Insect pathogenic fungus interacts with the gut microbiota to accelerate mosquito mortality

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Edited by Nancy A. Moran, University of Texas at Austin, Austin, TX, and approved May 4, 2017 (received for review March 2, 2017)

The insect gut microbiota plays crucial roles in modulating the interactions between the host and intestinal pathogens. Unlike viruses, bacteria, and parasites, which need to be ingested to cause disease, entomopathogenic fungi infect insects through the cuticle and proliferate in the hemolymph. However, interactions between the gut microbiota and entomopathogenic fungi are unknown. Here we show that the pathogenic fungus *Beauveria bassiana* interacts with the gut microbiota to accelerate mosquito death. After topical fungal infection, mosquitoes with gut microbiota die significantly faster than mosquitoes without microbiota. Furthermore, fungal infection causes dysbiosis of mosquito gut microbiota with a significant increase in gut bacterial load and a significant decrease in bacterial diversity. In particular, the opportunistic pathogenic bacterium *Serratia marcescens* overgrows in the midgut and translocates to the hemocoel, which promotes fungal killing of mosquitoes. We further reveal that fungal infection down-regulates antimicrobial peptide and dual oxidase expression in the midgut. Duox down-regulation in the midgut is mediated by secretion of the toxin oosporein from *B. bassiana*. Our findings reveal the important contribution of the gut microbiota in *B. bassiana*-killing activity, providing new insights into the mechanisms of fungal pathogenesis in insects.

Anopheles | gut microbiota | dysbiosis | entomopathogenic fungus | immunity

Mosquitoes transmit a wide range of pathogens that cause diseases such as malaria, dengue, yellow fever, and Zika, which have a devastating impact on human health (1). Although vector control via insecticides is a major tool for disease control, intensive use of insecticides poses risks to humans and the environment and creates intensive pressure for mosquitoes to develop resistance. Thus, alternative tools for mosquito control are urgently needed (2).

An environmentally friendly alternative to chemical insecticides is offered by entomopathogenic fungi (3, 4). Among them is *Beauveria bassiana* (Cordycipitaceae), which has been widely used for the biological control of agricultural insect pests (5) and insect vectors of human diseases, including mosquitoes (6). This fungus is equally effective at killing insecticide-resistant and insecticide-susceptible mosquitoes, and is considered a next-generation control agent against mosquitoes (7). However, the relatively slow action of fungal pathogens, compared with chemical insecticides, has hampered their widespread application (8). To develop approaches to accelerate the speed at which a fungal pathogen kills its host, a better understanding of fungus–mosquito interactions is critical.

The mosquito gut is colonized by diverse communities of commensal bacteria, the microbiota, that play important roles in host physiology, particularly in modulation of host immune response and the outcome of pathogen infection (9–11). The gut microbiota has been recognized as a virtual "organ," which is integrated into the biological system of the host and indispensable to its health (12–14). Coexistence between the insect and its microbiota is mostly harmonious, and in most cases is beneficial to the insect.

The protective roles of the gut microbiota against incoming intestinal pathogens have been studied in mosquitoes. In *Anopheles* and *Aedes*, removal of the gut microbiota with antibiotics renders the mosquito more susceptible to infection by the apicomplexan parasite *Plasmodium* and by the dengue virus (15–17). Gut bacteria protect their host insects against invading pathogens by stimulating the host immune response (16) or by producing antimalarial compounds (18).

However, recent studies have shown that the resident bacteria can also promote or assist the gut infection of incoming pathogens (19). The midgut bacterium *Serratia odorifera* enhances viral infection in *Aedes* (20) and *Anopheles* mosquitoes (21). Furthermore, pathogens can manipulate the microbiota to enhance infection. Abraham et al. have reported that the human pathogenic bacterium *Anaplasma phagocytophilum* (causative agent of anaplasmosis) appropriates the antibacterial protein of the tick vector, alters the host gut microbiota, and enables the pathogen to more efficiently colonize the tick (22).

Unlike viruses, bacteria and parasites, which need to be ingested to cause disease, pathogenic fungi primarily attack insects by penetrating the host integument and proliferating in the hemolymph (23). The interplay between the gut microbiota and fungal entomopathogens has not been examined. Outstanding issues include: Can gut microbiota protect insects from fungal infection? Do microbiota and fungal pathogens interact, or do they act independently? Can commensal gut bacteria become virulent when a fungal pathogen infects an insect? Understanding the tripartite interactions between mosquito host, resident microbiota, and fungal pathogen may yield new

Significance

As insecticide resistance is rapidly spreading, alternative tools for mosquito control are urgently needed. *Beauveria bassiana* is equally effective at killing insecticide-resistant and insecticide-susceptible mosquitoes. Better understanding of fungus–mosquito interactions is critical for improvement of its efficacy. Here we discover a contributory role for the gut microbiota in promoting fungal killing of mosquitoes via down-regulation of antimicrobial peptides and dual oxidase in the midgut. Fungal infection results in dysbiosis of mosquito gut microbiota by significantly increasing gut bacterial loads and decreasing bacterial diversity. In particular, fungal infection causes overgrowth and translocation of the opportunistic pathogen *Serratia marcescens* from the gut to the hemocoel, thus promoting mosquito death. Our study may lead to new strategies for biological control of mosquitoes.

