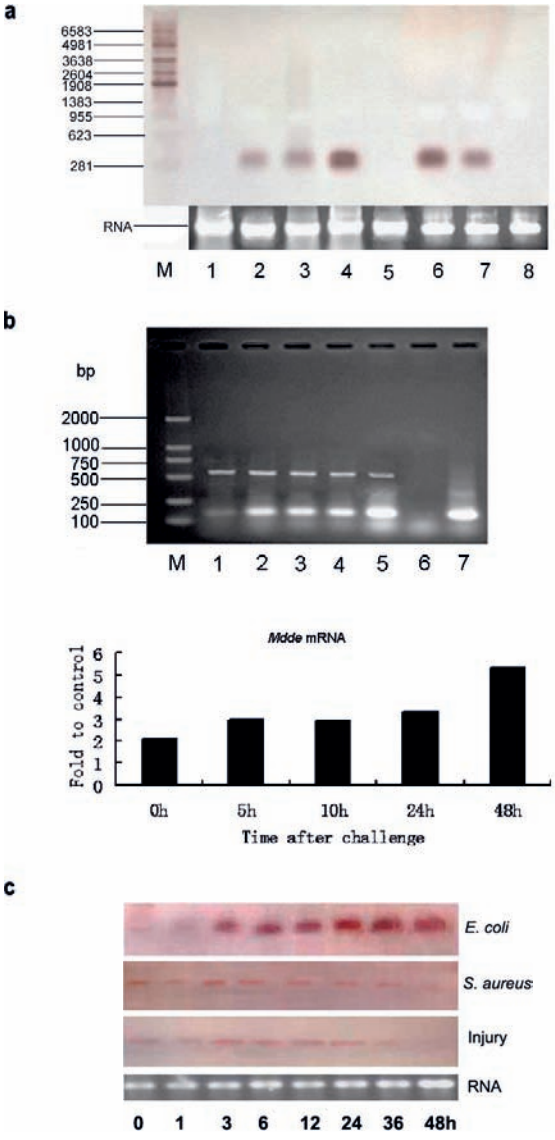

Figure 3. Temporal expression of *Mdde* in naive and bacteria-challenged houseflies. (*a*) Northern blot analysis of naive larvae and adults and immune-activated larvae and adults at various times after challenge. The 18S RNA was used as a loading control. Lane M: RNA marker; lane 1: naive larvae; lanes 2–4: larvae at 10, 24, and 48 h after bacterial challenge, respectively; lane 5: naive adults; lanes 6-8: adults at 10, 24, and 48 h after bacterial challenge, respectively. (*b*) Top: RT-PCR analysis of defensin expression in naive and bacteria-challenged larvae. Expression of *actin* was used as a control. PCR controls were performed with *Mdde*-pGEM-T easy plasmids as a template (lane 7). Lane M: DL-2000 DNA marker; lane 1: naive larvae; lanes 2–5: larvae at 5, 10, 24 and 48 h after bacterial challenge, respectively; lane 6: negative control. Bottom: Effects of the challenge with mixtures of *E. coli* and *S. aureus* on the *mdde* mRNA expression in the larvae of *M. domestica*, as measured by RT-PCR. 'Fold to control' refers to the ratios of OD of *Mdde* to those of *β-actin*. (*c*) Time-course analysis of defensin gene expression after infection by *E. coli* and *S. auereus*. A sterile needle was used for injury of control larva. Total RNAs extracted from larvae at different time intervals (1, 3, 6, 12, 36 and 48 h) after challenge were used for Northern blot analysis.

