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Investigations on the role of a lysozyme from the malaria vector *Anopheles dirus* during malaria parasite development

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Abstract

A cDNA encoding a lysozyme was obtained by rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) from females of the malaria vector *Anopheles dirus* A (Diptera: Culicidae). The 623 bp lysozyme (*AdLys c-1*) cDNA encodes the 120 amino acid mature protein with a predicted molecular mass of 13.4 kDa and theoretical pI of 8.45. Six cysteine residues and a potential calcium binding motif that are present in AdLys c-1 are highly conserved relative to those of c-type lysozymes found in other insects. RT-PCR analysis of the *AdLys c-1* transcript revealed its presence at high levels in the salivary glands both in larval and adult stages and in the larval caecum. dsRNA mediated gene knockdown experiments were conducted to examine the potential role of this lysozyme during *Plasmodium berghei* infection. Silencing of *AdLys c-1* resulted in a significant reduction in the number of oocysts as compared to control *dsGFP* injected mosquitoes.

Keywords

Lysozymes; muramidase activity; antimicrobial peptides; RNA interference; *Plasmodium*; malaria

1. Introduction

Malaria persists as one of the most serious insect-transmitted diseases in the world. *Anopheles dirus* is one of the main vectors of human malaria in Southeast Asia, including Thailand. The relationship between the mosquito and the parasite is complex as both reproduction and morphological development of the parasites occur within this host (Beier, 1998). Distinct stages of the parasites move between several tissue locations. Following

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ingestion of the infected blood meal, male and female gametocytes fuse to produce a zygote that develops to a motile ookinete that can actively traverse the midgut. The ookinete comes to rest at the interface of the midgut basal epithelium and the overlying basal lamina and develops to the oocyst stage. Within the oocyst, reproduction occurs and sporozoites begin to develop. Upon release from the oocyst, sporozoites move through the hemocoel and the dorsal vessel, and are then transported into the vicinity of the salivary glands. Sporozoites enter the glands and the canal and can then be transmitted to the next host. Understanding the molecular interactions occurring between mosquitoes and parasites during this complex life cycle might provide new avenues for malaria control (Garver et al., 2009; Li et al., 2005). In particular, the ability to manipulate the mechanisms that promote or that interfere with parasite development within the mosquito host could ultimately be used to control the transmission of this deadly disease (Hoffmann et al., 1999; Nürnberger and Brunner, 2002).

Mosquitoes can sense and counter microbial invasion via the IMD or Toll pathways (Meister et al., 2005). Inactivation of these pathways through targeted gene silencing improves the frequency of successful malaria parasite development on the midgut (Garver et al., 2009). Recently, it has been demonstrated that the natural bacterial community in the mosquito midgut plays a role in regulating the IMD and Toll pathways (Dong et al., 2009). Reduction of the midgut bacteria through antibiotic treatment resulted in lowered basal immunity (IMD and Toll activity) and subsequently, an increased susceptibility to *Plasmodium* infections following an infected blood meal (Dong et al., 2009). This work clearly indicates that the mechanisms of innate immunity interact with the parasites to suppress infection in a normal midgut; however many of the details of this phenomenon are unknown.

We have investigated a group of antimicrobial enzymes called lysozymes (Li et al., 2005; Li and Paskewitz, 2006; Paskewitz et al., 2008; Kajla et al., 2010). These enzymes are defined by the ability to hydrolyze the 1–4 glycosidic bonds between N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) of the peptidoglycan (PGN) present in the cell walls of bacteria. Lysozymes as antibacterial defense molecules have been well characterized in vertebrates (Jolles and Jolles, 1984; Markart et al., 2004) as well as in insects (Dunn, 1986; Hultmark, 1996; Kajla et al., 2010; Powning and Davidson, 1973; Jolles et al., 1979; Hultmark et al., 1980; Abraham et al., 1995). However, lysozymes have also recently been shown to inhibit melanization of non-biological targets in *Anopheles gambiae* (Li and Paskewitz, 2006) and to interact directly with the oocysts of *Plasmodium berghei* and *P. falciparum* in this mosquito (Kajla et al., 2011). These observations suggest that the functional roles of mosquito lysozymes may be broader than has been previously realized.

Because of the potential for affecting malaria infections either indirectly through regulation of the gut microbiota and basal immunity or through direct interactions with the oocysts, we initiated studies on lysozymes in a second important malaria vector. In this study, a full-length lysozyme was cloned and sequenced from *An. dirus*. Transcript abundance of this gene was analyzed using semiquantitative RT-PCR to compare different tissues and stages of mosquitoes. Gene silencing (RNA interference) experiments were conducted to examine the role of this lysozyme in mediating *P. berghei* infections in *An. dirus*. Our results suggest that AdLys c-1 plays a role in mediating the success of malaria parasites in this important vector.

2. Materials and methods

2.1. Mosquitoes and Plasmodium berghei parasites

Anopheles dirus A was reared under laboratory conditions in an insectary in an incubator set to 70–80% relative humidity and 25–27 °C at AFRIMS (Armed Forces Research Institute of Medical Sciences, Thailand) and at the Department of Entomology, University of

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Wisconsin- Madison, Wisconsin, USA according to previously described protocols (Paskewitz et al., 1999; Kajla et al., 2010 and 2011) with the modification that these mosquitoes were mated artificially.

A transgenic strain of *P. berghei* that was engineered to express green fluorescent protein (GFP) was obtained from MR4 (http://www.mr4.org/) and passaged in Balb/c mice as described in Kajla et al. (2011).