Author contributions: S.W. designed research; G. Wei, G. Wang, and H.C. performed research; Y.L. and F.L. contributed new reagents/analytic tools; G. Wei, Y.L., G. Wang, and S.W. analyzed data; and Y.L. and S.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The entire 16S rRNA gene sequence dataset reported in this paper has been deposited in the National Center for Biotechnology Information Sequence Read Archive (accession no. PRJNA371598).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1703546114/-/DCSupplemental.

insights into pathogen–insect interactions, and may assist in the development of new insect control strategies and disease interventions.

In the present study, we investigated the interplay between the pathogenic fungus *B. bassiana* and the gut microbiota of the mosquito *Anopheles stephensi*. We found that topical infection by *B. bassiana* down-regulated the mosquito midgut immune responses via production of the toxin oosporein, caused dysbiosis of gut microbiota and translocation of bacteria from the gut to the hemocoel, where they switched from asymptomatic gut symbionts to hemocoelic pathogens, and accelerated fungal killing of mosquitoes.

Results

Effect of the Gut Microbiota on *B. bassiana* Pathogenesis in Adult Mosquitoes. To investigate a possible role of the gut microbiota in fungal pathogenesis in the mosquito, axenic mosquitoes were generated via treatment with oral antibiotics. This treatment did not influence adult mosquito survival (24). The efficacy of elimination of midgut bacteria was confirmed by plating gut homogenates onto Luria–Bertani (LB) agar plates and performing PCR analysis using bacterial 16S ribosomal RNA (rRNA) gene universal primers (Fig. S1 and Table S1).

The insect bioassay was conducted using female mosquitoes with and without gut microbiota (nonaxenic and axenic, respectively). After topical inoculation with a *B. bassiana* conidial suspension, adult nonaxenic mosquitoes died significantly faster (median lethal time, LT_{50} 79.9 ± 3.2 h) than did axenic mosquitoes (LT_{50} 95.2 ± 3.1 h) ($P < 0.05$, *t* test) (Fig. 1*A*). This result suggests that the gut microbiota accelerates killing of mosquitoes by *B. bassiana*.

***B. bassiana* Infection Causes Dysbiosis of the Gut Microbiota.** We next tested whether fungal infection affected the homeostasis of mosquito gut microbiota by determining the cultivable bacterial loads in the midgut of the mosquitoes at 36 and 90 h post topical fungal infection (hpi). Bacterial load was significantly increased in *B. bassiana*-infected mosquitoes at 36 and 90 hpi compared with noninfected controls treated with 0.01% Triton X-100 (Fig. 1*B*). Quantitative PCR (qPCR) showed that total bacterial load in infected mosquitoes at 90 hpi was significantly higher (~3.7-fold higher) than in noninfected controls ($P < 0.001$) (Fig. 1*C*).

Based on 16S rRNA gene sequence analysis, the predominant cultivable bacterium had high similarity to *Serratia marcescens* (99% identity) (Fig. S2). We validated the proliferation of *S. marcescens* in the gut of infected mosquitoes using *Serratia*-specific 16S rRNA gene PCR primers (Table S1). Consistent with proliferation of the total gut bacteria, *S. marcescens* numbers significantly increased (by 3.4-fold, $P = 0.014$) in the infected mosquitoes at 90 hpi compared with noninfected controls (Fig. 1*D*). *S. marcescens* is a prevalent midgut bacterium in laboratory-reared and field-collected mosquitoes (25, 26), and can also be an opportunistic pathogen in mosquitoes under certain conditions (20, 27, 28).

To determine whether the proliferated bacteria contributed to the speed of the kill, we reintroduced the overproliferating *S. marcescens* recovered from the midgut of *B. bassiana*-infected mosquitoes into the midgut of axenic mosquitoes via sugar meals. We found that the reintroduction of *S. marcescens* restored mosquito susceptibility to fungal infection, in comparison with nonvirulent symbiotic bacterium *Asaia* sp.-treated mosquitoes (29) and PBS controls (Fig. S3).

We further assessed the dynamic composition and diversity of the midgut bacteria in the noninfected mosquitoes and the fungus-infected mosquitoes at 0, 12, 36, 60, and 84 hpi by deep sequencing of 16S rRNA genes (Table S2). In the noninfected mosquitoes, the midgut bacteria were diverse and dominated by bacteria of six phyla: Proteobacteria, Firmicutes, Actinobacteria, Bacterioidetes, Fusobacteria, and Cyanobacteria (Fig. 2*A*). The abundance of Proteobacteria and Firmicutes changed dynamically over time, possibly because of changes in mosquito physiology. The Proteobacteria *Acinetobacter*, *Photobacterium*, and *Asaia*, the Firmicutes *Streptococcus*, and the Actinobacteria *Rhodococcus* increased in abundance over time in the noninfected mosquitoes (Fig. 2*B*).

