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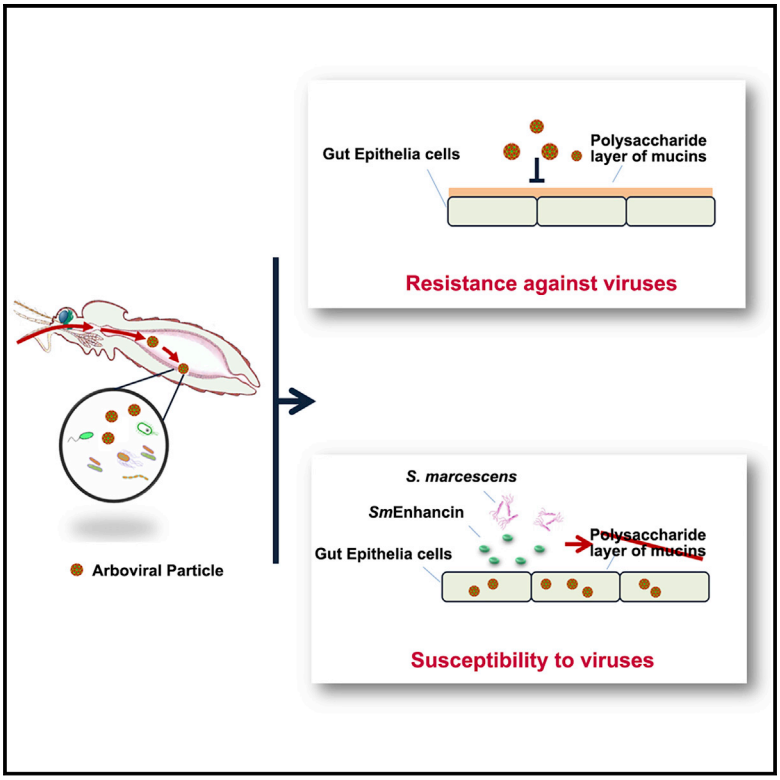
Cell Host & Microbe

A Gut Commensal Bacterium Promotes Mosquito Permissiveness to Arboviruses

Graphical Abstract



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In Brief

Wu et al. identified *Serratia marcescens* as a mosquito gut commensal bacterium critical for efficient arboviral acquisition. *S. marcescens* facilitates arboviral infection through secretion of a protein named *Sm*Enhancin. Colonization of *S. marcescens* in field-derived *Aedes* mosquitoes enhances vector competence. Gut residence of *S. marcescens* correlates with region-specific dengue prevalence.

Highlights

- The gut commensal *Serratia marcescens* promotes mosquito permissiveness to arboviruses
- *S. marcescens* facilitates arboviral infection via a secreted protein named *Sm*Enhancin
- *Sm*Enhancin digests gut membrane-bound mucins to enhance viral dissemination in mosquitoes
- *S. marcescens* enhances the susceptibility of field mosquitoes to dengue virus

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A Gut Commensal Bacterium Promotes Mosquito Permissiveness to Arboviruses

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SUMMARY

Mosquitoes are hematophagous vectors that can acquire human viruses in their intestinal tract. Here, we define a mosquito gut commensal bacterium that promotes permissiveness to arboviruses. Antibiotic depletion of gut bacteria impaired arboviral infection of a lab-adapted *Aedes aegypti* mosquito strain. Reconstitution of individual cultivable gut bacteria in antibiotic-treated mosquitoes identified *Serratia marcescens* as a commensal bacterium critical for efficient arboviral acquisition. *S. marcescens* facilitates arboviral infection through a secreted protein named *Sm*Enhancin, which digests membrane-bound mucins on the mosquito gut epithelia, thereby enhancing viral dissemination. Field *Aedes* mosquitoes positive for *S. marcescens* were more permissive to dengue virus infection than those free of *S. marcescens*. Oral introduction of *S. marcescens* into field mosquitoes that lack this bacterium rendered these mosquitoes highly susceptible to arboviruses. This study defines a commensal-driven mechanism that contributes to vector competence, and extends our understanding of multipartite interactions among hosts, the gut microbiome, and viruses.

INTRODUCTION

The intestinal tract of most metazoans harbors complex communities of microbes that influence host physiology, including nutrition, development, differentiation, immune regulation, and defense (Backhed et al., 2005). Nonetheless, the intestinal tract is a pivotal pathogen entry site that determines pathogen colonization and disease outcome. In mammals, the intestinal commensal bacteria are essential for host defense against many pathogens (Abt and Pamer, 2014) but can also be utilized by some pathogenic enteric viruses for gaining entry into the intestinal epithelia (Kane et al., 2011; Kuss et al., 2011). Mosquitoes are hematophagous insects that acquire viruses into their intestinal tracts by blood feeding. As natural vectors, mosquitoes bite virus-infected hosts and acquire viruses that circulate in the blood. The viruses subsequently infect the epithelial cells of the mosquito gut and spread systemically in mosquito tissues, thereby enabling mosquitoes to transmit the viruses (Cheng et al., 2016). When mosquitoes feed on a viremic host, the viruses in the host blood are ingested into the intricate ecological gut environment, where abundant commensal microbes reside (Ramirez et al., 2012, 2014). These microbes may regulate arboviral replication in the gut epithelia, and consequently transmission by mosquitoes.

The composition of gut microbial flora varies significantly among individuals (Osei-Poku et al., 2012). Recent accumulating evidence indicates that the commensal microbiome in the gut lumen plays pleiotropic roles in host-pathogen interplay. The mammalian intestinal commensal bacteria can hinder pathogen infections by competing for nutrients, producing inhibitory metabolites, and regulating antiviral immunity (Abt and Pamer, 2014; Belkaid and Hand, 2014). Nonetheless, lipopolysaccharides produced by the intestinal microbiota promote the association of poliovirus and reovirus with host cells, their replication, and systemic pathogenesis (Kuss et al., 2011). In addition, lipopolysaccharides of the intestinal microbiota can bind to a mouse retrovirus and promote viral transmission by inducing a TLR4-dependent immune evasion pathway (Kane et al., 2011). In mosquitoes, the gut microbiome can reduce the prevalence and number of *Plasmodium* via producing anti-parasitic effectors in *Anopheles gambiae* (Ramirez et al., 2014). Gut commensal bacteria limit viral infections by priming the host immune surveillance and secreting microbial metabolites (Hegde et al., 2015; Jupatanakul et al., 2014). Nonetheless, a recent study demonstrated that a *Talaromyces* fungus in the *Aedes aegypti* gut increased the mosquitoes' permissiveness to dengue virus (DENV) infection by modulating the digestive

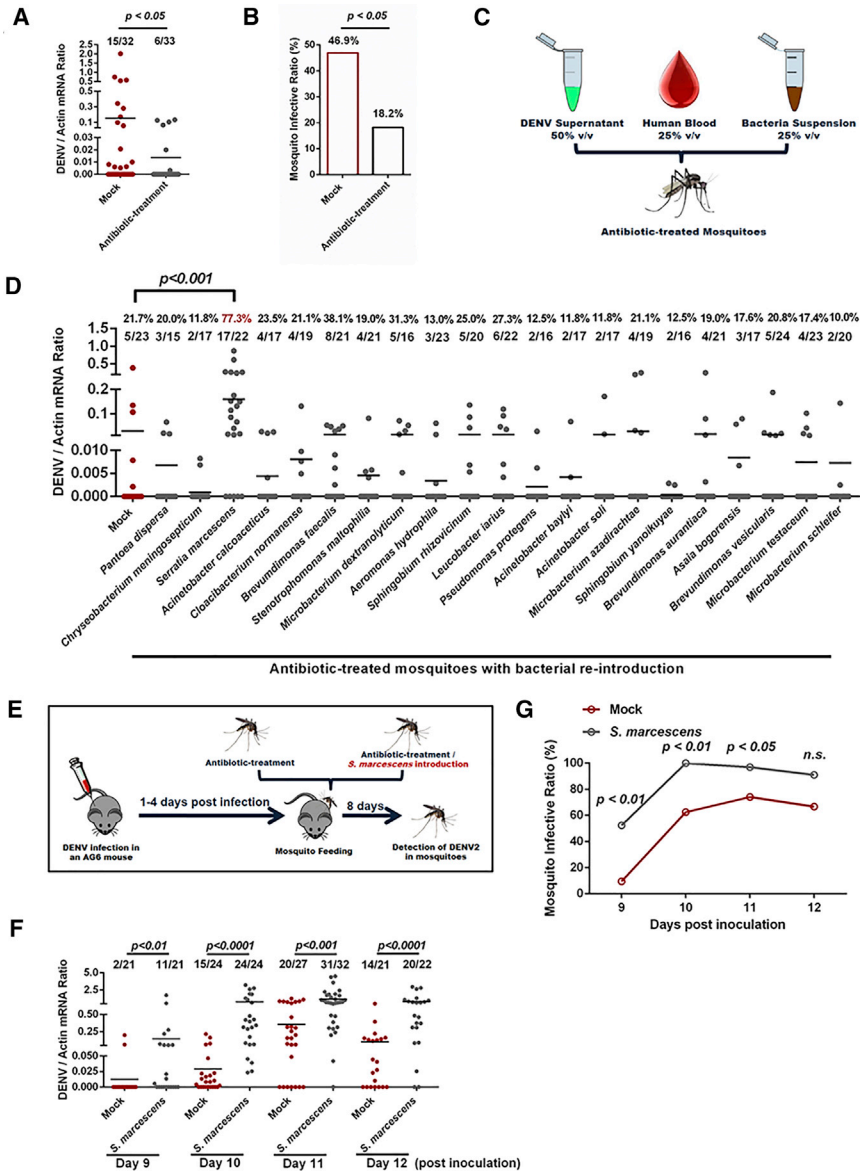

Figure 1. *S. marcescens* Facilitates Arboviral Infection of Mosquitoes

(A and B) Assessment of the antibiotic-mediated regulation of vector susceptibility to DENV of the *A. aegypti* Rockefeller strain. The *A. aegypti* Rockefeller strain was treated with antibiotics daily for 5 days. Mosquitoes with or without antibiotic treatment were fed a mixture containing human blood (50% v/v) and supernatant from DENV-2-infected Vero cells (50% v/v) via an *in vitro* blood feeding system.

(C and D) Determining the role of commensal bacteria in DENV infection of *A. aegypti*. (C) Schematic representation of the study design. A mixture containing human blood (25% v/v), the commensal bacteria suspension (25% v/v), and supernatant from DENV-2-infected Vero cells (50% v/v) was used to feed the antibiotic-treated Rockefeller *A. aegypti* via an *in vitro* blood feeding.