2.2. Isolation, cloning and sequencing of full length AdLys c-1 cDNA

For the production of cDNA, 5–10 *An. dirus* female mosquitoes were cold anaesthetized for five minutes and immediately processed for RNA isolation. Total RNA was extracted with TRIZOL® Reagent (Invitrogen, Carlsbad, CA) according to supplied instructions. RNA concentration was determined using spectrophotometry on a Nanodrop NT1000 (Thermo Fisher Scientific, Waltham, MA). Five hundred nanograms of total RNA were used to synthesize cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen). cDNA was stored at minus 20 °C until further use.

For amplification of *An. dirus* lysozyme, degenerate primers were designed based on conserved sequences found in mosquito c-type lysozymes. The selected primers used for amplification of the initial fragment of *AdLys c-1* were as follows: Forward I 5’- GCGCGG AATTCNGAYTGGATHTGYYTN G -3’; Forward II 5’- GCGCGGAATTCNGAY TGGGTHTGYYTNG -3’; Reverse 5’ GCGCGCAAGCTTANCCRTACCANGCRT T -3’.

cDNA synthesized from the previous step was used as a template with these primer sets (Forward I and Reverse or Forward II and Reverse) in PCR with cycle condition 35 cycles of 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing), and 72 °C for 1.5 min (extension). The resulting amplicons were purified and cloned into pGEM-T Easy Vector (Promega, Madison, WI) according to the manufacturer’s instructions and sequenced at the Biotechnology Center at the University of Wisconsin, Madison. The sequences were analyzed via BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the sequence of this amplified fragment as a c-type lysozyme.

To obtain a full-length clone, the 5’ and 3’- terminal ends of lysozyme cDNA were amplified using 5’ and 3’ RLM-RACE (GeneRacer™ kit, Invitrogen), respectively. The primers for RACE were designed based on the sequence of lysozyme fragment as follows: Lys-For I 5’-TTGTTCGTCGCCGAGGTGCTGAAT-3’; Lys-Rev I 5’-CAAGTGTGCCAAGCTGATATTCAAGCGC-3’; Lys-For II 5’-TATTCC GTAGTCGGTGGACCCGTTCT-3’; Lys-Rev II 5’-GTCTAACGATTGCAAGAT AGCGTGC-3’. In the first RACE PCR, the reaction mixture (50 μl total) comprised 38.5 μl of sterile water, 5 μl of 10X PCR buffer, 1 μl of dNTPmix (10 mM of each dNTP), 3 μl of 5’or 3’ GeneRacer™ Primer (10 μM), 1 μl of gene specific Primer (10 μM), and 0.5 μl of 5 U/μl of Advance *Taq* polymerase. The cDNA was amplified using 35 cycles of 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing), and 72 °C for 1.5 min (extension). The second PCR was prepared as the first PCR, except that GeneRacer™ 5’or 3’ Nested Primers and Lys-Rev II or Lys-For II were used for these reactions. Products were cloned and sequenced as described above.

Finally, a complete *AdLys c-1* cDNA was amplified using gene-specific end primers as follows: 5’-ATTCAGTGCCTGTGATACTTGCTG-3’ and 5’-AACGAATAACGTTCATTTTGTATTG-3’. The final PCR products were separated by electrophoresis on a 1.0% agarose gel and an amplified product of about 623 bp was detected, cloned and sequenced as described above. The sequence was analyzed by using

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BLAST and DNASTAR software (Lasergene, Madison, WI). The sequence of the full length clone was submitted to GenBank and assigned accession number EU622903.

A lysozyme phylogenetic tree was constructed to investigate the molecular phylogenetic relationships among lysozymes from *An. gambiae, An. stephensi, An. darlingi* and *Ae. aegypti*. Protein sequences were aligned using Clustal W. The phylogenetic tree was generated using a neighbor-joining program in Clustal X with the 'delete positions with gaps' and correct for multiple substitutions' settings on. The tree was bootstrapped 1000 times.

2.3. Plasmodium berghei infections

The protocol described in Kajla et al. (2011) was used for producing infections in *An. dirus* mosquitoes. The infections were monitored by visualizing GFP-expressing parasites under a fluorescent microscope.

2.4. dsRNA mediated silencing of AdLysc-1

The procedures for the production of dsRNA and injections in the mosquitoes were performed according to the protocol described in Kajla et al. (2010, 2011). Briefly, the template for generation of dsRNA for *AdLysc-1* was produced using one step PCR. Each primer included 15 bp of T7 promoter sequence plus *AdLys c-1* specific sequence: (5'-TAATACGACTCACTATAGGGTAATTTCACGCACACGCTAGCA-3') and 5'-TAATACGACTCACTATAGGGACTTGCTGTAGTGTGCTTCAGA- 3'). The *GFP* sequence (Fwd: 5'-TAATACGACTCACTATAGGGCGTGATCAAGCCCGACA-3', Rev: 5'-TAATACGACTCACTATAGGGCTTCGGCGTGCTC-3') was used to amplify a product from *phMGFP* vector (Promega, Madison, Wisconsin).

A QIAquick PCR Purification Kit (Qiagen Sciences, Maryland, USA) was used to purify the PCR product. The purified PCR product was used as template for transcription by using the MEGAscript™ RNAi kit (Ambion, Austin, Texas) following the manufacturer's instructions.

Zero to 1 d old mosquitoes were used for dsRNA injections. Female mosquitoes were injected with 1.4 µg/ 0.6 µl volume dsRNA for *AdLys c-1* or unrelated control *GFP*. This concentration of dsRNA was chosen based on pilot experiments in which different concentrations of dsRNA were injected in the mosquitoes for varying periods of time from 24 h to 4 days. The silencing effect was observed through 4 days after injections of dsRNA.