Fungal infection decreased the bacterial diversity in comparison with noninfected mosquitoes (Table S3). Starting at 36 hpi, the single phylum of Proteobacteria predominated in *B. bassiana*-infected mosquitoes (Fig. 2*A*). The composition and diversity of the midgut bacterial population changed markedly in mosquitoes after topical infection by *B. bassiana*, resulting in almost exclusive colonization by three genera of Proteobacteria: *Acinetobacter*, *Serratia*, and *Asaia*. *Serratia* overgrew in the fungus-infected mosquitoes, yet was not dominant in noninfected mosquitoes (Fig. 2*B*).

Principal coordinate analysis (PCA) of unweighted jack-knifed UniFrac distances of microbial communities showed that the first and second principal coordinates, which explained 34.4 and 12.1% of the variance in the data, respectively, separated infected mosquitoes from noninfected mosquitoes starting at 36 hpi (Fig. 2*C*). These results suggest that fungal infection can alter bacterial composition, reduce bacterial diversity, and result in dysbiosis of the gut microbiota.

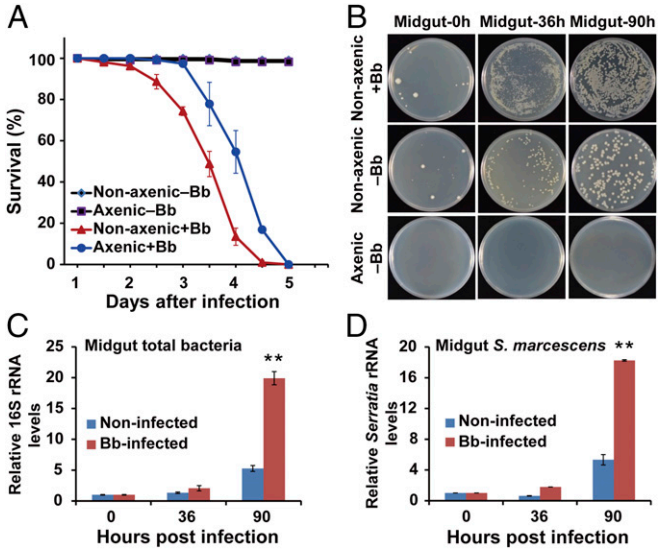

Fig. 1. Effect of gut microbiota on pathogenesis in *A. stephensi* infected by the fungus *B. bassiana* (Bb). (*A*) Survival of axenic (without microbiota) and nonaxenic (with microbiota) mosquitoes ($n = 50$) following topical infection (+Bb) or no topical infection (–Bb) with *B. bassiana*. (*B*) Load of midgut cultivable bacteria from Bb-infected nonaxenic, noninfected nonaxenic, and noninfected axenic mosquitoes ($n = 20$) at 0, 36, and 90 h post fungal infection. Bacterial load was determined by plating the homogenate of mosquito midguts with 10,000 dilution on LB agar plates. Representative images are shown. (*C* and *D*) Number of midgut total bacteria (*C*) and *S. marcescens* (*D*) in Bb-infected and noninfected mosquitoes ($n = 15$) at 0, 36, and 90 hpi; quantification was by 16S rRNA gene-based qPCR analysis. Three biological replicates were conducted. Error bars indicate SD. Double asterisks represent a significant difference determined by the Student's *t* test at $P < 0.01$.

Fungal Infection Promotes Translocation of Opportunistic Pathogenic Midgut Bacteria. Previous studies have shown that translocation of overgrowing bacteria from the gut to the hemocoel promotes infection and death (19, 30, 31). To determine whether fungal infection results in bacterial translocation from the gut to the hemocoel, the hemolymph from infected nonaxenic and axenic mosquitoes was collected and plated onto LB plates. In nonaxenic mosquitoes, 90 h after infection by *B. bassiana*, both *B. bassiana* and bacterial colonies were present in the hemolymph (Fig. 3*A*). In contrast, in noninfected nonaxenic mosquitoes and in infected axenic mosquitoes, no bacterial colonies were found in the hemolymph (Fig. 3*A*). The 16S rRNA gene sequence identified