(E–G) Gut colonization of *S. marcescens* promotes DENV acquisition from infected mice. (E) Schematic representation of the study design. An AG6 mouse was intraperitoneally infected with 1×10^5 PFU (plaque-forming units) of DENV-2. The mosquitoes fed on the same infected mouse, enabling them to acquire equal amounts of viruses.

(F and G) *S. marcescens* colonization enhanced the DENV infectivity (F) and prevalence (G) of *A. aegypti*.

(A, D, and F) The DENV-2 NGC strain was used for mosquito oral infection. Mosquito infectivity was determined by qPCR at 8 days post blood meal. The number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. A non-parametric Mann-Whitney test was used for the statistical analysis.

(A and D) The final DENV titer was 1×10^5 PFU/mL for oral infection. The results were reproduced and combined from 2 independent experiments.

(B and D) The percentage data at the top of each column are represented as the ratio of mosquito

(B, D, and G) Differences in the infectivity ratio were compared using Fisher's exact test.

(E–G) The data represent the DENV infectivity and prevalence of mosquitoes biting an AG6 mouse. The experiment was repeated with four AG6 mice with similar results.

enzymes and trypsin activity of the mosquito gut (Anglero-Rodriguez et al., 2017), suggesting complicated roles for the gut commensal microbiome in arboviral infection and transmission. In this study, we identify a gut bacterium, *Serratia marcescens*, that has a role in promoting arboviral infection of mosquitoes. *S. marcescens* facilitates arboviral infection through one of its secreted proteins, *Sm*Enhancin, which digests membrane-bound mucins on the mosquito gut epithelia to enhance viral dissemination across the gut barrier.

RESULTS

S. marcescens Facilitates Arboviral Infection of Mosquitoes

The *A. aegypti* Rockefeller strain, which is a well-established laboratory-adapted mosquito strain, showed a high DENV load and prevalence after infection via blood meal (Sim et al., 2013). However, removal of the gut bacteria through oral treatment with antibiotics (Figure S1A) resulted in a 10-fold decrease in DENV infectivity (Figure 1A) and 2- to 3-fold decrease in viral prevalence in mosquitoes (Figure 1B), suggesting that some bacteria in the gut of the Rockefeller strain might facilitate viral infection. To identify the specific bacteria, we isolated the cultivable bacteria from the gut using four different broths. Consequently, 21 bacterial species were identified from the midgut of the Rockefeller *A. aegypti* mosquitoes (Table S1). Next, we investigated the role of these commensal bacteria in DENV infection of *A. aegypti* (Figure 1C). The DENV load of the fed mosquitoes was determined 8 days post oral infection. Oral introduction of *S. marcescens* alone largely enhanced the infectivity and prevalence of DENV (Figure 1D) in the antibiotic-treated mosquitoes. This was readily reproduced in Zika virus (ZIKV) and Sindbis virus

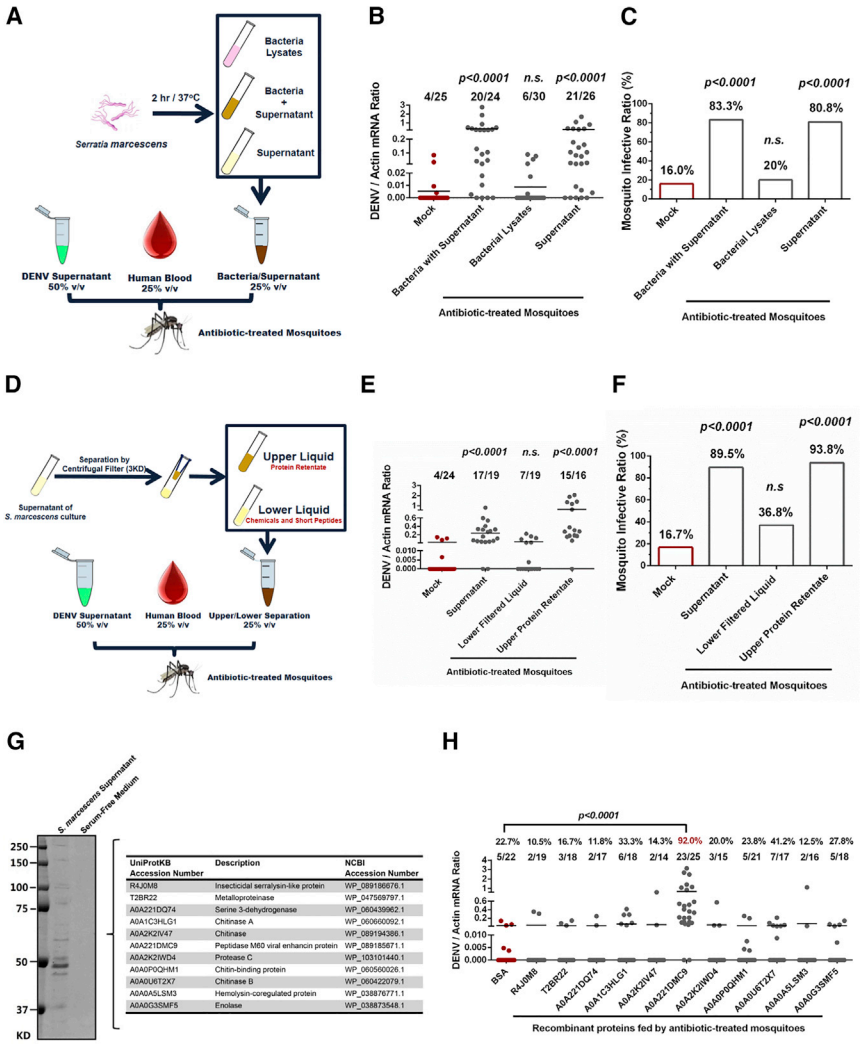

Figure 2. Identification of *S. marcescens* Secreted Effector Facilitating DENV Infection of *A. aegypti*

(A–C) *S. marcescens* secreted factor(s) enhanced DENV infection in *A. aegypti*. (A) Schematic representation of the study design. The *S. marcescens* suspension was separated into bacterial cells and culture supernatant by centrifugation. Either *S. marcescens* cell lysates or the culture supernatant (25% v/v) mixed with human blood (25% v/v) and supernatant from DENV-2-infected Vero cells (50% v/v) was used for oral feeding by the antibiotic-treated Rockefeller mosquitoes. Mosquitoes fed fresh medium or the bacterial suspension served as negative or positive controls, respectively.

(D–F) *S. marcescens* secreted protein(s) caused enhancement of DENV infection in *A. aegypti*. (D) Schematic representation of the study design. We mixed either the retentate (proteins) or the lower liquid (chemicals and short peptides) (25% v/v) with human blood (25% v/v) and DENV supernatant (50% v/v) for mosquito oral infection.

(G and H) Identification of *S. marcescens* Enhancin as the effector that promotes DENV infection. (G) The protein description was obtained from the UniProt and NCBI databases. The identified *S. marcescens* proteins were expressed and purified from *E. coli* cells. (H) A total of 10 μg of purified recombinant protein was mixed with fresh human blood (50% v/v) and DENV-2 supernatant (50% v/v) to orally infect the antibiotic-treated Rockefeller mosquitoes.

(B, E, and H) A total of 1 × 10^5 PFU/mL of the DENV-2 NGC strain was used for mosquito oral infection. Mosquito infectivity was determined by qPCR at day 8 post blood meal. The number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. A non-parametric Mann-Whitney test was used for statistical analysis.

(C, F, and H) The percentage data at the top of each column are represented as the ratio of mosquito infection. Differences in the infectivity ratios were compared using Fisher's exact test.

(B, C, and E–H) The results were reproduced and combined from two independent experiments.

(SINV) infection (Figures S1B–S1E), revealing that *S. marcescens* may be a bacterium regulating the susceptibility of the Rockefeller strain to arboviruses.

Next, we validated the role of *S. marcescens* in DENV infection using an *in vivo* animal feeding model. Type I/II interferon receptor-deficient (*ifnagr*$^{-/-}$) C57BL/6 (AG6) mice, which are an established mammalian model for DENV infection (Liu et al., 2016), were intraperitoneally infected with DENV-2 (Figure 1E). Similar to human infection, DENV replication in the AG6 mice resulted in a high viremia (Figure S1F). The infected mice were subjected to daily biting for 15 min on days 1–4 post infection, by antibiotic-treated Rockefeller *A. aegypti* reconstituted orally with or without *S. marcescens* (Figure 1E). The DENV prevalence and viral loads were higher in the *S. marcescens*-reconstituted than in the non-reconstituted mosquitoes (Figures 1F and 1G). These results indicate that *S. marcescens* enhances the mosquitoes' susceptibility to arboviral infection.

S. marcescens Facilitates Arboviral Infection via Its Secreted Enhancin

We aimed to understand how *S. marcescens* helped arboviruses infect mosquitoes. Bacteria are known to exploit their effectors, such as cellular components, metabolites, or secreted proteins, for interactions with their hosts for colonization. To identify the bacterial effector(s) that influences arboviral infections, we established a protein-free *S. marcescens* culture system with a commercial serum-free medium. *S. marcescens* grew well in this medium (Figure S2A). In this experiment, we seeded 1 OD (optical density) *S. marcescens* cells into the serum-free medium and incubated for 2 hr at 37°C. The cell-free culture supernatant was collected by centrifugation and filtration through a 0.22 μm filter unit, whereas cell lysates were generated by sonication. Either the bacterial cell lysate or the culture supernatant mixed with human blood and DENV supernatant was used for oral feeding by antibiotic-treated mosquitoes (Figure 2A). Those

mosquitoes fed with fresh medium or the whole bacterial suspension served as negative or positive controls, respectively. Oral introduction of the culture supernatant but not the bacterial lysate resulted in significant enhancement of the DENV infectivity (Figure 2B) and prevalence (Figure 2C) in the mosquitoes, indicating that an extracellular effector(s) secreted by *S. marcescens* was responsible for increased DENV infectivity. Next, we investigated whether the effector(s) was a secreted protein, small peptide, lipid, polysaccharide, or other metabolite. Therefore, the culture supernatant was separated using a 3 kDa centrifugal filter. We mixed either the upper retentate (mainly proteins) or the lower liquid filtrate (small-molecule compounds and short peptides) with human blood and DENV supernatant for mosquito oral feeding (Figure 2D). Intriguingly, oral introduction of the retentate rather than the filtrate enhanced viral infectivity to a level similar to that of the whole culture supernatant (Figures 2E and 2F), indicating that the effector(s) might be a protein(s) secreted by *S. marcescens*. Subsequently, the protein components in the upper retentate were separated by SDS-PAGE and then identified by mass spectrometry (Figure 2G). The highly abundant secretable proteins (Figure 2G) were expressed and purified in an *E. coli* expression system (Figure S2B). Of all the proteins tested, only the peptidase M60 viral Enhancin significantly enhanced DENV infection of *A. aegypti* (Figure 2H). We named this protein as *S. marcescens* Enhancin (*Sm*Enhancin) for convenience throughout the study. *Sm*Enhancin facilitated the DENV infectivity (Figure 3A) and prevalence (Figure 3B) in *A. aegypti* in a dose-dependent manner. Thus, we have identified *Sm*Enhancin, which is a secreted *S. marcescens* protein, as the major effector that renders mosquitoes highly susceptible to arboviral infection.