In these experiments about 40–50 mosquitoes were injected with dsRNA for *AdLys c-1* or *dsGFP*. Three days after dsRNA injections, mosquitoes were starved of sugar for about 2–4 h before blood feeding on a mouse infected with the GFP-expressing strain of *P. berghei*. This time period allows for the mosquitoes to recover from the injury caused by injections. Mosquitoes that failed to feed on the mouse were removed within 24 h. Four to five days later, midguts from each batch were dissected and the number of oocysts counted using fluorescence microscopy.

Five mosquitoes were harvested to check the efficacy of the knockdown using RT-PCR as described below. For this, the RNA isolation and cDNA was prepared as described above in section 2.2. This cDNA was used in the RT-PCR reaction for amplification of *AdLys c-1*. Specific primer sets for RT-PCR for *AdLys c-1* were as follows: 5-GCTGAGGCCAAGAAATTCAG-3 and 5-AGCAGCTTTTTGCACGCTAT-3. All experiments were repeated three times.

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2.5. Semiquantitative RT-PCR

Semiquantitative RT-PCR was performed to assess relative transcript abundance in the different developmental stages and in the adult female mosquitoes following RNAi. The different life stages and tissues tested were: Eggs, larvae (first through 4th instars), pupae, freshly emerged males and females at day zero and five day old adult males and females. In all cases 5–10 whole animals were taken for analysis. Various tissues from larvae (4th instar) and adult (2–3 d old females) mosquitoes included head, thorax, abdomen, fat bodies, midguts, malphigian tubules, and ovaries from naïve as well as from blood fed mosquitoes. From larvae, the hindguts and gastric caeca were also tested for the presence of *AdLys c-1*.

Total RNA was isolated from five *An. dirus* mosquitoes from respective samples/treatments using MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the supplied instructions. Total RNA was treated with RQ1 DNAse (Promega, Madison, WI) at a concentration of 1 unit/μg RNA preparation to remove genomic DNA. The concentration of RNA was measured with micro-spectrophotometry (Nanodrop NT1000, Thermo Fisher Scientific, Waltham, MA). Five hundred nanograms of total RNA were used to synthesize cDNA using a High-Capacity cDNA Archive Kit (Applied Biosystems, Carlsbad, California) The resulting cDNA was stored at −20°C until use.

Primers based on the *An. dirus* cDNA sequence (GenBank: accession no. EU622903) and *RPS7* (Salazar et al., 1993) were designed using Oligoperfect software (Invitrogen). The S7 ribosomal gene was used to normalize the template. Final concentrations of reagents used for RT-PCR were 1X buffer, 200 μM dNTPs, 0.5 μM each primer, 0.2 μl (4 units) Advantage Taq polymerase (Clontech Laboratories Inc., CA, USA). PCR cycle conditions were: an initial denaturation at 95°C for 3 min; then repeated cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s; and a final extension step at 72°C for 10 min. For *AdLys c-1*, 35 cycles were used while 25 cycles were used for *S7*. PCR products were separated on 1% agarose gels and stained with ethidium bromide. The gels were exposed to UV-transillumination and images were captured on a gel-imaging system. For the dsRNA knockdown experiments, signal intensities of bands were quantified by densitometric analysis using the Quantity One Software (version 4.6.8) from Bio-Rad Laboratories (Bio-Rad, Hercules, CA). Fold change in expression was calculated by dividing the ratios of ds*GFP/S7* control with the ds*AdLys c-1/S7* ratios.

3. Results

3.1. Features of AdLys c-1 and comparison to other reported mosquito lysozymes

A full-length lysozyme was cloned and sequenced from *An. dirus*. The sequence information was submitted to GenBank (EU622903). The lysozyme (*AdLys c-1*) cDNA is 623 nt in length (Fig. 1). The SIGNAL P 3.0 program was used to predict the location of signal peptides. The PROTEAN program (DNASTAR) was used to estimate the theoretical pI and molecular mass of the predicted mature protein. For AdLys c-1, the signal peptide cleavage was shown between amino acids 20 and 21 leaving a mature protein of 120 amino acids. The predicted molecular mass of the mature protein is 13.4 kDa and the theoretical pI is 8.45.

C-type lysozymes are characterized by the presence of conserved amino acids at the catalytic cleft as well as eight cysteine residues. These features were examined in *AdLys c-1* sequence. Two critical amino acids, E32 and D49 that are necessary for muramidase enzymatic activity were identified. These two amino acids also were identified in Lys c-1 and c-2 from *An. gambiae* as well as in lysozymes from *An. stephensi* and *An. darlingi* (Fig. 2) and define a group of catalytically active mosquito enzymes. Mammals and birds contain a group of calcium binding c-type lysozymes (Prager and Jolles, 1996). In a previous study,

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both Lys c-3 and c-8 of *An. gambiae* displayed similarity to the binding site in the vertebrate calcium-binding lysozymes (Prager and Jolles, 1996; Li et al., 2005). In *AdLys c-1* sequence, a similar site (DDDITDD) was identified.