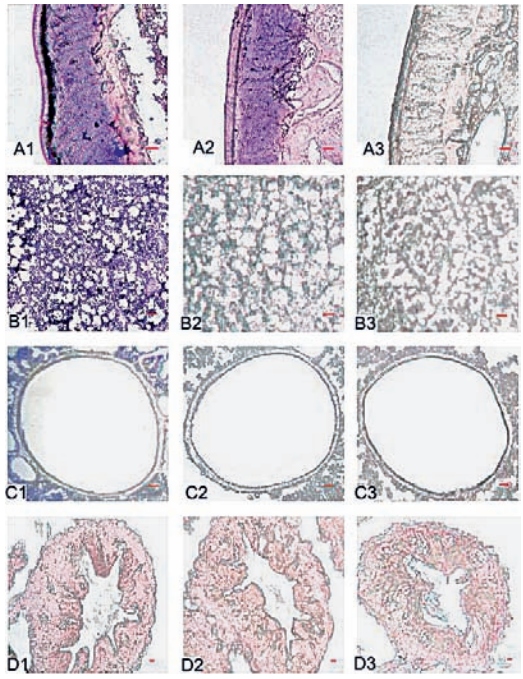

Figure 4. *In situ* hybridization analysis of the *Mdde* transcripts in larva of the housefly 24 h post challenge. (*a*) Body wall, (*b*) fat body, (*c*) tracheae, and (*d*) midgut. '1', '2', and '3' represent challenged tissue, unchallenged tissue, and negative controls, respectively. Bars = 10 μm.

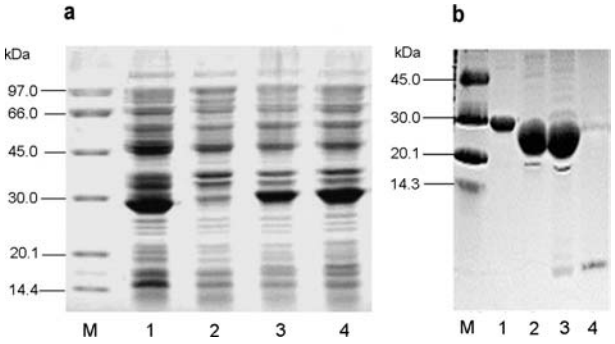

Figure 5. Recombinant expression of Mdde in *E. coli*. (*a*) Screening of the expressive clones of the *m-Mdde*/pGEX-4T-1 in *E. coli* (12.5% SDS-PAGE). Lane M: protein molecular marker; lane 1: bacterial proteins of a transformant with pGEX-4T-1 after induction; lane 2: bacterial proteins with *m-Mdde*/pGEX-4T1 before induction by IPTG; lanes 3 and 4: bacterial samples of two transformants with *m-Mdde*/pGEX-4T-1 after induction by IPTG respectively. (*b*) Purification and cleavage of the fusion protein GST-*Mdde* by thrombin (15% SDS-PAGE). Lane M: Rainbow-colored protein molecular weight markers (Amersham); lane 1: 30-kDa protein of GST-Mdde; lane 2: 26-kDa protein of GST; lane 3: cleaved GST-Mdde fusion protein by thrombin; lane 4: partial purified rMdde.

a liquid inhibition assay *in vitro* against *E. coli* and *S. aureus,* but relatively low activity was detected (data not shown). The rabbit antiserum made from fusion protein GST-Mdde could specifically recognize Mdde expressed in *Pichia*.

P. pastoris expression and purification of rMdde

Semi-logarithmic phase transformants were induced with 0.5% methanol for a prolonged period of time. Aliquots of the culture supernatant, withdrawn at 24 h intervals, were subjected directly to tricine SDS-PAGE analysis followed by staining with Coomassie brilliant Blue (Fig. 6a). A major protein band of approximately 4.6 kDa was observed after induction, which was named rMdde. The amount of rMdde increased with time after induction, up to approximately 4 days. Western blot analysis revealed that the protein was recognized specifically by rabbit antiserum raised against the GST-Mdde expressed in *E. coli*, demonstrating that the *P. pastoris*-expressed peptide was indeed Mdde (Fig. 6b). Thus, mature *Mdde* was successfully secreted into the culture medium.