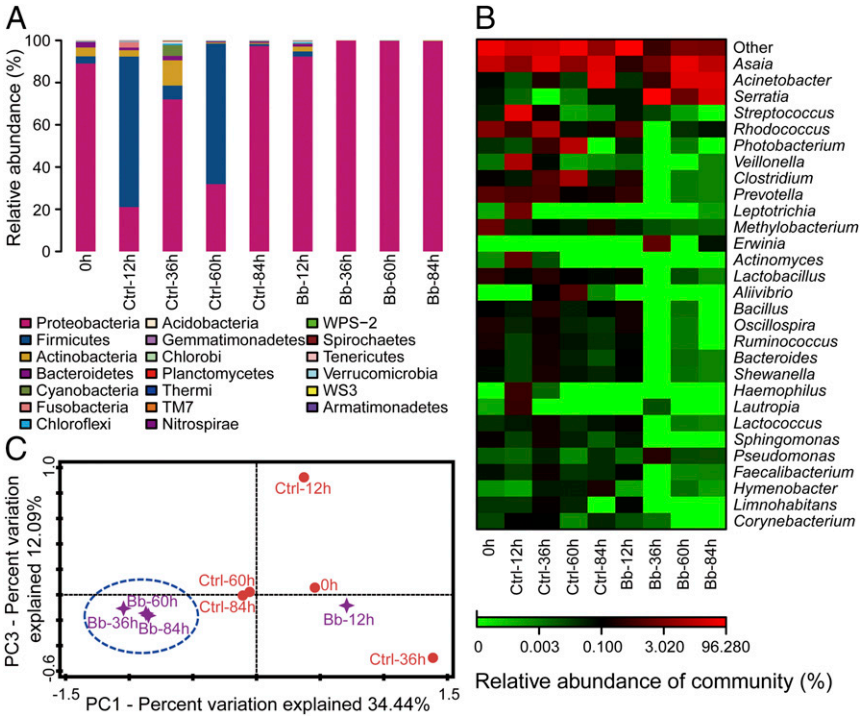

Fig. 2. Fungal infection alters the composition of gut microbiota in mosquitoes. (*A*) Histogram showing temporal changes, at the phylum level, in noninfected (Ctrl; Triton treatment as control) and Bb-infected mosquitoes ($n = 40$) over 84 h. (*B*) Heat map showing temporal changes, at the genus level, in Ctrl and Bb-infected mosquitoes. (*C*) Principal component analysis of unweighted jack-knifed UniFrac distances of microbial communities from Ctrl and Bb-infected mosquitoes.

the predominant bacterium isolated from the hemolymph of *B. bassiana*-infected mosquitoes as *S. marcescens*, which overgrew in the mosquito midgut after fungal infection (Fig. 1*D*). These results indicated that fungal infection leads to translocation of *S. marcescens* from the midgut to the hemocoel at 90 hpi.

There was no obvious antagonism between the translocating *S. marcescens* and *B. bassiana* grown on LB plates (Fig. 3*A*). Next, we examined in vivo whether the translocating bacteria affected the fungal colonization of the mosquito hemocoel. qPCR with fungus-specific GPD gene primers showed no significant differences in fungal load between nonaxenic and axenic mosquitoes (Fig. 3*B*), suggesting that translocation of bacteria into the hemocoel does not affect fungal proliferation.

Further, we tested whether *S. marcescens* isolated from the fungus-infected mosquito hemolymph could cause systemic infection. Injection of 100 CFUs of the isolated *S. marcescens* directly into the mosquito hemocoel caused up to 94.5% mortality within 1 d. In contrast, injection of 100 CFUs of the avirulent symbiont *Asaia* sp. into the hemocoel caused only 3.6% mortality within 5 d (Fig. 3*C*). These data show that fungal infection results in translocation of opportunistic bacteria such as *S. marcescens* from the midgut to the hemocoel, where they switch from asymptomatic gut symbionts to hemocoelic pathogens and facilitate fungal killing of mosquitoes.

Fungal Infection Down-Regulates Immune Gene Expression in the Midgut. Given the dysbiosis of gut microbiota in fungus-infected mosquitoes, we reasoned that decreased immune responses might account, in part, for the bacterial overproliferation. To test this hypothesis, we used qPCR to assess changes in the expression profiles of five effector genes encoding antimicrobial peptides (AMPs), chosen based on their roles in mosquito midgut immunity and controlling bacterial proliferation (32).

In the midgut, in the absence of fungal infection, effector genes encoding Gambicin 1 (GAM1), Defensin 1 (DEF1), Cecropin 1 (CEC1), Attacin (ATT), and FBN9 (Fibrinogen-related protein family) were expressed at high levels (Fig. 4*A*), a likely reflection of enhanced basal immunity induced by the resident bacteria. After 36 and 90 hpi with *B. bassiana*, all five effector genes in the midgut were significantly down-regulated by approximately twofold (Fig. 4*A*). Conversely, in the midgut of axenic mosquitoes and in the absence of fungal infection, all AMP genes were expressed at low levels, and were significantly up-regulated at 36 h after fungal infection.