A phylogenetic tree was constructed to investigate the molecular phylogenetic relationships among lysozymes from *An. dirus*, *An. gambiae*, and additional members that were identified in *An. stephensi, An. darlingi*, and *Ae. aegypti* (Fig. 3). A neighbor joining analysis in ClustalX was used to determine the closest associations among mosquito lysozymes. AdLys c-1 grouped into a clade of lysozymes from anopheline mosquitoes. This clade includes Lys c-1 and Lys c-2 from *An. gambiae* as well as enzymes from *An. stephensi* and *An. darlingi* (Fig. 3). All of these enzymes share the two critical active site amino acids, while other mosquito lysozymes have lost one or both of these.

3.2. Temporal and tissue expression profiles of AdLys c-1 transcript levels

AdLys c-1 expression was examined using RT-PCR at various developmental stages and in different tissues from both larval and adult *An. dirus* mosquitoes (Fig. 4). The expression of *AdLys c-1* in these RT-PCR assays was normalized against the ribosomal S7 gene. *AdLys c-1* was expressed in all developmental stages. It appeared to be highly expressed in later stages of larval development as compared to eggs and earlier stages (Fig. 4A). In larval tissues, *AdLys c-1* appeared high in salivary gland, fat body, and hindgut but not in the midgut (Fig. 4B). Interestingly, *AdLys c-1* transcripts were very abundant in the larval caecum, a potential site of residence for bacteria. In the adults, *AdLys c-1* transcripts were detected in most of the tested tissues with highest expression in the thorax, salivary glands and ovaries (Fig. 4C).

3.3. Presence of AdLys c-1 was found to promote the development of Plasmodium berghei

Transient depletion using dsRNA-mediated gene silencing is an effective and widely used approach in the mosquito for assessing the potential role of a target gene of interest and its effect on the development of *Plasmodium* parasites (Blandin et al., 2002; Kajla et al., 2011). The injection of *dsLys c-1* successfully suppressed the systemic expression of *AdLys c-1* in three replicate experiments (2.8–5.3 fold; Fig. 5). dsRNA mediated silencing of *AdLys c-1* also resulted in a significant reduction ($p<0.05$, Kruskal-Wallis test) in the number of developing oocysts four days after the feeding of an infectious blood meal as compared with *dsGFP*-injected mosquitoes (Fig. 6 and 7). The morphology of parasites did not differ between *AdLys c-1* knockdown and control *dsGFP* injected mosquitoes. Moreover, melanization of parasites did not occur in any of the three experiments (data not shown). Thus, knockdown of *AdLys c-1* did not affect this immune process. The reduced parasite number was most evident in the 3rd replicate (Fig. 7) which correlated with the strongest reduction in the *AdLys c-1* transcript.

4. Discussion

Our results support an agonistic role of *AdLys c-1* in the response of mosquitoes during *P. berghei* infection. Knockdown of this gene resulted in a significant reduction in the number of oocysts. This suggests that *AdLys c-1* may play roles in mosquito biology that assist the development of the *Plasmodium* parasites.

It is possible that this agonistic role relates to regulation of the gut bacterial community. A number of studies have shown an increased lysozyme expression in the mosquitoes after challenge with bacteria (Fujimoto et al., 2001; Lee and Brey, 1995; Sun et al., 1991; Gao and Fallon, 2000; Hernandez et al., 2003; Bedoya et al., 2005; Kajla et al., 2010). In blood fed mosquitoes the bacterial population increases significantly within the midgut (Beier,

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1998, DeMaio et al., 1996; Pumpuni et al., 1993, 1996; Bedoya et al., 2005). Recently Dong et al. (2009) showed that the presence of gut microbiota indirectly impacted the susceptibility of the mosquitoes to become infected with malaria parasites apparently via modulation of the basal immune response. It is conceivable that in the absence of AdLys c-1, certain types of gut bacteria might grow at a higher rate and could upregulate basal immunity and/or directly injure parasites, preventing their development. However, silencing of lysozyme c-1 in *An. gambiae* did not result in a significant increase in the total number of culturable midgut bacteria (Kajla et al., 2011). Blandin et al. (2002) reported that knockdown of another antibacterial protein, defensin, in *An. gambiae*, did not cause significant differences in oocyst morphology or the frequency distribution of oocyst numbers although an altered microbial community would be predicted as a result of this manipulation. Further work will be needed to determine the interaction between AdLys c-1 and other antibacterial proteins, the midgut microbial community and the malaria parasites within mosquitoes.

Lysozyme c-1 has also been shown to inhibit melanization of abiotic foreign targets in *An. gambiae* (Li and Paskewitz, 2006). Recent work showed that *An. gambiae* lysozyme c-1 physically associates with *P. berghei* oocysts (Kajla et al., 2011). This suggested the possibility that the presence of mosquito lysozyme c-1 on the surface of the oocyst could protect it from melanization. However, we did not observe melanization of parasites following silencing of the *AdLys c-1* gene.

Because mosquito lysozymes belong to an expanded gene family (Li et al., 2005; Bedoya et al., 2005), we are also interested in investigating the biological functions of each member of the group. As a first step towards that goal, we analyzed the expression profiles for *AdLys c-1* across stages and tissues of *An. dirus*. *AdLys c-1* was expressed in all developmental stages. Surprisingly, the larval gastric caecae exhibited very high levels of transcript abundance. The function of the mosquito gastric caecum is not known but it may harbor microorganisms that promote digestion or protect the gut from colonization by other, more pathogenic bacteria. The presence of *AdLys c-1* in this tissue suggests that the protein may be present, perhaps to regulate the bacterial community either in the caecum or in the midgut itself. Alternatively, the gastric caecum may secrete lysozyme c-1 into the midgut where it might participate in regulation of the microbiota. How larval immunity differs from adults and whether larval exposure to bacteria can affect immune status of the subsequent adult are relevant questions that should be examined in more detail.