After 4 days of methanol induction, cultures were harvested and the supernatants were collected for purification of rMdde by CM-Sepharose CL-6B cation exchange chromatography. The purity of rMdde was assessed by tricine SDS-PAGE (Fig. 6a). The purified rMdde was transferred to a PVDF membrane and sequenced up to eight cycles. The N terminus began with a Tyr-Val-Glu-Phe sequence, which represents the C terminus of the α-factor signal portion, and the following four residues Met-Ala-Thr-Cys were completely consistent with those of rMdde. High cell density-fed batch cultures in shake-flasks produced 70 mg/l secreted protein, consisting mostly of soluble and active rMdde.

The molecular mass measured by MALDI-TOF for the recombinant Mdde with the N-terminal tetrapeptides of α-factor from pPIC9K vector was 4659.99 Da (theoretical mass 4665.37 Da, without disulfide bridge formation). The difference between the theoretical mass and the measured mass may have been due to the loss of six hydrogens, as the six cysteines of the Mdde molecule formed three disulfide bridges. The measured molecular mass was 4659.99 Da and was very close to the theoretical mass with the three disulfide bridges (4659.37), suggesting its integrity (Fig. 7).

To evaluate the antimicrobial activity of *P. pastoris*-expressed rMdde, liquid growth inhibition assays were carried out against Gram-positive and Gram-negative bacteria and against fungi. The MIC values obtained with rMdde are summarized in Table 1. The rMdde was active against Gram-positive and Gram-negative bacteria with an MIC of 0.9–5 μM, but was less active against fungi (MIC > 45 μM).

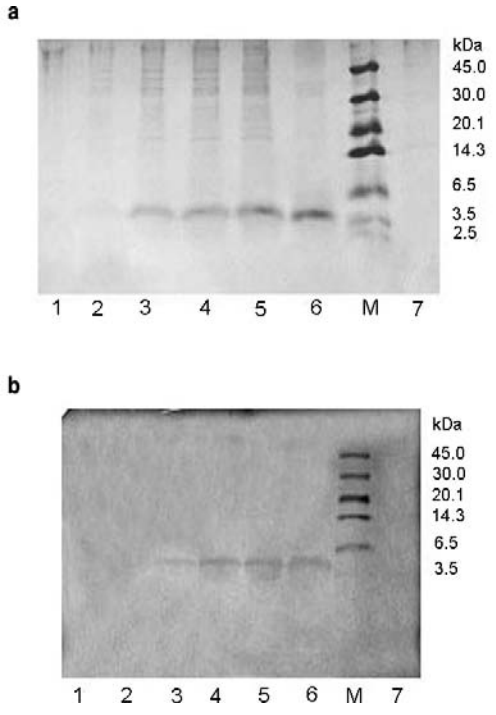

Figure 6. (*a*) Tricine SDS-PAGE analysis of the medium of the shake-flask culture expressing *Mdde* in *P. pastoris*. An aliquot of the medium was removed at 24-h intervals after methanol induction. The samples were separated by 15% tricine SDS-PAGE and stained with Coomassie blue. Lane 1: the medium of *P. pastoris* with *Mdde* genes before methanol induction; lanes 2–5: the medium of *P. pastoris* with *Mdde* genes from 1 to 4 days after methanol induction, respectively; lane 6: purified rMdde; lane 7: rainbow-colored protein molecular weight markers (Amersham); lane 8, the medium of *P. pastoris* without insert of *Mdde* from 4 days of expression after methanol induction. (*b*) Immunoblot analysis of Mdde expressed in yeast with polyclonal rabbit anti-GST-CHP antiserum. Lane 1: the medium of *P. pastoris* with the *Mdde* gene before methanol induction; lanes 2–5, the medium of *P. pastoris* with *Mdde* genes from 1 to 4 days after methanol induction; lane M: rainbow-colored protein molecular weight markers (Amersham); lane 7: the medium of *P. pastoris* without the insert of *Mdde* 4 days after methanol induction.

