Anopheles stephensi dual oxidase maintains microbial homeostasis in blood fed midgut

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ABSTRACT

The presence of bacteria in mosquito gut is mainly involved in the food digestion. After blood meal they proliferate and are protected from immune attacks by the formation of gut barriers. Simultaneously, it is also required that the gut microbial population should not over proliferate to cause any deleterious effect on the host and thus there is the need of maintaining gut microbiota homeo-stasis. Here, we demonstrate that *A. stephensi* dual oxidase (AsDUOX) is not only protecting the gut bacteria through barrier formation but it is also responsible for balancing their population after the blood meal. The transcriptional analysis revealed that AsDUOX is highly induced in the blood fed midgut and its silencing significantly increased the bacteria in these midguts. However, the AsDUOX silencing has non-significant effect on the mortality of mosquito. This is due to the induction of an array of anti-bacterial immune genes in silenced midguts. These findings revealed that the multiple levels of immune responses are functional to control the bacterial population in the blood fed midgut. We hypothesized that manipulating the microbial homeostasis will introduce new frontiers in blocking the malaria transmission as the gut bacteria have been reported as suppressors of the *Plasmodium* development.

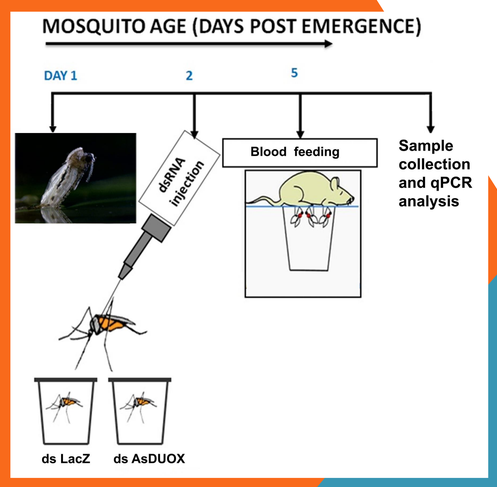

Figure 1. Silencing procedure.

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INTRODUCTION

Insects, like other metazoa, have been shown to harbour numerous bacteria in the gut. These bacteria have role in host development, food digestion and energy extraction, defence of natural enemies and maturation as well as development of the immune system. Therefore it is needed to protect beneficial bacteria from the immune responses however, at the same time insuring no over-proliferation of these bacteria.

In the blood feeding insect it is not a simple task, as bacteria extensively proliferates after blood feeding. So the gut system allows gut–microbe protection as well as elimination of any over proliferative or detrimental microbe(s). Till date, merely few mechanisms have been identified in the insect that adjust the immune response in the gut that maintains microbial homeostasis. DUOX gene from other insect is reported as gene of barrier formation to create low immunity zone as well as acts like an anti-bacterial molecule.

Here, we demonstrate that *A. stephensi* dual oxidase (AsDUOX) is not only protecting the gut bacteria through barrier formation but it is also responsible for balancing their population after the blood meal. Silencing of this gene increases the bacterial load in the gut. Hence, this will introduce new frontiers in blocking the malaria transmission through manipulation of gut bacteria.

METHODS AND MATERIALS

Mosquito Rearing
Anopheles stephensi mosquitoes were reared in insectory at 28°C, 80% relative humidity (RH) and 12h light:dark cycle.

Blood feeding and sample collection
For these experiments 3-4 days old females were allowed to fed on anesthetized mice to collect the blood fed samples. Midguts (Mg) is dissected from rest of the body parts (called carcass, Cc), collected separately in RNAlater and stored at -80ºC till further use.

dsRNA synthesis and Gene silencing
A fragment from AsDUOX gene was amplified from *Anopheles stephensi* and was cloned into the pCR®II-TOPO® vector and T7 promoters were incorporated at the ends of this fragment. This fragment was used to synthesize double-stranded RNA (dsRNA) with MEGAscript RNAi kit (Ambion, Austin, TX, USA). The dsRNA was further purified with water and concentrated to 3 µg/µl using a Microcon YM-100 filter (Millipore). A similar strategy was used to synthesize LacZ dsRNA. Gene silencing experiments were performed injecting 69nL of 3 ug/uL solution of dsRNA dsLacZ as control and dsAsDUOX as test (Figure 1). These females are allowed to feed on anesthetized mice to collect blood fed (BF) midgut and carcass separately as before.

RESULTS

AsDUOX gene is induced in blood fed midguts:
Result presented in figure 2 showed that AsDUOX expression is ~100 folds higher in sugar fed carcasses against the midguts. However, this gene is induced ~7 folds in blood fed midguts with no change in carcasses. The time kinetics revealed that AsDUOX expression peak at 6h after blood feeding in midgut (figure 3). Interestingly, the expression kinetics of 16S rRNA and AsDUOX in blood fed midguts revealed a week negative correlation (figure 3).

AsDUOX regulates gut bacterial density:
RNAi mediated silencing of AsDUOX gene greatly increased the bacterial density in blood fed midguts (figure 4 and 5). This results showed the anti-bacterial role of AsDUOX in *Anopheles stephensi* midguts.

An array of immune genes is induced in AsDUOX silenced gut to regulate endogenous bacterial load:
Silencing of AsDUOX increases the gut bacteria. However, this has a non-significant effect on mosquito mortality. This is due the induction of an array of immune genes in silenced midguts against the increased bacterial load (figure 6 and 7).