The patterns of *AdLys c-1* transcription are similar to the temporal and tissue expression profiles of *Lysc-1* from *An. gambiae*. *AgLys c-1* and *Lys c-2* were inducible when bacteria were introduced into the mosquito (Li et al., 2005). Because the phylogenetic analysis indicated that *AdLys c-1* is closely related to these proteins, we expect that *AdLys c-1* will be similarly involved in responses to bacteria.

In conclusion, we found that the presence of *An. dirus Lys c-1* was important to the development of *Plasmodium* parasites as has been reported for *An. gambiae Lys c-1* (Kajla et al., 2011). Future investigations on the relationships among mosquito lysozymes, midgut bacteria and malaria parasites will be required to understand the intricacies of these complex relationships.

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Fig. 1.

Complementary DNA and translated amino acid sequences of *An. dirus Lys c-1* (GenBank accession number EU622903). The start and stop codons and the putative signal peptide are indicated in bold. The predicted signal cleavage site is indicated by the vertical arrow.

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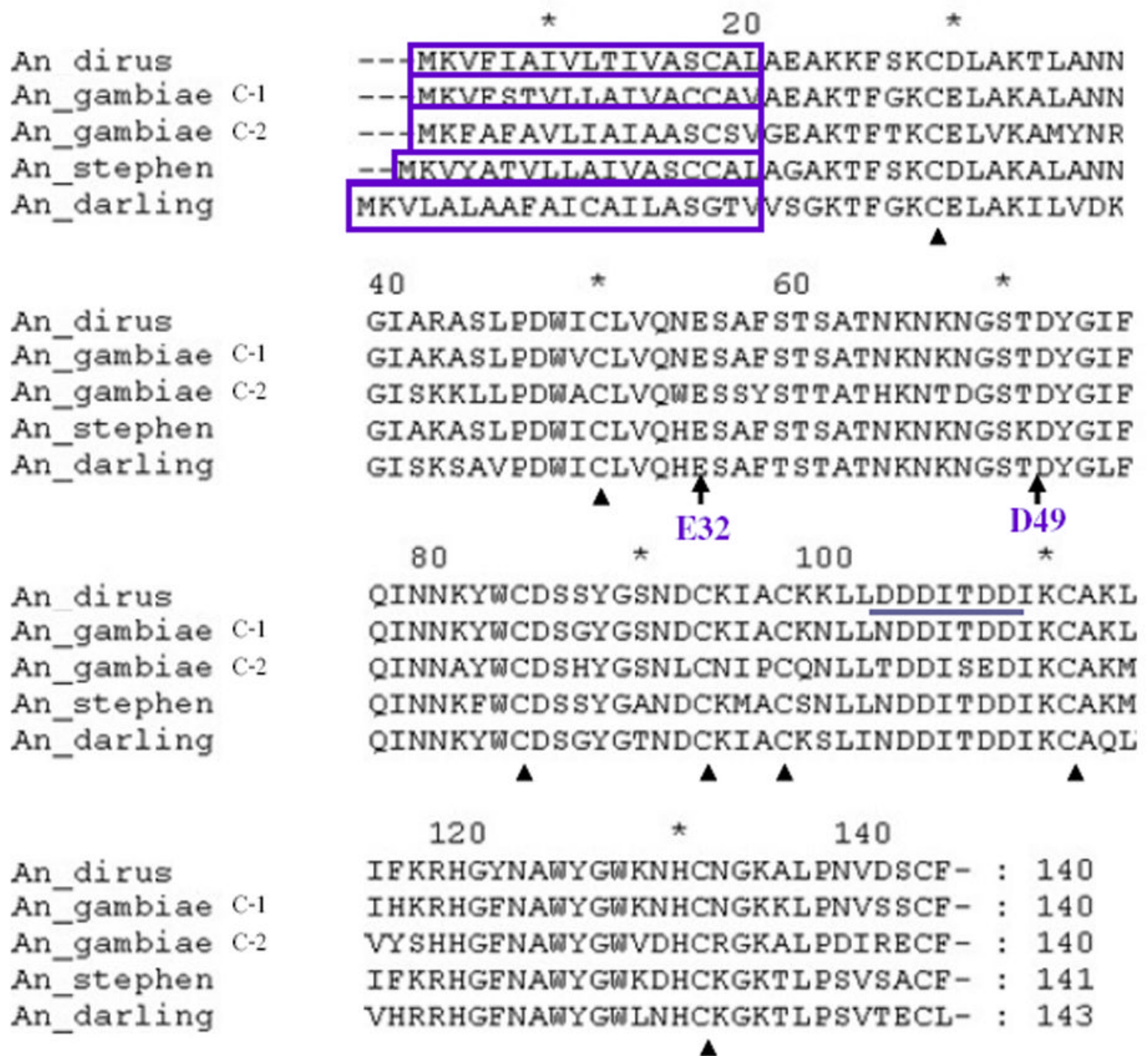

Fig. 2.

Alignment of deduced amino acid sequences of *AdLys c-1* from *An. dirus* and other *Anopheles* mosquitoes. The predicted signal peptides are boxed. Conserved cysteines are marked with black triangles. E32 and D49, the two amino acids that are essential for muramidase function are denoted by black arrows. The potential calcium binding site (DDDITDD) from *An. dirus* Lys c-is underlined.