Next, we generated a murine polyclonal antiserum against *Sm*Enhancin (Figure S3A). In agreement with the aforementioned results, gut colonization of *S. marcescens* boosted DENV infection of antibiotic-treated-mosquitoes, which was blocked by a polyclonal antiserum against *Sm*Enhancin (Figures 3C and 3D). To validate the role of *Sm*Enhancin in DENV infection, we knocked out the *SmEnhancin* gene from *S. marcescens* via homologous recombination using a suicide T vector pLP12 (Figures S3B and S3C). The mutant strain with the *SmEnhancin* deletion (Δ*Sm*Enhancin-*S. marcescens*) did not express *Sm*Enhancin in the culture medium (Figure 3E). Nonetheless, the *SmEnhancin* deletion did not alter bacterial *in vitro* growth (Figure 3F) or colonization of mosquito guts (Figure 3G). Notably, Δ*Sm*Enhancin-*S. marcescens* completely failed to enhance DENV infection (Figures 3H and 3I) in *A. aegypti*, demonstrating that the *S. marcescens*-mediated effect on DENV infectivity was solely attributed to *Sm*Enhancin. We also investigated the role of *Sm*Enhancin in other arboviral infections in mosquitoes. Oral introduction of purified recombinant *Sm*Enhancin enhanced ZIKV and SINV infections in *A. aegypti* (Figures S3D–S3G). Consistently, wild-type *S. marcescens* but not Δ*Sm*Enhancin-*S. marcescens* conferred the mosquitoes with greater susceptibility to ZIKV and SINV infection than the mock controls (Figure S3H), indicating that *Sm*Enhancin influences host physiology rather than acting on viruses *per se* to promote viral infection.

Since SINV is naturally transmitted by *Culex* mosquitoes, we assessed the role of *S. marcescens* in SINV infection of *Culex pipiens pallens*, with the same experimental procedures shown in Figure 1C. Intriguingly, oral introduction of *S. marcescens* did not alter the infectivity and prevalence of SINV in antibiotic-treated *C. pipiens pallens* (Figure S3I). Similarly, feeding *Culex* mosquitoes with purified *Sm*Enhancin did not have any effect on SINV infectivity (Figure S3J). These results suggest that the *S. marcescens*-mediated viral enhancement is specific to *Aedes* mosquitoes.

*Sm*Enhancin Facilitates Viral Infection by Digesting Mucins on the Mosquito Gut Epithelia

Enhancins, which were first identified in baculoviruses such as *Trichoplusia ni* granulovirus and *Mamestra configurata* nucleopolyhedrovirus, degrade the mucins of the insect peritrophic matrix and enable the viruses to easily penetrate the gut epithelia (Peng et al., 1999; Toprak et al., 2012; Wang and Granados, 1997). In addition, some bacterial species secrete Enhancin-like proteins (Fang et al., 2009; Parkhill et al., 2001). *Bacillus thuringiensis*, which belongs to the *Bacillus cereus* group, encodes an Enhancin-like peptide with 20%–30% identity to the baculoviral Enhancins. The *Bacillus* Enhancin digests the intestinal mucins of *T. ni* and *Helicoverpa armigera* larvae, resulting in destruction of the insect peritrophic matrix (Fang et al., 2009). Therefore, we speculated that *Sm*Enhancin might digest the mucin layer on the gut epithelia, thereby enhancing gut permeability to arboviruses in mosquitoes. To address this possibility, we identified 12 genes encoding mucin domains in the *A. aegypti* genome through sequence comparison (Figure 4A; Table S2). Of these genes, six *A. aegypti mucin* genes (*Aamucins*) were highly expressed in the mosquito midgut (Figure 4B). To investigate the roles of these six *Aamucins* in DENV infection, we individually silenced the genes with double-stranded RNA (dsRNA). Three days post dsRNA delivery, the mosquitoes were infected through an *in vitro* membrane blood meal. Knockdown of both *Aamucin-6* and *Aamucin-11* enhanced DENV infection (Figures 4C and 4D) in *A. aegypti*. Notably, these two genes encode the only two *Aa*mucins with a transmembrane region (Figure 4A), suggesting that the membrane-bound rather than the secreted *Aa*mucins may play a refractory role against DENV infection of mosquitoes via a blood meal. Next, we assessed whether *Aa*mucins were substrates of *Sm*Enhancin. *Aa*mucin-3 and *Aa*mucin-11, which represented the secreted and membrane-bound *Aa*mucins, respectively, were cloned and expressed in *Drosophila* S2 cells (Figure 4E). However, the recombinant *Aa*mucin-6 did not express in *Drosophila* S2 or mosquito C6/36 cells. Both *Aa*mucin-3 and *Aa*mucin-11 were digested completely by *Sm*Enhancin to smaller molecular weight forms (Figure 4E), which was consistent with a previous observation (Wang and Granados, 1997). To exclude the cleavage caused by non-specific effects, the heat-inactivated *Sm*Enhancin was then incubated with *Aa*mucins. The native *Sm*Enhancin served as a positive control. Heat inactivation of *Sm*Enhancin fully abolished the digestion of *Aa*mucins (Figure S4A). Accumulating evidence has suggested that the mucin-degrading enzyme activity of Enhancin is Zn^{2+} dependent (Luo et al., 2014; Wang and Granados, 1997). Incubation with EDTA inhibited the *Sm*Enhancin-mediated cleavage of *Aa*mucin-3 and *Aa*mucin-11, while supplementation of $ZnSO_4$ rescued the *Aa*mucin digestion (Figure S4B), further validating the specificity of *Aa*mucin cleavage

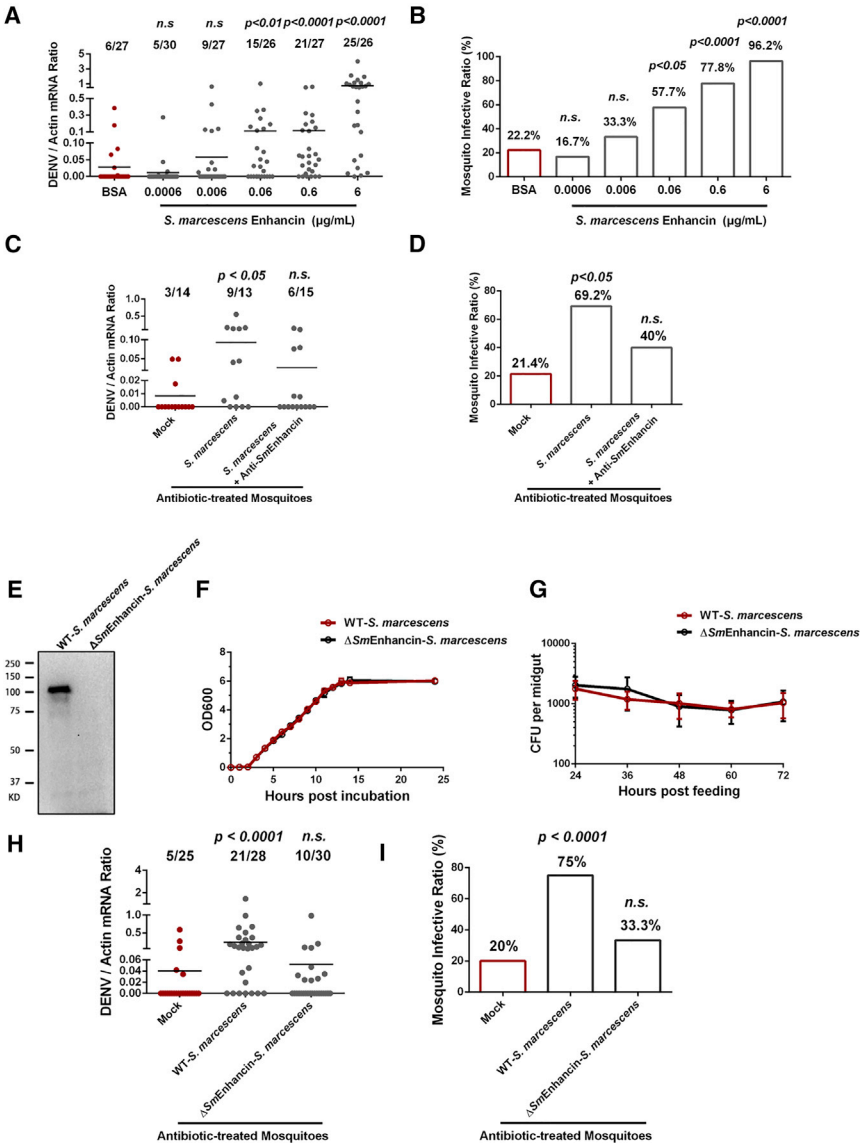

Figure 3. *S. marcescens* Facilitates Arboviral Infection via Its Secreted Enhancin

(A and B) Oral introduction of purified *Sm*Enhancin enhanced DENV infection. Serial concentrations of purified *Sm*Enhancin were mixed with fresh human blood (50% v/v) and DENV-2 supernatant (50% v/v) to orally infect the antibiotic-treated Rockefeller mosquitoes.

(C and D) Feeding the polyclonal antiserum against *Sm*Enhancin impaired the *S. marcescens*-mediated DENV susceptibility. A total of 10 μL murine *Sm*Enhancin antiserum was mixed with *S. marcescens* suspension. The bacterial suspension with or without the *Sm*Enhancin antiserum (25%) was mixed with fresh human blood (25% v/v) and DENV-2 supernatant (50% v/v) for oral feeding by the antibiotic-treated Rockefeller mosquitoes.

(E–I) The *S. marcescens* mutant strain with an *Sm*Enhancin deletion abolished the enhanced DENV infection in *A. aegypti*.

(E) Knockout of *SmEnhancin* aborted *Sm*Enhancin expression in *S. marcescens*. The supernatant was collected after 24 hr of culture for western blot detection by a murine *Sm*Enhancin antiserum.