In the carcass, the expression pattern of the effector genes was similar in nonaxenic and axenic mosquitoes (Fig. 4*B*). All of the effector genes were significantly up-regulated at 36 hpi and then declined at 90 hpi, even though the gut microbiota such as *S. marcescens* had translocated to the hemocoel of the nonaxenic mosquitoes (Fig. 3*A*). These observations suggest that immune responses in the carcass of the mosquito are not influenced by midgut bacteria during fungal infection. Taken together, these data demonstrate that fungal infection strongly suppressed the expression of immune effector genes in nonaxenic mosquito midguts, which might cause dysbiosis of the midgut microbiota.

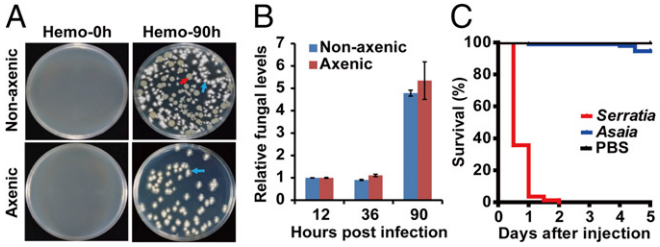

Fig. 3. Translocation of gut bacteria to mosquito hemocoel after topical infection with *B. bassiana*. (*A*) Growth of bacteria and fungi from the hemolymph of nonaxenic and axenic mosquitoes at 0 and 90 hpi with *B. bassiana*. The red arrow indicates bacterial colonies. The blue arrows indicate fungal colonies. (*B*) qPCR-based quantification of fungal load in nonaxenic and axenic mosquitoes ($n = 15$) at 12, 36, and 90 hpi. Fungal levels are expressed as that of fungal *gpd* mRNA relative to *A. stephensi* ribosomal protein S7 (AsS7) mRNA. (*C*) Survival of mosquitoes ($n = 100$) following injection of 100 CFUs of *S. marcescens*, 100 CFUs of *Asaia* sp., or PBS (control) into the hemolymph. Experiments were performed in three replicates with similar results. Error bars indicate SD.

The number of bacteria in the midgut is also controlled by reactive oxygen species (ROS) produced by dual oxidase (Duox) in the insect midgut epithelium (24). To explore this aspect in our experimental system, we further tested the expression patterns of *Duox* in the midgut of mosquitoes infected with *B. bassiana*. Expression of *Duox* was significantly higher in the midguts of nonaxenic mosquitoes than in axenic mosquitoes (Fig. 5*A*). However, following fungal infection, *Duox* expression was significantly down-regulated at 36 hpi in the nonaxenic mosquitoes and significantly up-regulated in the midgut of axenic mosquitoes (Fig. 5*A*). In contrast, after fungal infection, there was no significant difference in the expression of *Duox* in the carcasses of axenic and nonaxenic mosquitoes (Fig. S4).

After fungal infection, the diminished *Duox* expression was consistent with the weaker ROS signal (Fig. 5*B*) and lower hydrogen peroxide (H_2O_2) production (Fig. 5*C*) in the midgut of nonaxenic mosquitoes. To validate the role of ROS in the control of gut bacteria, we silenced *Duox* by systemic injection of *Duox* double-stranded RNA (dsDuox). The dsDuox silencing reduced midgut *Duox* mRNA levels by 79% (Fig. 5*D*) and markedly promoted proliferation of midgut bacteria (Fig. 5*E*), including *S. marcescens* (Fig. 5*F*). A high dose of dsDuox (170 ng) significantly reduced the survival of nonaxenic mosquitoes but did not impact survival of axenic mosquitoes (Fig. 5*G*). These data suggest that fungal infection causes dysregulation of AMPs and Duox in the midgut, which in turn might result in dysbiosis of the midgut microbiota.

***B. bassiana* Produces the Toxin Oosporein, Which Down-Regulates *Duox* Expression in the Midgut.** The fungal pathogen *B. bassiana* produces oosporein, which can down-regulate expression of antifungal peptide genes in the insect fat body (33). To test whether oosporein can inhibit midgut immune responses, we generated an oosporein-nonproducing mutant strain, *Bb*Δ*ops*, by disrupting the oosporein synthase 1 gene (*Bbops1*; required for oosporein biosynthesis) in *B. bassiana*. We found that deletion of *Bbops1* resulted in a significant decrease in fungal virulence against adult female mosquitoes ($P < 0.05$) (Fig. 6*A*). qPCR analysis showed that expression of the AMP genes *def1* and *cec1* was not significantly