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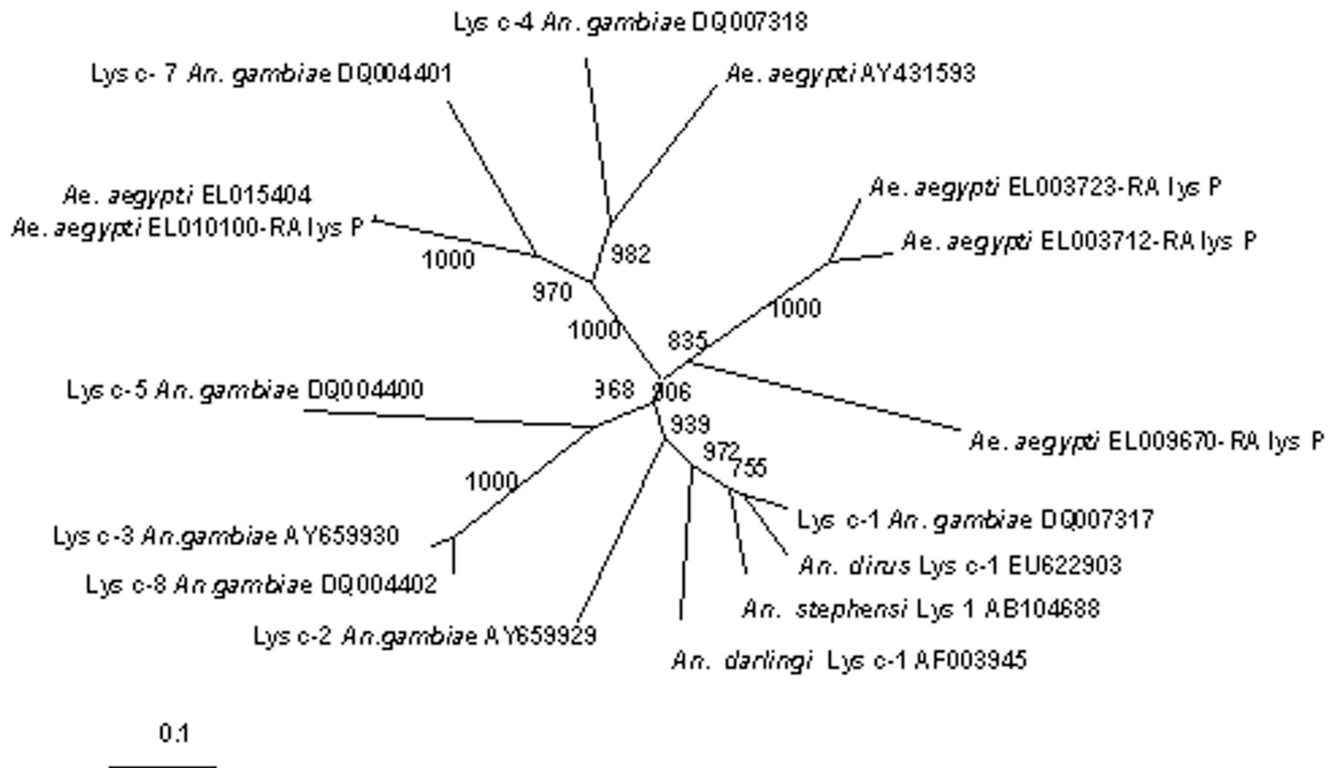

Fig. 3.

Phylogenetic analysis of the c-type lysozyme proteins from *An. dirus, An. gambiae, Ae. aegypti, An. stephensi*, and *An. darlingi*. Numbers at the nodes shows bootstrap proportions (BP) on 1000 replicates. Only BPs over 75% are shown. The GenBank accession numbers for each sequence are provided.

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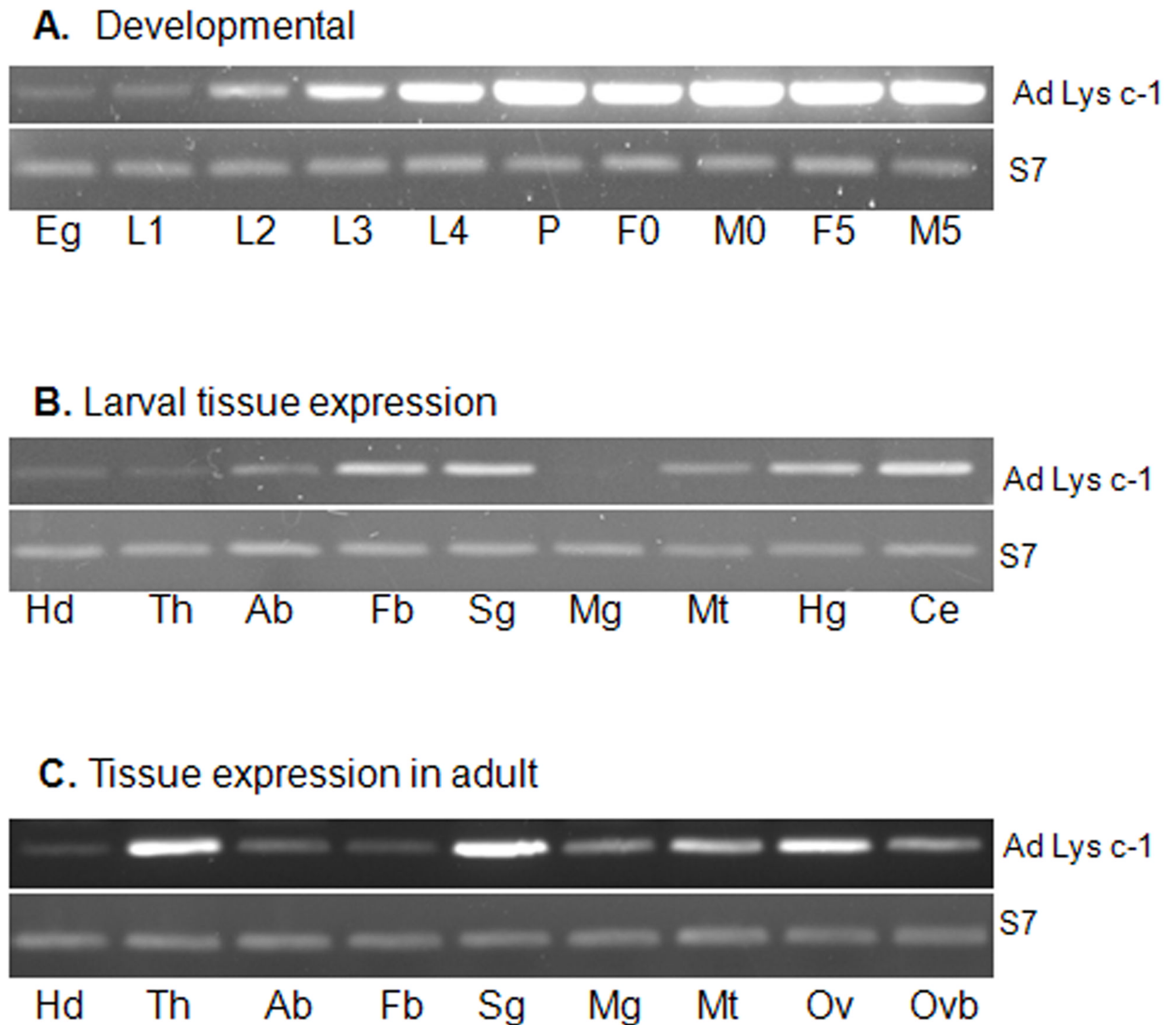