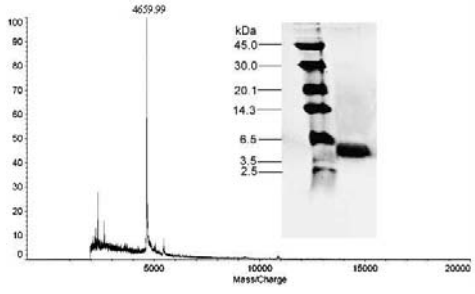

Figure 7. MALDI-TOF mass spectrum of purified rMdde. The inset shows a 15% tricine SDS-PAGE of purified rMdde (right lane) stained with Coomassie blue and rainbow-colored protein molecular weight markers (left lane).

Table 1. rMdde antibacterial activity and minimal growth inhibition concentration (MIC).

Microorganisms	MIC (μM)
Gram-positive bacteria	
Staphylococcus aureus	5.0
Bacillus megaterium	5.6
Bacillus cereus	1.8
Bacillus subtilis	0.9
Bacillus thuringiensis subsp. Israelensis	>22
Bacillus thuringiensis subsp. Kurstaki	>22
Micrococcus luteus	1.4
Gram-negative bacteria	
Escherichia coli	5.0
Klebsiella pneumoniae	5.0
Fungi	
Pythium aphanidermatum	>45
Colletotrichum lagenarium	>45
*Piricularia oryza*e	>45

Discussion

The present study isolated and sequenced cDNA encoding *Mdde* and analyzed its expression in different tissues and at different times following bacterial challenges. Defensin was expressed in *E. coli* BL21 and *Pichia pastoris*, purified respectively, and its antimicrobial activity was tested.

The defensin family is a group of ubiquitous AMPs found in plants, invertebrates, and vertebrates. The encoding protein of the insect defensin gene usually consists of a signal peptide region, prodefensin, and the mature peptide. With pre-prodefensin sequences found in insects, there is a defined signal peptide region, a prodefensin region that terminates with a KR cleavage site. This conserved cleavage site is common among insects [20, 21]. Based on comparisons of defensins, there appears to be strong conservation of the mature peptide sequence among all organisms, particularly the location of the six cysteine residues that form three disulfide bridges. Most likely, such conservation exists due to the importance of defensins as key components in insects' immune response that has allowed them to thrive in environments full of potential pathogens. However, the pre-pro region of housefly defensins are more variable than in other reported defensins. These data suggest that, although the mature peptide region has been highly conserved through evolutionary time, the pre-pro region of this peptide has been modified during evolution.

The time course of transcription of the housefly defensin was determined 0–48 h after inoculation with two kinds of bacteria. *Mdde* is contained within a 430-bp message that is detectable in naive housefly and increases in strength 3 h after inoculation of Gram-negtive bacterium (*E. coli*) and reaches a higher level from 36 to 48 h. Tran-

script levels of *Mdde* are not significantly changed after Gram-positive bacterial challenge (*S. aureus*). These findings indicate that the housefly defensin is constitutively expressed at low levels and is up-regulated following Gram-negative bacterial challenge due to immune system activation.

The pattern of induction of defensin gene in housefly is somewhat similar with that of *Drosophila*. The transcription of the *Drosophila* defensin is marked by a strong inducibility by Gram-negative bacterium (*E. coli*) and a weak inducibility by Gram-positive bacterium (*M. luteus*). There was also low level of constitutive transcription of the *Drosophila* defensin gene [22]. Actually, the expression profile of defensin gene is not exactly same between the two insects. The transcription reached highest level at 3 h and almost no signal at 24 h post challenge of Gram-negative bacterium, and slightly increased at 6 h and decreased at 12 h post injection of Gram-positive bacterium in adult *Drosophila*.