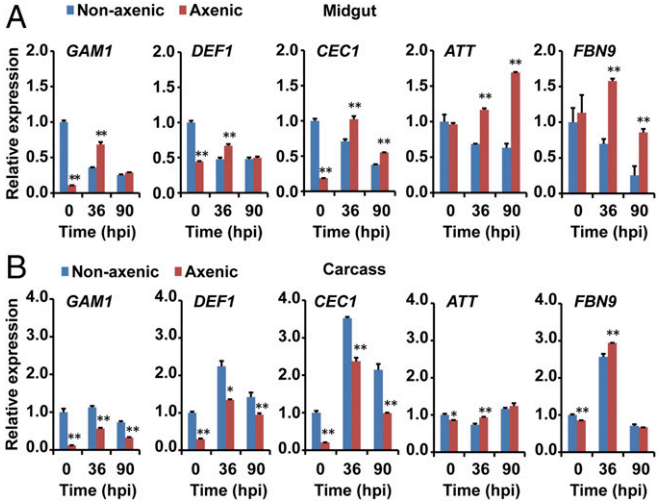

Fig. 4. Expression of five AMPs in the midgut is down-regulated after topical infection with *B. bassiana*. qPCR analysis of expression levels of AMPs in the (*A*) midgut and (*B*) carcass of nonaxenic and axenic mosquitoes (n = 20) at 0, 36, and 90 hpi. Gene expression of each sample was normalized to that of nonaxenic mosquitoes at time 0 (taken as 1). Three biological replicates were conducted. Error bars indicate SD. Single and double asterisks represent a significant difference determined by the Student's t test at $P < 0.05$ and $P < 0.01$, respectively.

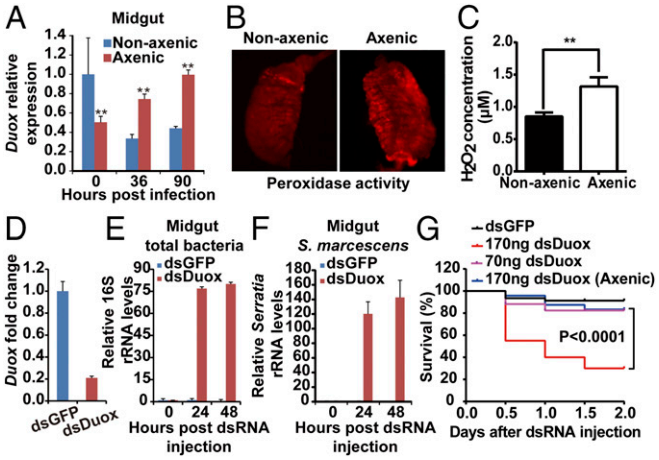

Fig. 5. Expression of mosquito *Duox* following fungal infection and effect of *Duox* silencing on midgut bacterial load and host survival. (*A*) *Duox* mRNA levels in the midgut of nonaxenic and axenic mosquitoes (n = 20) at 0, 36, and 90 hpi with *B. bassiana*. (*B*) Fluorescence staining for peroxidase activity. (*C*) H_2O_2 concentration in the midgut of nonaxenic and axenic mosquitoes (n = 5) infected by *B. bassiana* at 60 h. (*D*) Midgut *Duox* silencing efficiency in mosquitoes (n = 20) injected with 70 ng of dsGFP or dsDuox. (*E* and *F*) Effect of *Duox* silencing on midgut total bacterial load (*E*) (n = 15) and *S. marcescens* (*F*) at 0, 24, and 48 h post dsRNA injection (n = 15); levels are relative to readings at time 0 taken as 1. (*G*) Survival of *Duox*-silenced nonaxenic and axenic mosquitoes (n = 50) injected with different amounts of dsDuox. Experiments were performed in three biological replicates. Error bars indicate SD. Double asterisks represent a significant difference determined by the Student's t test at $P < 0.01$.

different in the midgut of mosquitoes infected by *B. bassiana* WT and by the *Bb*Δ*ops* mutant (Fig. S5). However, in the carcass, at 72 hpi, expression of *def1* and *cec1* was significantly lower in mosquitoes infected with *B. bassiana* WT than in those infected by the mutant *Bb*Δ*ops*.

Duox expression was significantly lower in the midgut of mosquitoes infected by *B. bassiana* WT than in those infected by *Bb*Δ*ops* (Fig. 6*B*). In contrast, *Duox* expression in the carcass was not significantly different in mosquitoes infected by *B. bassiana* WT or by the *Bb*Δ*ops* mutant (Fig. 6*C*). Accordingly, midgut bacterial load at 24 and 72 hpi was significantly higher in mosquitoes infected by *B. bassiana* WT than in those infected by *Bb*Δ*ops* (Fig. 6*D*). Taken together, our data suggest that *B. bassiana* produces the toxin oosporein to mediate interactions with the mosquitoes' immune systems. It appears that oosporein is involved in the down-regulation of *Duox* in the midgut and suppression of AMP genes in the carcass, which might result in dysbiosis of the midgut bacteria and promotion of fungal killing of mosquitoes.

Discussion

Entomopathogenic fungi gain access to the hemocoel cavity through the external cuticle, where they take up nutrients, produce toxins, destroy host cells, and eventually kill their hosts (23). Historically, fungal infections have primarily been studied as interactions between the fungus and the host insect, without consideration of interactions with the gut microbiota. To address this gap in knowledge, we have investigated the role of the gut microbiota in the interactions of the pathogenic fungus *B. bassiana* with its mosquito hosts. We now report that the fungus interacts with the gut microbiota to promote mosquito death. Reintroduction of gut bacteria into axenic mosquitoes enhanced the susceptibility of the mosquitoes to fungal infection.