Fig. 4.

RT-PCR expression analyses of *AdLys c-1* in developmental and tissue samples from *An. dirus.* The ribosomal protein S7 was used to normalize the samples. (A) Developmental: Eg=eggs, L1, L2, L3, L4= 1st, 2nd, 3rd, and 4th instar larvae respectively, P=pupae, F0=adult females on day of emergence, M0=adult males on day of emergence, F5=adult females 5 days after emergence, M5=adult males 5 days after emergence. (B) Larval tissue expression: Hd=head, Th=thorax, Ab=abdomen, Mg=midgut, Sg=salivary gland, Fb=fat body (abdominal body wall), Mt=malpighian tubules, Hg=Hindgut, Ce=Caecum. (C) Tissue expression in adult: Hd=head, Th=thorax, Ab=abdomen, Mg=midgut, Sg=salivary gland, Fb=fat body (abdomal body wall), Mt=malpighian tubules, Ov=ovary OvB=ovary post blood feeding.

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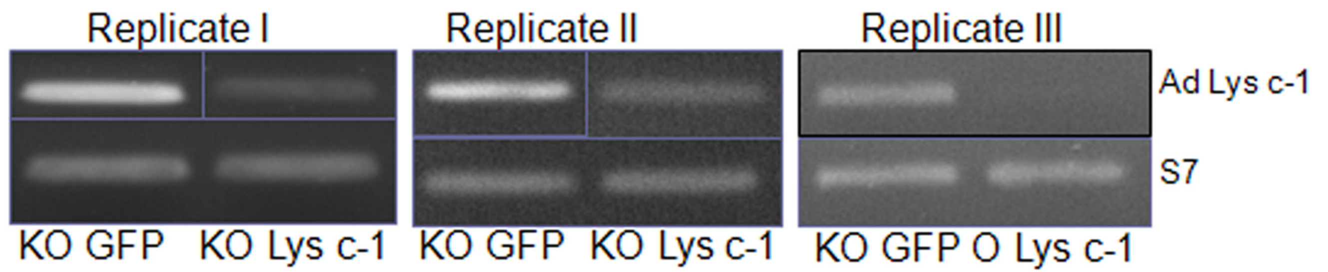

Fig. 5.

RT-PCR verification of RNA interference-mediated gene silencing. A ribosomal S7 primer set was used as reference gene for comparisons. Inhibition of expression of AdLys c-1 was most efficient in the 3rd replicate. KO GFP = knock down of GFP, KO *Lys c-1* = knock down of *AdLys c-*