The expression profile of defensin gene is different from that of *Md-cecropin* [9]. No constitutive expression of cecropin gene was detected by Northern blot and RT-PCR. The expression signal was detected 5 h post bacterial (mixture of *E. coli* and *S. aureus*) injection. No signal was detected by Northern analysis and only a weak signal appeared in the RT-PCR analysis 48 h post injection.

Although transcription of most insect immune peptides has been reported to occur in insects' fat bodies, there is growing evidence that other tissues may also be involved in the immune response. For example, the constitutive expression of some AMPs is found in specific *Drosophila* tissues, such as the salivary glands or the reproductive tract [23]. Defensin transcripts are found in the midgut of *Aedes aegypti* [20], *Anopheles gambiae* [24], *Stomoxys calcitrans* [25] and *Rhodnius prolixus* [26] and in the salivary gland of *An. gambiae* [27]. The cecropin transcripts are found in cuticular cells of *Bombyx mori* after abrading the cuticle [28]. The present work did not find the transcripts of defensin in the midgut or salivary gland by *in situ* hybridization. However, the results of *in situ* hybridization and RT-PCR suggest that the defensin transcripts were constitutively expressed in the epidermis of the body wall and were inducibly expressed in the fat body.

Defensin isoforms differing by one to several amino acids have been identified from several insect species [26, 29–31]. A single defensin gene exists in *Drosophila melanogaster* [32]. Two defensin genes encoding three defensin proteins A, B, and C (A and B represent allelic variants) with small differences in one or two amino acid residues have been identified in the mosquito *A. aegypti* [20, 30]. *R. prolixus*, an insect vector of Chagas disease, possesses three defensin genes that show relatively large differences [26]. The present findings suggest the existence of a single defensin in the housefly.

Previous work has purified and expressed defensins in *E. coli* and *P. pastoris* [33–35]. The present results indicate that Mdde was successfully expressed as a recombinant peptide in the prokaryocyte and the eukaryocyte expression system. The rMdde was highly expressed in *E. coli* BL21 representing 30–34% of total cell proteins. The purified rMdde expressed in *E. coli* had low activity against bacteria. This may be due to the formation of inappropriate disulfide bonds. This interpretation is not consistent with the result for human β-defensin 3 (hBD3) reported by Wu et al. [36], who suggest that disulfide bonds alignment may not be critical for hBD3 antimicrobial activity. We know that all insect defensins have the same cysteine pairing: Cys1–Cys4, Cys2–Cys5 and Cys3–Cys6 and adopt a 'cysteine-stabilized $\alpha\beta$' (CS$\alpha\beta$) scaffold [37]. The three disulfide bonds are required for the stable structure. Most of the vertebrate defensins may adopt a β-sheet conformation with triple strands ($\beta\beta\beta$) and the human β-defensin 2 and 3 were reported to adopt an $\alpha\beta\beta\beta$ scaffold [38]. Another difference between the hBD3 and Mdde is the positive charges: hBD3 contains significantly more positively charged residues (net charge +11) than that of mature Mdde (net charge +3). The AMPs preferentially target prokaryotic cells because bacterial membranes contain large amounts of negatively charged phospholipids. The hBD3 contains more positive charges and it may easily react with bacterial membranes even though it adopts an inappropriate 3-D structure. The Mdde has fewer positive charges and it may need the appropriate 3-D structure for its antibacterial function.

Thus, the yeast *P. pastoris* expression system was used to produce active soluble rMdde. It displayed high antibacterial activities yet almost no activity against fungi. Thus, rMdde appears to be an effective AMP that can be successfully produced at high levels in yeast and may be a potential antimicrobial candidate for practical use.

Prior and present work indicates the presence of at least three AMPs in the housefly: defensin, cecropin [9] and attacin [39], all of them inducible in the fat body. However, defensin is constitutively expressed in the epidermis of the body wall, potentially to fight pathogen infection. Further work needs to be done to find more effector molecules of innate immunity of the housefly, to determine how the fly perceives infection, and to determine how different pathogens trigger distinct responses.

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