In the present study, fungal infection caused the opportunistic bacterial pathogen *S. marcescens* to outgrow others and translocate from the gut to the hemocoel. Whereas *S. marcescens* persists in the mosquito midgut without causing apparent illness,

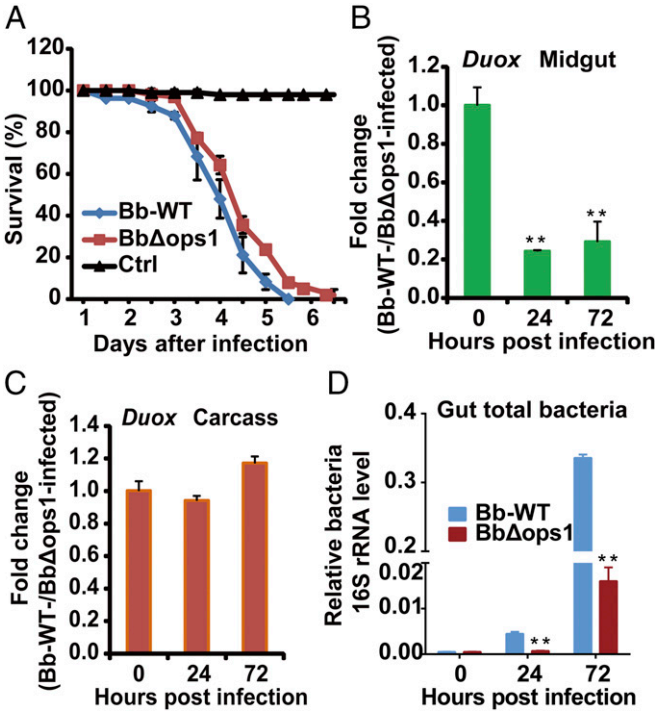

Fig. 6. Effect of oosporein on fungal virulence, mosquito *Duox* expression, and midgut bacterial growth. (*A*) Survival of adult female *A. stephensi* (n = 50) following topical infection with *B. bassiana* WT or *BbΔops1*; control mosquitoes were not infected. (*B* and *C*) Effect of *Bbops1* disruption on mRNA levels of *Duox* in the midgut (*B*) (n = 20) and carcass (*C*) of mosquitoes (n = 20) following topical infection with *B. bassiana* WT and *BbΔops1*. (*D*) Effect of *Bbops1* disruption on midgut total bacteria determined by 16S rRNA gene-based qPCR analysis (n = 15). Experiments were conducted in three biological replicates. Error bars indicate SD. Double asterisks represent a significant difference determined by the Student's t test at $P < 0.01$.

injection of *S. marcescens* into the hemocoel leads to rapid death (Fig. 3*C*), indicating that *S. marcescens* switches from being an asymptomatic gut symbiont to a hemocoelic pathogen following fungal topical infection. Additionally, oral ingestion of a large amount of *S. marcescens* induced an elevated rate of mosquito mortality following fungal topical inoculation. *S. marcescens* is also a pathogenic bacterium to *Drosophila* both by oral infection to the gut and injection into the hemocoel (28). It was previously shown that the commensal midgut microbiota contributes to lepidopteran mortality induced by the pathogenic bacterium *Bacillus thuringiensis* (30, 31). Moreover, Caccia et al. reported that mortality of the cotton leafworm *Spodoptera littoralis* was increased by the gut bacteria *Serratia* and *Clostridia* species invading the body cavity through toxin-induced epithelial lesions (31). In *Anopheles*, the symbiotic bacterium *Asaia* is responsible for inhibiting *Wolbachia* transmission but antibiotic microbiome perturbation enables *Wolbachia* transmission (34). Examples of the contributory role of gut microbiota to invading intestinal pathogens are also found among vertebrates. For example, the gut commensal microbiota promotes viral infection directly, by activating the immunosuppressive cytokine, or indirectly, by stimulating the proliferation of target cells (35–37).

Bacterial overproliferation is limited by the delicate balance between the commensal gut microbiota and the immune system of the mosquito host. Our data show that mosquito midgut commensal bacteria trigger a basal level of immunity that enhances the expression of AMPs, which in other studies has been shown to be mainly through the IMD pathway (27) and to control the proliferation of the bacterial population (16, 35, 38). Constitutive activation of the gut immune response is detrimental to insect health. Thus, regulatory mechanisms that dampen the basal immune response are required to avoid unhealthy excesses and prevent chronic lethal reactions (24), but the immune system must remain responsive to acute infectious challenges (39). Our results show that the mosquito's systemic immune response is significantly induced but that the midgut immune response in nonaxenic mosquitoes is significantly down-regulated at 36 hpi. These data suggest that mosquitoes might regulate their immune response to prioritize the fight against acute fungal infection. Such prioritizing modulation of the immune response might be a factor that causes dysregulation of the midgut immune response following topical fungal infection. A recent study by Barreaux et al. showed that the immune response of the *Anopheles gambiae* mosquito becomes dramatically induced by a small number of injected Sephadex beads, and that melanization is prioritized for one bead rather than distributed over all beads (40). However, dysbiosis of midgut microbiota might also be caused by other factors. Damage to the gut by *B. bassiana* could change gut physiology, which leads to dysbiosis and translocation of gut microbes to the hemocoel, as indicated in the case of the pathogenic bacterium *B. thuringiensis* (31).