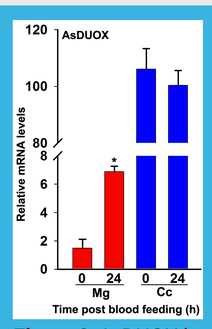

Figure 2. AsDUOX is induced by blood feeding.

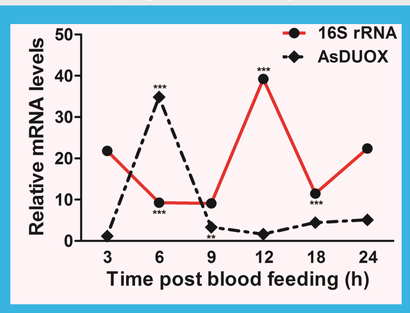

Figure 3. AsDUOX gene expression shows negative correlation with midgut microbial density.

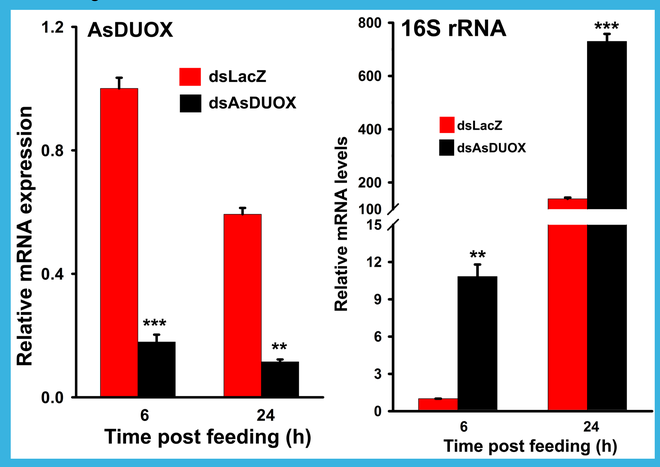

Figure 4. Analysis of RNAi mediated gene silencing efficiency of AsDUOX gene in BF midguts.

Figure 5. AsDUOX regulates the microbial density blood fed midguts.

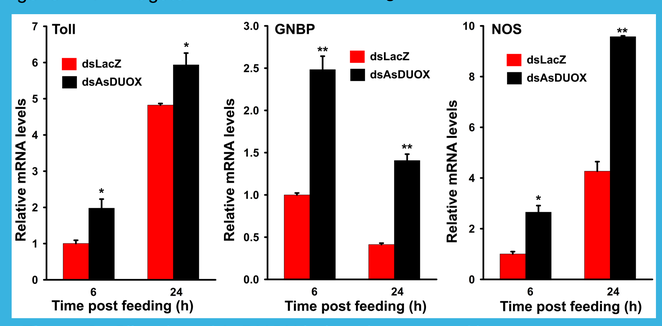

Figure 6. Silencing of AsDUOX induced an array of immune genes to regulate the endogenous bacterial load in gut.

DISCUSSION

In this study, we investigated the role of AsDUOX in *Anopheles stephensi* midguts. The result showed that AsDUOX is a blood induced gene in the midguts. This gene showed the negative correlation of expression with 16S rRNA post blood meal. The AsDUOX gene is previously reported as one of the gene required for the crosslinking of gut barrier to create low immunity zone, so that gut bacteria can proliferate without mounted by immune responses. The DUOX gene in other insects is reported to regulate the microbial load. Therefore, *A. stephensi* dual oxidase (AsDUOX) is one of the molecules that is responsible for balancing the bacterial population in the blood fed midguts. This fact is further supported by the data that silencing of AsDUOX gene significantly increased the bacterial load in these guts. These results can be exploited to develop methodology that can adjust the immune response in the gut and hence determining vector competence.

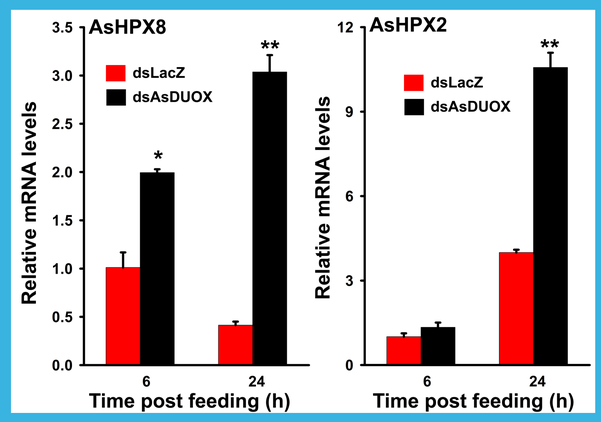

Figure 7. Silencing of AsDUOX induced other anti-bacterial peroxidase to regulate the endogenous bacterial load in gut.

CONCLUSIONS

We concluded that AsDUOX is a blood induced gene in midguts. This gene has an important role in maintain the gut microbial homeostasis after blood feeding. Silencing of AsDUOX in blood fed midguts significantly increases the bacterial load and hence AsDUOX is an anti-microbial gene of the innate immunity system. Further studies will decipher the molecular mechanism of AsDUOX gene, which will allow to regulate the gut bacteria and new methodology to suppress *Plasmodium* development.

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