(F and G) *SmEnhancin* deletion did not influence bacterial growth in LB broth (F) or colonization in the midgut of *A. aegypti* (G). The data are presented as the mean ± SEM. (F) *S. marcescens* was seeded in LB broth at an OD_{600} of 0.001. Bacterial growth was determined by OD measurement over a time course. (G) *S. marcescens* with OD_{600} of 1 was fed to the antibiotic-treated mosquitoes. Gut colonization by the *S. marcescens* cells was assessed by counting over a time course after oral bacterial feeding.

(H and I) Knockout of *SmEnhancin* in *S. marcescens* resulted in abolished enhancement of DENV infection in *A. aegypti*. Either the wild-type (WT)- or the Δ*Sm*Enhancin-*S. marcescens* suspension (25% v/v) was mixed with human blood (25% v/v) and supernatant from DENV-2-infected Vero cells (50% v/v) and used for oral feeding by the antibiotic-treated Rockefeller mosquitoes. Mosquitoes fed fresh broth served as the mock controls.

(A, C, and H) The DENV-2 NGC strain was used for mosquito oral infection. Mosquito infectivity was determined by qPCR at 8 days post blood meal. The number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. A non-parametric Mann-Whitney test was used for the statistical analysis.

(B, D, and I) The percentage data at the top of each column are represented as the ratio of mosquito infection. Differences in the infectivity ratio were compared using Fisher's exact test.

(A–D, H, and I) A total of 1×10^5 PFU/mL DENV-2 NGC strain was used for oral infection. The results were reproduced and combined from two independent experiments.

by *Sm*Enhancin. Heavy O-linked glycosylation is the main post-translational modification of mucins, which form a thick polysaccharide layer to separate the gut luminal contents from epithelia (Govindarajan et al., 2012). Enhancin-mediated digestion of mucins is dependent upon their polysaccharide chains (Noach et al., 2017). We next assessed the glycosylation of *Aa*mucins using O-glycosidase. Treatment with O-glycosidase reduced the molecular weights of both *Aa*mucin-3 and *Aa*mucin-11 (Figure S4C). Intriguingly, removal of polysaccharide modification by O-glycosidase fully abolished the cleavage of *Aa*mucin-3 and *Aa*mucin-11 by *Sm*Enhancin (Figure S4C), indicating the essential role of glycosylation in Enhancin-mediated mucin digestion. To further assess the role of *Sm*Enhancin in the *in vivo* condition, we then introduced *Sm*Enhancin into the *A. aegypti* midgut via a blood meal including DENV, with the same experimental procedures as in Figure 2H. Mosquitoes fed an equal amount of BSA with DENV and blood served as the mock controls. The mosquitoes were sectioned for periodic acid-Schiff staining at 2 hr post oral feeding (Godoy et al., 2015). Compared with the mock gut, the polysaccharide layer of mucin on the brush border (Godoy et al., 2015) was almost eliminated in the *Sm*Enhancin-treated gut (Figure 4F), further validating that *Sm*Enhancin facilitated viral infection by digesting mucins on the mosquito gut epithelia.

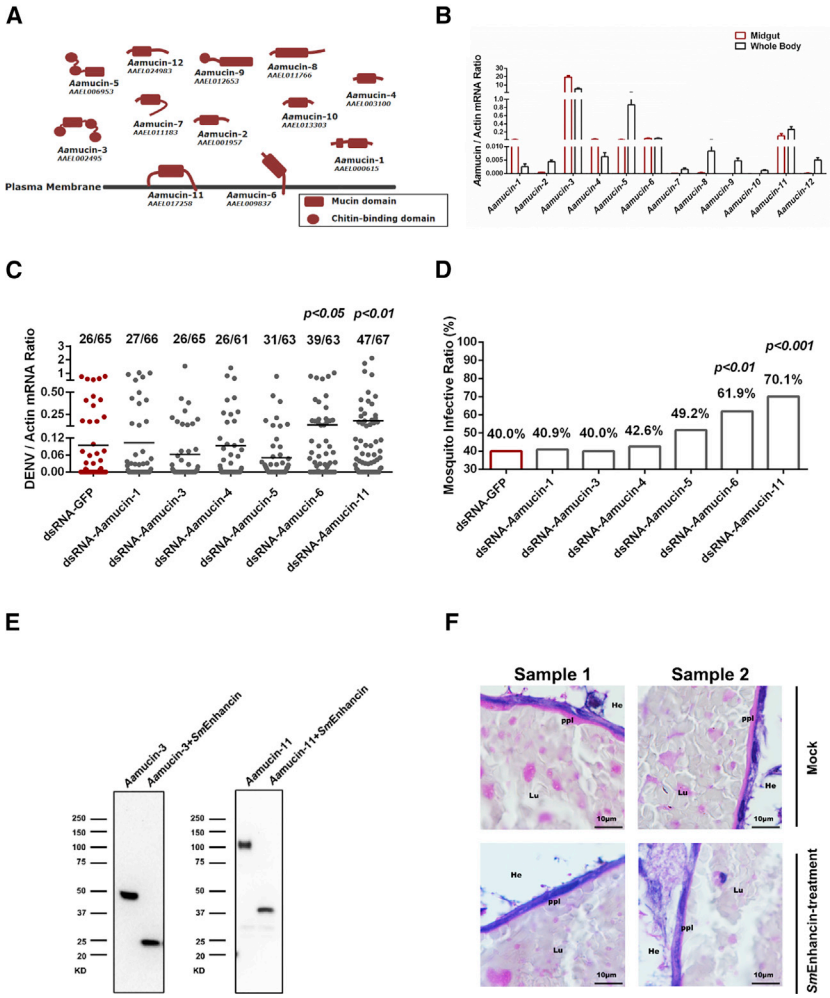

Figure 4. *Sm*Enhancin Facilitates Viral Infection by Digesting Mucins on the Mosquito Gut Epithelia

(A) Identification of 12 genes encoding mucin domains in *A. aegypti* via sequence comparison.

(B) Expression of *Aamucins* in the midgut and whole body of *A. aegypti*. The qPCR primers are summarized in Table S4. The gene quantities were normalized against *A. aegypti actin* (*AAEL011197*). The data are presented as the mean ± SEM.

(C and D) Determining the role of gut-expressed *Aamucins* during DENV infection of *A. aegypti*.

(C) A total of 1 × 10^5 PFU/mL DENV-2 NGC strain was used for the mosquito oral infection. Mosquito infectivity was determined by qPCR at 8 days post blood meal. The number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. A non-parametric Mann-Whitney test was used for the statistical analysis.

(D) The percentage data at the top of each column are represented as the ratio of mosquito infection. Differences in infectivity ratio were compared using Fisher's exact test.

(E) The *in vitro* digestion of *Aa*mucin by *Sm*Enhancin. *Aa*mucin-3 and *Aa*mucin-11 were expressed in *Drosophila* S2 cells. A total of 5 μg/mL purified recombinant *Sm*Enhancin was incubated with either the conditional supernatant with *Aa*mucin-3 or the cell lysates with *Aa*mucin-11 for 2 hr. The *Aa*mucins were detected by western blotting with an anti-V5 monoclonal antibody.

(F) *Sm*Enhancin-mediated digestion of mucin on the gut epithelium of *A. aegypti*. *Sm*Enhancin was mixed with fresh human blood (50% v/v) and the DENV supernatant (50% v/v) for oral infection of the antibiotic-treated mosquitoes. Mosquitoes feeding on an equal amount of BSA served as the mock controls. The mosquitoes were sectioned for periodic acid-Schiff (PAS) staining at 2 hr post oral feeding. Scale bar represents 10 μm. Lu, gut lumen; He, hemolymph; ppl, PAS-positive layer.

(B–F) The results were reproduced by at least two independent experiments.

Specificity of Bacterial Enhancins in Facilitating Viral Infection in Mosquitoes

Many bacterial species are able to secrete Enhancin-like proteins (Fang et al., 2009; Parkhill et al., 2001). According to the current literature, we identified seven additional gut commensal bacteria in mosquitoes belonging to *Bacillus*, *Enterobacter*, *Enterococcus*, and *Klebsiella* spp. that expressed *Enhancins* (Figure 5A). These bacterial Enhancins share 29%–37% identity with *Sm*Enhancin (Figure 5A). Next, we synthesized these *Enhancin* genes from seven gut commensal bacteria and subsequently expressed and purified these Enhancins in *E. coli* (Figure S5). In contrast to *Sm*Enhancin, oral introduction of the other seven bacterial Enhancins failed to enhance the DENV infectivity (Figure 5B) and prevalence (Figure 5C), indicating the specificity of *Sm*Enhancin in the enhancement of arboviral infection in mosquitoes. Next, we assessed the ability of these bacterial Enhancins to digest *Aa*mucins. The six bacterial Enhancins from *S. marcescens*, *B. cereus*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Klebsiella oxytoca*, and *Klebsiella michiganensis* degraded *Aa*mucin-3 (Figure 5D, left). Nonetheless, only *Sm*Enhancin was capable of efficiently cleaving *Aa*mucin-11 (Figure 5D, right). The aforementioned gene-silencing study indicated that only mucins with a transmembrane domain, such as *Aa*mucin-6 and *Aa*mucin-11, played refractory roles against arboviral infection (Figures 4C and 4D), suggesting that *Sm*Enhancin but not the other bacterial Enhancins specifically damages membrane-bound mucins, thereby facilitating arboviral infection in the mosquito gut epithelia.