The suppression of immune responses by invading fungi has been attributed to the combined activity of enzymes and immunosuppressive toxins (41). Entomopathogenic fungi produce a large array of secondary metabolites that are toxic to insects, such as bassianolide, beauvericin, beauverolides, cordycepin, destruxins, and oosporein. Most of these are required for full fungal virulence, via weakening the host immune defenses or damaging the muscular system (23, 42). The destruxins produced by *Metarhizium robertsii* induce flaccid paralysis and visceral muscle contraction by targeting the Ca^{2+} channel in insects (43–45). A recent study showed that the toxin oosporein produced by *B. bassiana* promotes fungal infection by inhibiting polyphenol oxidase activity and down-regulating expression of antifungal peptide genes in the insect fat body (33). Our study reveals that the toxin oosporein specifically mediates down-regulation of *Duox* expression in the midgut, which reduces midgut ROS production. Duox-dependent ROS generation plays a major role in gut immunity and the control of gut-associated bacteria (24, 46). In *Drosophila*, opportunistic pathogenic bacteria can be discriminated and controlled by triggering the Duox-dependent gut immunity (47, 48). Down-regulation of *Duox* in the midgut may cause the opportunistic pathogen *S. marcescens* to outgrow other commensal bacteria.

In conclusion, we have discovered a contributory role for the gut microbiota in promoting fungal killing of mosquitoes. We propose a model in which a fungal pathogen interacts with the midgut microbiota to accelerate mosquito death via down-regulation of antimicrobial peptides and dual oxidase in the midgut (Fig. S6). The down-regulated midgut immune responses might account, in part, for microbiota dysbiosis, and bacterial translocation from the gut to the hemocoel results in the acceleration of mosquito death by *B. bassiana*. These findings provide new insights into the mechanisms of fungal pathogenesis in insects. Understanding of fungus–insect–microbiota interactions may lead to new strategies for biological control of mosquitoes, and consequently the prevention of vector-borne disease transmission.

Materials and Methods

Mosquito Rearing and Antibiotic Treatment. *A. stephensi* (Dutch strain) mosquitoes were maintained as previously described (49). Axenic mosquitoes were generated via oral antibiotic treatment as previously described (24). Experimental details can be found in *SI Materials and Methods*.

Fungal Infection. To conduct fungal infection, adult female *A. stephensi* were sprayed with fungal conidia suspension (5×10^8 conidia per mL). Mosquitoes sprayed with sterile 0.01% Triton X-100 were used as control. Experimental details can be found in *SI Materials and Methods*.

Deep Sequencing. At each of five time points (0, 12, 36, 60, and 84 h) after fungal infection, mosquitoes were dissected to collect midguts. The bacterial

DNA was purified using Gentra Puregene Yeast/Bact. Kit B (Qiagen). The V3 and V4 variable regions of the 16S rRNA gene were amplified and sequenced on the Illumina MiSeq platform. Experimental details can be found in *SI Materials and Methods*.

Quantitative Real-Time PCR Analysis. To quantify gene expression, qRT-PCR was performed using SYBR dye technology. Experimental details can be found in *SI Materials and Methods*. Primers are shown in Table S1.

In Vivo Detection of Reactive Oxygen Species. ROS production in intact midguts was measured using the intracellular ROS-sensitive fluorescent dye dihydroethidium. Experimental details can be found in *SI Materials and Methods*.

dsRNA-Mediated Gene Silencing. To conduct RNAi-mediated gene silencing, mosquitoes were injected with 70 or 140 ng of dsDuox. Control mosquitoes were injected with dsGFP. Experimental details can be found in *SI Materials and Methods*.

***B. bassiana OpS1* Gene Disruption.** The *Bbops1* gene was disrupted in *B. bassiana* Bb252 by homologous recombination using *Agrobacterium tumefaciens*-mediated transformation (50). Experimental details can be found in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Prof. Marcelo Jacobs-Lorena at the Johns Hopkins University School of Public Health for critical comments on the manuscript. We also thank Dr. Sarah Poynton at the Johns Hopkins Editing Referral Service, William H. Welch Medical Library, Johns Hopkins University School of Medicine for editorial assistance. This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant XDB11010500) and One Hundred Talents Program of the Chinese Academy of Sciences (Grant 2013OHTP01).

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