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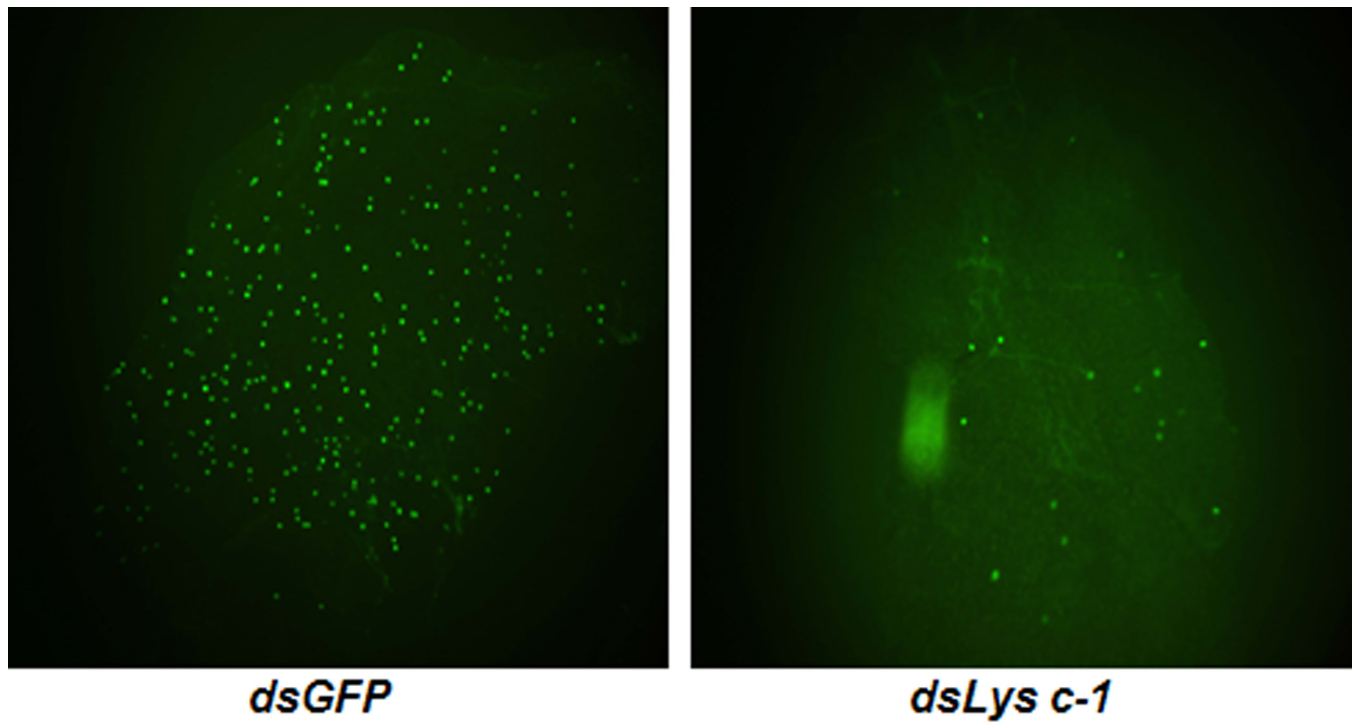

Fig. 6.
Comparison of *An. dirus* midguts infected with a GFP-expressing strain of *P. berghei* after the mosquitoes were injected with *dsGFP* or *dsAdLys c-1*. At 4 days after infection, midguts from each batch were dissected and the number of oocysts was counted using fluorescence microscopy. The number of parasites in *dsAdLys c-1* KO mosquitoes was dramatically reduced when compared with control *dsGFP* mosquitoes.

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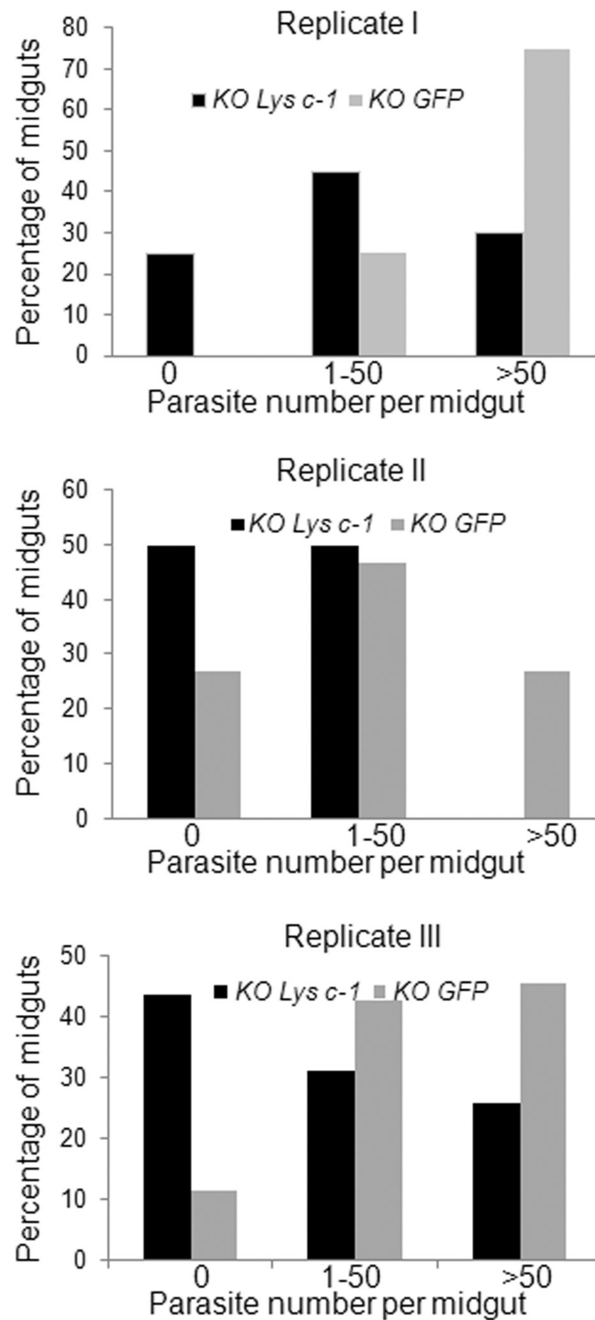

Fig. 7.

The effect of *AdLys c-1* knockdown on number of *P. berghei*.

At 4 days after infection, midguts from each batch were dissected and the number of oocysts counted using fluorescence microscopy. Results of three independent replicates are presented. (KO GFP = knock down of *GFP*, KO *Lys c-1* = knock down of *AdLys c-1*, Parasite number per midguts 1= 0, 2= 1–50, 3= >50).