S. marcescens is a bacterial species with many strains of varying virulence, morphology, and pathogenicity. For instance, some strains produce a characteristic red pigment and are less virulent than the non-pigmented strains (Kamble and Hiwarale, 2012). Therefore, we investigated whether the pivotal pro-arboviral feature of *S. marcescens* was associated with strain virulence and *SmEnhancin* expression level. In five *S. marcescens* strains collected from soil, water, patients, and field mosquitoes (Table S3), the *SmEnhancin* mRNA (Figure 5E) and protein (Figure 5F) were readily detectable. All five *S. marcescens* strains had an effect similar to that of the wild-type strain (isolated from the Rockefeller mosquitoes) on the DENV infectivity (Figure 5G) and prevalence (Figure 5H) in the antibiotic-treated

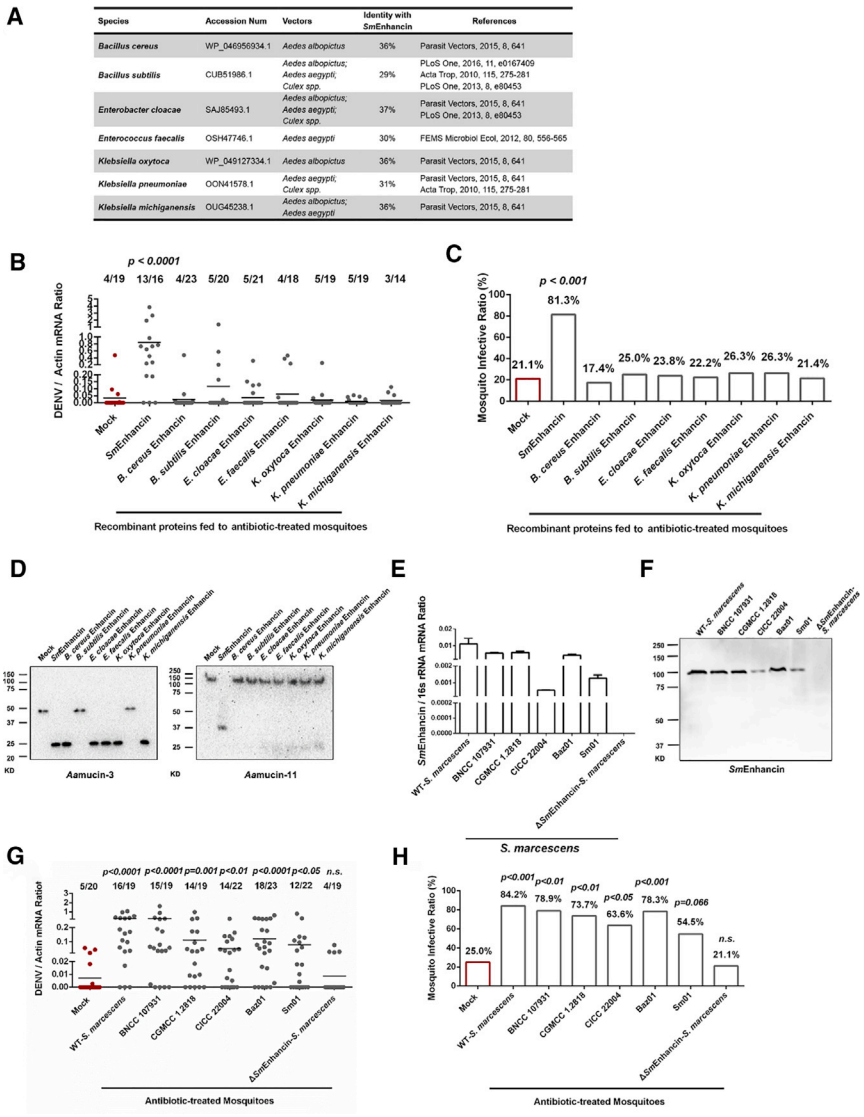

Figure 5. Specificity of Bacterial Enhancins in Facilitating DENV Infection in Mosquitoes

(A) Mosquito gut commensal bacteria with *Enhancins* expression.

(B and C) Specificity of bacterial Enhancins in DENV infection of *A. aegypti*. A total of 10 μg of each bacterial Enhancin was mixed with human blood (50% v/v) and DENV supernatant (50% v/v) to feed the antibiotic-treated Rockefeller *A. aegypti*. Eight days post blood meal, the DENV infectivity (B) and prevalence (C) in mosquitoes were determined by qPCR.

(D) *In vitro* digestion of *Aa*mucin by various bacterial Enhancins. *Aa*mucin-3 and *Aa*mucin-11 were expressed in *Drosophila* S2 cells. A total of 5 μg/mL recombinant *Sm*Enhancin was incubated with *Aa*mucins for 2 hr. The *Aa*mucins were detected by western blotting with an anti-V5 monoclonal antibody. The results were reproduced by 3 independent experiments.

(E and F) Expression of *Sm*Enhancin by various *S. marcescens* strains.

(E) *SmEnhancin* gene expression was detected by qPCR and normalized to the *S. marcescens* 16S rRNA. The data are presented as the mean ± SEM.

(F) The supernatant was collected after 24 hr of culture for western blot detection by a murine *Sm*Enhancin antibody.

(G and H) Assessing the role of various *S. marcescens* strains in DENV infection of *A. aegypti*. The *S. marcescens* suspensions (25% v/v) were mixed with fresh human blood (25% v/v) and DENV-2 supernatant (50% v/v) for oral feeding to the antibiotic-treated Rockefeller mosquitoes. Eight days post blood meal, the DENV infectivity (G) and prevalence (H) in mosquitoes were determined by qPCR.

(B and G) A total of 1×10^5 PFU/mL DENV-2 NGC strain was used for mosquito oral infection. The number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. A non-parametric Mann-Whitney test was used for the statistical analysis.

(C and H) The percentage data at the top of each column are represented as the ratio of mosquito infection. Differences in infectivity ratio were compared using Fisher's exact test.

(B, C, and E–H) The results were reproduced and combined from at least 2 independent experiments.

A. aegypti. These results demonstrate that the property of *S. marcescens* is strain independent.

S. marcescens Enhances the Vector Competence of Field Mosquitoes for Arboviruses

The capacity of mosquitoes to maintain and transmit arboviruses, defined as vector competence, varies dramatically with strain and species (Bennett et al., 2002). The aforementioned studies indicate that *S. marcescens* is a critical bacterium for regulating the mosquito susceptibility to arboviral infection. Indeed, *S. marcescens* is an indigenous gut commensal bacterium identified in field-caught mosquitoes frequently (Anglero-Rodriguez et al., 2017; Ramirez et al., 2012). We next assessed whether *S. marcescens*-mediated regulation of the gut microbial ecology might influence the vector competence of low-passage field-derived mosquitoes. Five low-passage field *Aedes* strains were exploited for the investigation, which were collected from Hainan (the *A. aegypti* Hainan strain); Tainan, Taiwan (the *A. aegypti* Taiwan Tainan strain); Jiangsu (the *Aedes albopictus* Jiangsu strain); Guangzhou (the *A. albopictus* Guangzhou strain); and Foshan (the *A. albopictus* Foshan strain). Both Hainan and Jiangsu are not dengue-prevalent regions (Gao et al., 2010; Lai et al., 2015). However, Guangzhou, Foshan, and Tainan are districts with recent dengue epidemics (Chuang et al., 2018; Wang et al., 2016; Xiao et al., 2016). In the *A. albopictus* Foshan, *A. albopictus* Guangzhou, and *A. aegypti* Taiwan Tainan strains, 8.3%–17.7% of the colonies of gut cultivable bacteria were *S. marcescens*. Nonetheless, *S. marcescens* was barely detectable from the guts of both the *A. aegypti* Hainan and the *A. albopictus* Jiangsu strains (Figure 6A). The *S. marcescens* inhabiting the mosquito gut may be acquired either from the natural environment or through maternal transmission. Indeed,

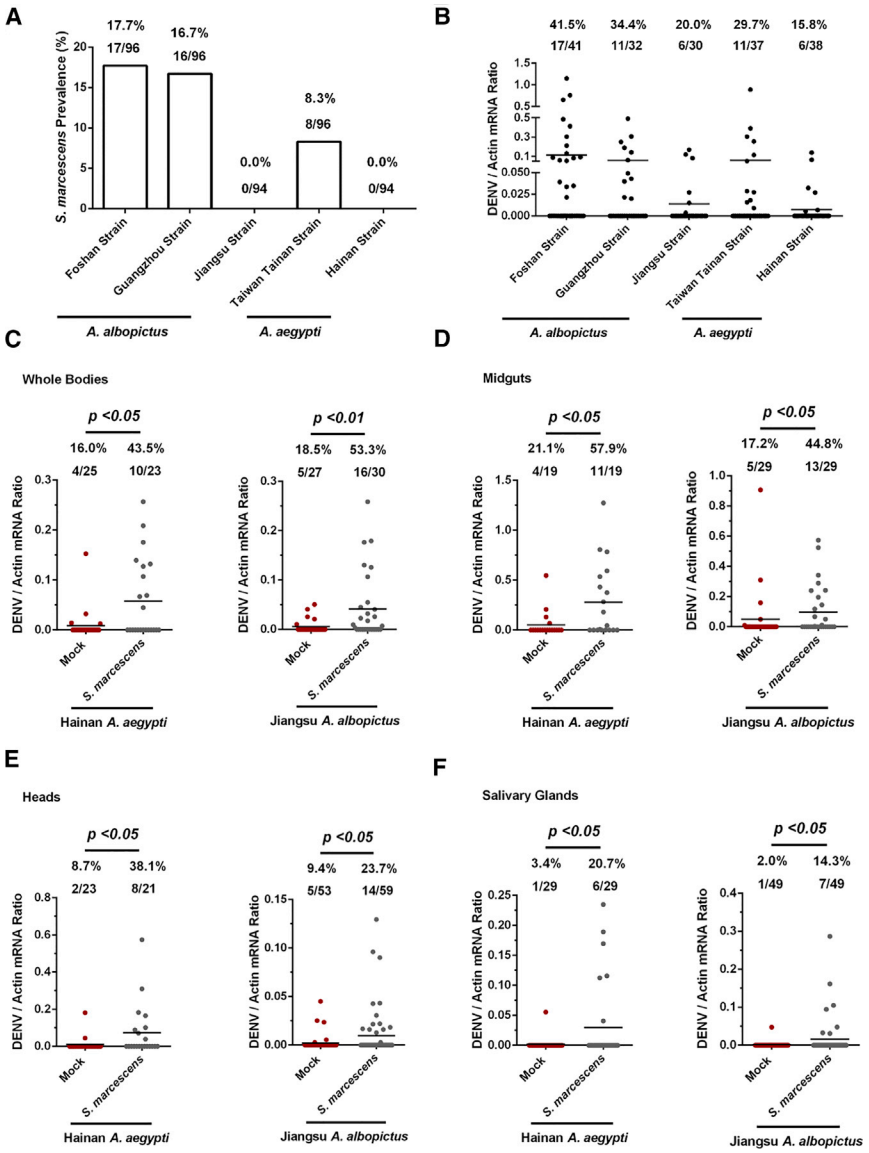

Figure 6. Gut Colonization by *S. marcescens* Regulates the Vector Competence of Field-Derived Mosquitoes

(A) Assessment of *S. marcescens* prevalence in five field-derived *Aedes* strains. Cultivable gut bacteria were isolated on LB plates for bacterial identification. The number of *S. marcescens* colonies relative to the total number of bacterial colonies is shown at the top of each column. The data for the upper colony numbers are represented as the percentage of *S. marcescens* colonies.

(B) Assessment of the susceptibility of various field mosquito strains to DENV infection. A mixture containing human blood (50% v/v) and supernatant from DENV-2-infected Vero cells (50% v/v) was used to feed the different mosquito strains via an *in vitro* blood feeding system.

(C–F) Gut colonization by *S. marcescens* regulates the vector competence of field-derived mosquitoes. Both the *A. aegypti* Hainan strain (left) and the *A. albopictus* Jiangsu strain (right) were used for the experiment. The midguts, heads, and salivary glands were dissected on days 7, 14, and 14 after oral feeding, respectively. The whole mosquitoes were evaluated at 8 days post blood meal. The viral loads were measured by qPCR. The DENV ratios of whole mosquitoes (C), midgut infection (D), head dissemination (E), and salivary gland infection (transmission) (F) were calculated by the positive number divided by the total number.

(B–F) A total of 1×10^5 PFU/mL DENV-2 NGC strain was used for mosquito oral infection. Gene quantities were normalized against *A. aegypti actin* (*AAEL011197*) or *A. albopictus actin* (*AALF010408*). The number of infected mosquitoes relative to the total number of mosquitoes is shown at the top of each column. The data for the upper mosquito numbers are represented as the percentage of mosquito infection. A non-parametric Mann-Whitney test was used for the statistical analysis. The results were reproduced and combined from at least two independent experiments.

S. marcescens can be vertically transmitted within a mosquito lineage (Wang et al., 2017). The amount of *Sm*Enhancin in the guts of field-derived Foshan, Guangzhou, and Taiwan Tainan mosquitoes was comparable to that of antibiotic-treated mosquitoes reconstituted with *S. marcescens*, as shown through immunoblotting (Figure S6A). Subsequently, we assessed the susceptibility of these field-derived mosquito strains to DENV infection. The three field-derived *Aedes* strains positive for *S. marcescens* from the dengue endemic regions presented with a higher DENV load and prevalence than the strains free of *S. marcescens* from non-endemic Hainan and Jiangsu (Figure 6B). Next, the two mosquito species from non-endemic regions were reared with sucrose meals containing *S. marcescens* for 5 days. Compared with the rare existence of *S. marcescens* in the non-endemic field mosquitoes, the *S. marcescens* abundance was significantly enhanced after the bacterial sucrose meal (Figure S6B). The mosquitoes were starved for 24 hr, and subsequently infected with DENV via a blood meal. The presence of abundant *S. marcescens* increased the ratios of DENV-positive whole mosquitoes (Figure 6C), midguts (Figure 6D), heads (Figure 6E), and salivary glands (Figure 6F) in both of the field stains, indicating a potential association between the gut-inhabiting *S. marcescens* and the dengue prevalence in *Aedes* mosquitoes.

DISCUSSION

Our recent work reveals the intricate interplay between disease vector mosquitoes and the gut microbiome (Pang et al., 2016; Xiao et al., 2017). In the current study, we demonstrate that the gut commensal bacterium *S. marcescens* alone is sufficient to enhance vectorial susceptibility to arboviruses in the *Aedes* mosquitoes. The bacterium increases mosquito permissiveness to viruses through *Sm*Enhancin, which digests the membrane-bound epithelial mucins and thus disrupts the physical barrier to the viruses. Gut colonization of

S. marcescens by oral introduction renders the field-derived mosquitoes that lack this bacterium highly susceptible to arboviruses. Indeed, *S. marcescens* is a commensal bacterium that is frequently identified in the guts of lab-reared and field-caught mosquitoes (Gusmao et al., 2010; Ramirez et al., 2012). In addition to inhabiting the gut lumen, *S. marcescens* also colonizes the diverticulum of *A. aegypti* (Gusmão et al., 2007) and the ovaries of *Anopheles stephensi* (Wang et al., 2017), suggesting its close association with mosquito physiology. As a gut commensal bacterium, *Serratia* spp. have been found to influence the invasion of intestinal pathogens. The diversity among *S. marcescens* strains defines the *Plasmodium* transmission capacity by mosquitoes (Bando et al., 2013). *S. marcescens* may translocate from the midgut to the hemocoel, thereby accelerating mosquito mortality from an insect pathogenic fungus, *Beauveria bassiana* (Wei et al., 2017). In addition, *Serratia odorifera*, a gut bacterium isolated from the field *A. aegypti* mosquitoes, increases mosquito susceptibility to DENV-2 infection. The underlying mechanism might involve specific interactions between a secreted *S. odorifera* polypeptide, the mosquito prohibitin, and DENV-2 virions (Apte-Deshpande et al., 2012). The effect of *S. odorifera* on DENV-2 infection is thus unlikely to be applicable to other arboviruses.

In the present study, we reveal a mechanism by which a *Serratia* sp. influences a broad range of arboviral infections. *S. marcescens* enhances permissiveness of the *Aedes* mosquitoes to arboviruses (Gao, 2018) through the secretion of *Sm*Enhancin, which degrades membrane-bound mucins on the gut epithelium, thereby facilitating arboviral infection in mosquitoes. Indeed, the Enhancin protein was primarily identified from *Baculoviridae*, a family of arthropod-specific viruses (Peng et al., 1999; Wang and Granados, 1997). Intriguingly, the homologs of baculoviral Enhancin, which presents a conserved role in the digestion of intestinal mucins, are widely expressed in some bacteria, such as species in the *Bacillus*, *Yersinia*, and *Serratia* genera (Fang et al., 2009; Parkhill et al., 2001). For example, the *B. thuringiensis* Enhancin digests the intestinal mucins of *T. ni* and *H. armigera* larvae (Fang et al., 2009). In contrast to the constant existence of the peritrophic matrix in non-hematophagous insects, the formation of the peritrophic matrix in the mosquito gut is strictly induced in response to the blood meal (Kumar et al., 2010). Intriguingly, the absence of the peritrophic matrix had no effect on the DENV dissemination in *A. aegypti* (Kato et al., 2008). It is possible that DENV invasion into the gut epithelium might be completed before the formation of the peritrophic matrix induced by the blood meal. In agreement with this notion, dsRNA-mediated knockdown of secreted *Aamucins*, which are essential components of the peritrophic matrix in mosquitoes (Devenport et al., 2006), did not alter DENV infection in *A. aegypti*. We observed similar results for several secreted mucins. Although the mRNA of the secreted mucins is expressed prior to blood feeding, the protein production of the secreted mucins in peritrophic matrix is induced by the blood meal (Devenport et al., 2006; Hegedus et al., 2009). By contrast, the membrane-bound *Aa*mucins form a constitutive highly glycosylated barrier to separate the gut lumen materials from epithelial cells (Shen et al., 1999). Given that the knockdown of membrane-bound *Aamucin-6* and *Aamucin-11* enhanced DENV infection in mosquitoes, we speculate that the glycosylated layer of membrane-bound *Aa*mucins, but not the peritrophic matrix with secreted *Aa*mucins, constitutes a physical barrier against viral entry in mosquitoes. In addition, we found that multiple gut commensal bacteria in mosquitoes express Enhancin-like proteins. Although these Enhancins are generally able to digest a secreted *Aa*mucin, only *Sm*Enhancin cleaves membrane-bound *Aa*mucin-11. This observation may underlie the unique role of *S. marcescens*, but not other gut commensal bacteria, in facilitating arboviral infection in mosquitoes.

Our study indicates that *S. marcescens*-mediated SINV enhancement is specific to the *Aedes* spp., rather than the *Culex* mosquitoes. The *Sm*Enhancin-mediated enhancement of mosquito susceptibility is attributed to the digestion of mucins on the gut epithelium. Indeed, mucins are a group of proteins characterized by multiple O-glycosylated regions referred to as the mucin domain (Toprak et al., 2012). Mucins present high diversity not only in their amino acid composition, but also their glycosylation patterns (Strous and Dekker, 1992). The distinct sequence and modification patterns may result in a strict specificity of mucin cleavage by Enhancins, which is evidenced by the resistance of *Aa*mucin-11 to Enhancins of other bacterial species than *S. marcescens*. We speculate that *Sm*Enhancin may be unable to digest the *Culex* mucins that play resistant roles against arbovirus infection, thereby exerting no effect on SINV infection in the *Culex* mosquitoes.

The three field-derived *Aedes* strains positive for *S. marcescens* from the dengue endemic regions were more susceptible to DENV than the strains free of *S. marcescens* from non-endemic areas. Given that the gut-inhabiting *S. marcescens* rendered mosquitoes permissive to arboviruses, we hypothesize that the existence of *S. marcescens* might be correlated to the virus presence in field mosquitoes. Indeed, the previous literature suggests a potential relevance of *S. marcescens* to dengue prevalence. The presence of *S. marcescens* was identified in *Aedes* mosquitoes by 16S rRNA sequencing in Brazil, Panama, and Guangzhou, China, where dengue is endemic (David et al., 2016; Ramirez et al., 2012; Wang et al., 2018). A *Serratia* species, *Serratia nematodiphila*, was also identified in the *Aedes* mosquito gut in a dengue endemic region of Thailand (Thongsripong et al., 2018). *S. nematodiphila* encodes an Enhancin that shares 100% sequence coverage and 97% identity with *Sm*Enhancin, suggesting a similar role of *S. nematodiphila* Enhancin in arbovirus infection in mosquitoes. These pieces of evidence suggest that *S. marcescens* is likely to be associated with dengue prevalence. Our studies suggest that eradication of *S. marcescens* in the *Aedes* populations might reduce DENV or other arboviral transmission and prevalence in nature. This could be feasible through either spreading *S. marcescens*-specific phages (Teng et al., 2018) or utilizing specific bactericidal materials against *S. marcescens* growth in dengue endemic environments (Phadke et al., 2002).

In this study, we reveal that a specific gut commensal bacterium, *S. marcescens*, promotes mosquito permissiveness to arboviruses and defines the underlying mechanism. In addition to insects, mammalian digestive tracts are also covered with a heavily glycosylated mucous layer that prevents pathogens from accessing the intestinal epithelia (Strous and Dekker, 1992). Enhancin-like genes are encoded by many intestinal

commensal bacteria in human and mouse. It is therefore plausible that Enhancin may also regulate the infection of enteric pathogens in mammalian hosts. Understanding the molecular mechanisms governing gut microbiota-virus interactions may lead toward prospective strategies against viral infection and transmission.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Acquisition of Human Blood
  - Mice
  - Mosquitoes
  - Viruses
  - Cells
  - Bacteria
- METHOD DETAILS
  - Isolation and Characterization of Mosquito Midgut Bacteria
  - Mass Spectrometry
  - Protein Expression and Purification
  - Antiserum
  - Generation of Antibiotic-Treated Mosquitoes and Re-introduction of Bacteria
  - Membrane Blood Feeding
  - Mosquitoes Feeding on Mice
  - Viral Genome Quantitation by TaqMan qPCR
  - Virus Titration in Mouse Blood
  - Mucin Digestion Assay
  - Gene Silencing in Mosquitoes
  - Measurement of the Gut Bacteria by 16S rDNA qPCR
  - Construction of the *SmEnhancin* Knockout *S. marcescens* Mutant Strain
  - Mucin Staining
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and four tables and can be found with this article online at https://doi.org/10.1016/j.chom.2018.11.004.

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AUTHOR CONTRIBUTIONS

G.C. designed the experiments and wrote the manuscript; P. Wu performed the majority of the experiments and analyzed the data. P.S. helped with mosquito dissection. K.N. helped with animal experiments. Y.Z., M.S., C.X., and H.L. facilitated the experiment on mosquito infection. Q.L., T.Z., X.C., and H.Z. provided the field-derived mosquitoes. P. Wang contributed to experimental suggestions and strengthened the writing of the manuscript. All authors reviewed, critiqued, and provided comments on the text.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-V5 HRP antibody	Thermo Fisher Scientific	Cat# R961-25; RRID: AB_2556565
Bacterial and Virus Strains		
S. marcescens BNCC107931	Be Na Culture Collection	Cat#BNCC107931
S. marcescens CGMCC1.2818	China General Microbiological Culture Collection Center	Cat#1.2818
S. marcescens CICC22004	China Center of Industrial Culture Collection	Cat#22004
S. marcescens Sm01	this paper	N/A
S. marcescens Baz01	this paper	N/A
Δ*Sm*Enhancin-*S. marcescens*	this paper	N/A
DENV-2 (New Guinea C strain, *AF038403.1*)	Zhu et al. (2017)	N/A
SINV (*U90536.1*)	Zhu et al. (2017)	N/A
ZIKV (GZ01 strain, *KU820898.1*)	Zhu et al. (2017)	N/A
E. coli β2163	Luo et al. (2015)	N/A
Chemicals, Peptides, and Recombinant Proteins		
Penicillin-Streptomycin	Thermo Fisher Scientific	Cat#15070-063
O-glycosidase	New England Biolabs	Cat#P0733
Effectene transfection reagent	Qiagen	Cat#301425
TALON metal affinity resin	Clontech	Cat#635501
Antibiotic-antimycotic	Invitrogen	Cat#15240-062
SeaPlaque	Lonza	Cat#50101
Critical Commercial Assays		
Multisource RNA miniprep kit	Axygen	Cat#AP-MN-MS-RNA-250
Periodic acid Schiff (PAS) stain kit (Mucin stain)	Abcam	Cat#ab150680
iScript cDNA synthesis kit	Bio-Rad	Cat#170-8890
MEGAscript T7 transcription kit	Invitrogen	Cat#AM1334
Bacteria DNA kit	TIANamp	Cat#dp302-02
Deposited Data		
Raw and analyzed data	This paper; Mendeley Data	https://doi.org/10.17632/65fjp9yymt.1
Experimental Models: Cell Lines		
Vero cell	ATCC	Cat#CCL-81; RRID: CVCL_0059
Drosophila S2 cell	Invitrogen	Cat#R69007
Experimental Models: Organisms/Strains		
Mice: AG6 (C57BL/6 mice deficient in type I and II interferon (IFN) receptors)	Institute Pasteur of Shanghai (Chinese Academy of Sciences)	N/A
Mice: BALB/c	Vital River Laboratories, Beijing, China	Cat#211
Mosquitoes: *Aedes aegypti* (the Rockefeller strain)	Zhu et al. (2017)	N/A
Mosquitoes: *Aedes aegypti* (the Taiwan Tainan and Hainan strains), *Aedes albopictus* (the Guangzhou, Foshan and Jiangsu strains) and *Culex pipiens pallens* (the Beijing strain)	this paper	N/A
Oligonucleotides		
See Table S4 for primers	this paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
pLP12	Luo et al. (2015)	N/A
pLP12-*enh*	this paper	N/A
pMT/BiP/V5-His A vector	Invitrogen	Cat#V4130-20
Software and Algorithms		
Prism 6	GraphPad Software	https://www.graphpad.com/
Blast+	N/A	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Image Lab 4.0	Bio-Rad	www.bio-rad.com/zh-cn/product/image-lab-software?ID=KRE6P5E8Z
Other		
Mouse polyclonal antiserum of *Sm*Enhancin	This paper	N/A
Ultra-15 centrifugal filter concentrator	Amicon	Cat#UFC900396
Filter unit (0.22 μm)	Millipore	Cat#SLGP033RB
VP-SFM	Gibco	Cat#11681020
DMEM	Gibco	Cat#11965118
Schneider's *Drosophila* Medium	Gibco	Cat#21720024
Liver broth	Oxoid	Cat#CM0077
Yeast extract	Oxoid	Cat#LP0021
Fetal bovine serum	Gibco	Cat#10099141
GlutaMAX	Gibco	Cat#35050061
Protein assay dye	Bio-Rad	Cat#500-0006
Hemotek membrane feeding system	Hemotek	Cat#6W1

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Gong Cheng (gongcheng@mail.tsinghua.edu.cn).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Acquisition of Human Blood

The blood used for mosquito feeding was taken from healthy volunteers (41 males, 40 females, 20-30 years of age) who were provided with written informed consent. The collection of human blood was conducted with the approval of the local ethics committee at Tsinghua University.

Mice

The animal work was performed in accordance with the guidelines of the Experimental Animal Welfare and Ethics Committee of Tsinghua University. C57BL/6 mice deficient in type I and II interferon (IFN) receptors (AG6 mice) were donated from Institute Pasteur of Shanghai (Chinese Academy of Sciences) and maintained at Tsinghua University. Eight-week-old male AG6 mice were used for virus transmission assay. Six-week-old female BALB/c mice, purchased from Vital River Laboratories in China, were used for antisera generation. All mice were housed in ventilated cages (maxima six mice per cage) in a specific-pathogen-free barrier facility at Tsinghua University. The mice were maintained on a 12/12-hour light/dark cycle, 22-26°C with sterile pellet food and water *ad libitum*.

Mosquitoes

The *A. aegypti* (the Rockefeller, Taiwan Tainan and Hainan strains), *A. albopictus* (the Guangzhou, Foshan and Jiangsu strains) and *C. pipiens pallens* (the Beijing strain) mosquitoes were maintained in a low-temperature, illuminated incubator (Model 818, Thermo Electron Corporation) in a biosafety level 2 (BSL2) facility at Tsinghua University. The rearing conditions were 28°C, 80% humidity with 12/12-hour light/dark cycle. Mosquito larvae were raised with standard diet, which consisted of a 0.1% solution of three parts liver broth (CM0077, Oxoid) and two parts yeast extract (LP0021, Oxoid). Male and female adult mosquitoes were maintained in a cage with unlimited access to water and sugar (raisin for standard diet). Female mosquitoes aged 7-10 days after eclosion were subjected for further investigation.

Viruses

DENV-2 (New Guinea C strain, *AF038403.1*), SINV (*U90536.1*) and ZIKV (GZ01 strain, *KU820898.1*) were grown in Vero cells at 37°C with 5% CO_2 in VP-SFM medium (11681020, Gibco) for blood meals. The DENV, SINV and ZIKV were titrated by a plaque assay.

Cells

Vero cells, which were originally isolated from a female *Cercopithecus aethiops* kidney, were purchased from ATCC. Vero cells were cultured at 37°C with 5% CO_2 in DMEM (11965118, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (10099141, Gibco), 2 mM GlutaMAX (35050061, Gibco) and 1% antibiotic–antimycotic (15240-062, Invitrogen). *Drosophila* S2 cells, which were originally isolated from male *Drosophila melanogaster* embryos, were purchased from Invitrogen. *Drosophila* S2 cells were cultured at 28°C in Schneider's *Drosophila* medium (21720024, Gibco) with 10% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic. The cell lines purchased from ATCC and Invitrogen are accompanied by authentication documents verifying the identity according to their short tandem repeat profiles and that they are mycoplasma free.

Bacteria

The *S. marcescens* BNCC107931 strain was purchased from the Be Na Culture Collection, *S. marcescens* CGMCC1.2818 strain was purchased from the China General Microbiological Culture Collection Center, *S. marcescens* CICC22004 strain was purchased from the China Center of Industrial Culture Collection, *S. marcescens* Sm01 strain was donated by Professor Sibao Wang from the Chinese Academy Sciences, and *S. marcescens* Baz01 strain was isolated from the field-derived *A. aegypti*. Single colonies of *S. marcescens* were inoculated into LB broth and incubated with shaking at 37°C. After overnight incubation, *S. marcescens* was centrifuged and the bacterial pellet was resuspended as 1:10 sub-cultures into fresh VP-SFM medium, and cultured for an extra 3-4 hrs for blood feeding. For measurement of bacteria growth curve, the overnight culture was re-inoculated to LB broth at an OD_{600} of 0.001.

METHOD DETAILS

Isolation and Characterization of Mosquito Midgut Bacteria

The mosquitoes were surface-sterilized in 75% ethanol and then rinsed with PBS buffer twice for midgut dissection. The midguts were ground in 200 μl sterile PBS for bacterial isolation. The bacteria isolations were categorized via the 16S rRNA gene sequencing and comparisons (Dinparast Djadid et al., 2011; Ramirez et al., 2012). Culture condition of each bacteria isolation is summarized in Table S1.

Mass Spectrometry

S. marcescens was washed three times with PBS and suspended in 10 ml of VP-SFM medium. After incubation for 2 hrs at 37°C, the bacteria were removed by centrifugation and filtration using a 0.22 μm filter unit (SLGP033RB, Millipore). The protein component of bacteria supernatant was concentrated using Ultra-15 centrifugal filter concentrator (UFC900396, Amicon) and subjected to SDS-PAGE. Fresh VP-SFM medium was used as a negative control. The whole gel lane was excised and analyzed by liquid chromatography-mass spectrometry (LC-MS) at the Protein Chemistry Technology Core, Tsinghua University. The MS readouts were searched against the protein sequence database of *S. marcescens* at the UniProt Database using the Mascot software. The secreted proteins with a score $\geq$150 were included in the subsequent investigation.

Protein Expression and Purification

The genes identified from mass spectrometry were amplified from *S. marcescens* cDNA and cloned into the pET-28a (+) expression vector. Recombinant proteins were induced to express in the *E. coli* BL21 DE3 strain using 500 μM IPTG for inclusion bodies and 100 μM IPTG for protein expressed at soluble form. The metalloproteinases (R4J0M8, T2BR22 and A0A221DQ74) were induced by 500 μM IPTG for 5 hrs at 37°C to generate inclusion bodies. The inclusion bodies were washed with lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 5% Triton X-100 and 1 mM DTT) for 3 times and 2 M urea for 1 time, and then dissolved in 8 M urea and dialyzed overnight in renaturation buffer (Zhang et al., 2012). The activity of re-natured metalloproteinases was validated by a casein degradation assay (Zhang et al., 2012). The other proteins were induced by 100 μM IPTG overnight at 16°C to generate soluble forms and purified with the TALON metal affinity resin (635501, Clontech). The proteins were eluted with 300 mM imidazole, and subsequently dialyzed in PBS buffer (pH 7.4). The protein concentration was measured using a Protein assay dye (500-0006, Bio-Rad) and the protein purity was checked with SDS-PAGE.

The *Enhancin* genes from *B. cereus*, *B. subtilis*, *E. cloacae*, *E. faecalis, K. oxytoca, K. pneumoniae* and *K. michiganensis* were commercially synthesized and then cloned into the pET-28a (+) expression vector and expressed in the *E. coli* BL21 DE3 strain. The soluble proteins were induced by 100 μM IPTG overnight at 16°C to generate soluble forms and purified with the TALON metal affinity resin.

Antiserum

The recombinant *Sm*Enhancin protein was purified with the TALON metal affinity resin and emulsified in complete Freund's adjuvant, and injected subcutaneously into six-week-old female BALB/c mice of 80 μg/animal/injection. Animals were boosted twice at 2-week

intervals with the same dose of antigen in incomplete Freund's adjuvant. Sera were collected 10 days after the last boost. The antiserum specificity was measured by Western blotting with the *S. marcescens* culture supernatant.

Generation of Antibiotic-Treated Mosquitoes and Reintroduction of Bacteria

Mosquitoes were provided with cotton balls moistened with a 10% sucrose solution including 20 units of penicillin and 20 μg of streptomycin per ml (15070-063, Thermo Fisher Scientific) for 5 days to remove gut bacteria (Pang et al., 2016; Ramirez et al., 2012, 2014; Xiao et al., 2017). The mosquitoes were starved for 24 hrs to allow the antibiotics to be metabolized prior to the *in vitro* membrane blood feeding. Removal of gut bacteria was confirmed by a colony forming unit assay. For reintroduction of bacteria via blood meal or sugar meal, the given bacteria were used at a final concentration of OD_{600}=1.

Membrane Blood Feeding

Fresh human blood was collected from healthy volunteers and collected with anticoagulant tubes. Serum and blood cells were separated by centrifugation at 1,000 *g* for 10 min. The plasma was collected and heat-inactivated at 56°C for 1 hr, while blood cells were washed in PBS for three times. The cells were resuspended with heat-inactivated plasma. Based on different experimental settings, purified proteins, bacteria suspension, bacterial cell lysates or culture supernatant was mixed with viruses and the treated blood for mosquito oral feeding via a Hemotek membrane feeding system (6W1, Hemotek). Engorged female mosquitoes were anaesthetized at 4°C for 20 min, and then transferred into new containers and maintained under standard conditions. Mosquito infectivity was determined by qPCR or a plaque assay.

Mosquitoes Feeding on Mice

An AG6 mouse was intraperitoneally infected with 1×10^5 p.f.u. of DENV-2. The infected mouse was subjected to daily biting from days 1 to 4 post-animal infection by antibiotic-treated Rockefeller *A. aegypti* with or without *S. marcescens* oral introduction. For each individual experiment, the mosquitoes fed on the same infected mouse, enabling to acquire the equal amount of viruses. The mouse blood-fed mosquitoes were reared for an additional 8 days to determine their DENV burdens.

Viral Genome Quantitation by TaqMan qPCR

The mosquitoes and their tissues were homogenized and total RNA was isolated using the Multisource RNA miniprep kit (AP-MN-MS-RNA-250, Axygen) and cDNA reverse-transcription was conducted with the iScript cDNA synthesis kit (170-8890, Bio-Rad). Viral genomes were quantified via qPCR amplification of DENV, SINV and ZIKV genes. The primers and probes used for this analysis are shown in Table S4. The detection limit of viral genome / *actin* mRNA ratio is 0.002. Each dot in the figure represents a mosquito and each line represents the mean value of the group. The viral loads were normalized against either *A. aegypti actin* (*AAEL011197*) or *A. albopictus actin* (*AALF010408*).

Virus Titration in Mouse Blood

Blood samples were collected from the tail veins of infected mice in 0.4% sodium citrate and centrifuged for 6,000 *g* for 5 min at 4°C for plasma isolation. The presence of infectious viral particles in the plasma was determined by a plaque assay. For plaque assays, ten-fold serial dilutions of plasma (dilutions of 10^{-2} to 10^{-6}) were added in duplicate to cultured Vero cells in 6-well plates and incubated for 1 hr at 37°C. After discarding the inoculum and washing twice with PBS, the cells were overlaid with 1% SeaPlaque (50101, Lonza) in DMEM supplemented with 2% heat-inactivated fetal bovine serum. Plates were incubated at 37°C in 5% CO_2 for 5 days. Plates were fixed with 4% formalin for at least 1 hr, and plaque-forming units were visualized by staining with 2.5% crystal violet for 10 min at room temperature.

Mucin Digestion Assay

Aamucin-3 was cloned into the pMT/BiP/V5-His A vector (V4130-20, Invitrogen) and transfected into *Drosophila* S2 cells with the Effectene transfection reagent (301425, Qiagen) according to the manufacturer's instructions. *Aa*mucin-3 protein expression was induced by 500 μM copper sulfate. The supernatant was collected 2 days after induction for the degradation assay. *Aa*mucin-11 expression was quite inefficient in the pMT/BiP/V5-HisA expression system. To increase expression, the *Aamucin-11* gene was cloned into the pAC5.1-eGFP-V5-His A plasmid (modified from pAC5.1/V5-His A, V4110-20, Invitrogen). The recombinant *Aa*mucin-11-eGFP protein was expressed in *Drosophila* S2 cells. The cell lysates were collected 2 days after transfection, and then subjected to the degradation assay. The cloning primers are shown in Table S4. To assess the *Sm*Enhacin-mediated digestion of *Aa*mucins, either *Aa*mucin-3 or *Aa*mucin-11 was incubated with 5 μg/ml of *Sm*Enhancin at 37°C for 2 hrs. To assess the glycolsylation of *Aa*mucins, O-glycosidase (P0733, NEB) was incubated with *Aa*mucins for 15 hrs at 37°C according to the manufacturer's instructions. The degradation of *Aa*mucins was examined by Western blotting with an anti-V5 HRP antibody (R961-25, Invitrogen).

Gene Silencing in Mosquitoes

Double-stranded RNA (dsRNA) for gene silencing was synthetized using MEGAscript T7 transcription kit (AM1334, Invitrogen). Female mosquitoes were anaesthetized on a cold tray and 1 μg per 300 nl of dsRNA was microinjected into their thoraxes. The injected mosquitoes were allowed to recover for 3 days under standard rearing conditions and subsequently used for oral infection. The gene silencing efficiency was assessed by qPCR. The primers used for dsRNA synthesis and gene detection are shown in Table S4.

The detection limit of viral genome / *actin* mRNA ratio is 0.002. Each dot in the figure represents a mosquito and each line represents the mean value of the group. Gene quantities were normalized against *A. aegypti actin* (*AAEL011197*).

Measurement of the Gut Bacteria by 16S rDNA qPCR

The surface of a mosquito was sterilized with 70% ethanol and washed twice with sterile PBS. For each individual group, a total of 20 midguts from the mosquitoes was carefully removed from the mosquito abdomen under aseptic conditions. Total DNA was extracted with the Bacteria DNA kit (dp302-02, TIANamp) for 16S rDNA amplification with a pair of universal primers (Table S4). The burden of the gut microbiota was normalized to *A. aegypti actin* (*AAEL011197*) or *A. albopictus actin* (*AALF010408*).

Construction of the *SmEnhancin* Knockout *S. marcescens* Mutant Strain

The generation of the gene knockout mutant in *S. marcescens* was described previously (Soo et al., 2005). Briefly, the *SAY*-MF1 and *SAY*-MR1 primers were used to amplify a *SmEnhancin* upstream fragment and the *SAY*-MF2 and *SAY*-MR2 primers were used to amplify a *SmEnhancin* a downstream fragment. Both fragments were spliced together using overlap extension PCR and then cloned into a pLP12 suicide vector (Luo et al., 2015) named pLP12-*enh*. The *E. coli* β2163 cells transfected with pLP12-*enh* were screened on LB plates containing chloromycetin (20 μg/ml), diaminopimelic acid (0.3 mM) and 0.3% D-glucose. Then, the pLP12-*enh* was transferred to *S. marcescens* via a highly efficient *E. coli* β2163 conjugation system. The transconjugants were obtained by screening on LB plates with chloromycetin (20 μg/ml) and 0.3% D-glucose, but without diaminopimelic acid, which is necessary for *E. coli* β2163 growth. Subsequently, the recombination-mediated gene knockout was triggered by adding 0.4% L-arabinose. The Δ*SmEnhancin-S. marcescens* cells were validated by PCR with the *SAY*-TF-1and *SAY*-TR-1 primers. The primers are shown in Table S4.

Mucin Staining

The mosquito abdomens were dissected and fixed in 4% paraformaldehyde at 4°C prior to paraffin embedding and sectioning. The paraffin-embedded mosquito abdomens were stained with a Periodic acid-Schiff (PAS) stain kit (Mucin stain) following the instructions (ab150680, Abcam). The stained slides were photographed under a microscope (Eclipse 90i, Nikon) coupled with a Nikon digital camera at the Laboratory Animal Research Center at Tsinghua University.

QUANTIFICATION AND STATISTICAL ANALYSIS

For measuring bacterial growth curve and *A. aegypti* gene expression assay, data were shown as mean±SEM. For mosquito blood feeding on mice, "n" representing mice number was indicated in the figure legend. For mosquito infection assays, the number of mosquitoes was directly indicated in the figures. Animals were randomly allocated into different groups. Mosquitoes that died before measurement were excluded from the analysis. The investigators were not blinded to the allocation during the experiments or to the outcome assessment. No statistical methods were used to predetermine the sample size. For viral genome quantitation by TaqMan qPCR, the detection limit of viral genome / *actin* mRNA ratio is 0.002. For all data, one dot represents one mosquito and each line represents the mean value of the group. Descriptive statistics have been provided in the figure legends. A *Kruskal-Wallis* analysis of variance was conducted to detect any significant variation among replicates. If no significant variation was detected, the results were pooled for further comparison. All analyses were performed using the GraphPad Prism statistical software.

DATA AND SOFTWARE AVAILABILITY

Raw and analyzed data have been deposited to Mendeley Data and are available at https://doi.org/10.17632/65fjp9yymt.1.