

Fighting malaria with engineered symbiotic bacteria from vector mosquitoes

Sibao Wang^a, Anil K. Ghosh^a, Nicholas Bongio^b, Kevin A. Stebbings^{b,1}, David J. Lampe^b, and Marcelo Jacobs-Lorena^{a,2}

^aDepartment of Molecular Microbiology and Immunology, Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205; and ^bDepartment of Biological Sciences, Duquesne University, Pittsburgh, PA 15282

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The most vulnerable stages of *Plasmodium* development occur in the lumen of the mosquito midgut, a compartment shared with symbiotic bacteria. Here, we describe a strategy that uses symbiotic bacteria to deliver antimalaria effector molecules to the midgut lumen, thus rendering host mosquitoes refractory to malaria infection. The *Escherichia coli* hemolysin A secretion system was used to promote the secretion of a variety of anti-*Plasmodium* effector proteins by *Pantoea agglomerans*, a common mosquito symbiotic bacterium. These engineered *P. agglomerans* strains inhibited development of the human malaria parasite *Plasmodium falciparum* and rodent malaria parasite *Plasmodium berghei* by up to 98%. Significantly, the proportion of mosquitoes carrying parasites (prevalence) decreased by up to 84% for two of the effector molecules, scorpine, a potent antiparasitic peptide and (EPIP)₄, four copies of *Plasmodium* enolase–plasminogen interaction peptide that prevents plasminogen binding to the ookinete surface. We demonstrate the use of an engineered symbiotic bacterium to interfere with the development of *P. falciparum* in the mosquito. These findings provide the foundation for the use of genetically modified symbiotic bacteria as a powerful tool to combat malaria.

Anopheles gambiae | malaria control | paratransgenesis | transmission blocking

Malaria is one of the most lethal infectious diseases. Close to half of the population of the world is at risk, about 300–500 million contract the disease annually, and about 1.2 million people die of malaria every year (1). Continuous emergence of mosquito insecticide resistance and parasite drug resistance, combined with the lack of an effective malaria vaccine, severely limits our ability to counteract this intolerable burden (2, 3). New weapons to fight the disease are urgently needed.

Unlike the other two major infectious diseases (AIDS and tuberculosis) that are transmitted directly from person to person, transmission of *Plasmodium*, the causative agent of malaria, strictly depends on the completion of a complex developmental cycle in vector mosquitoes (4). A mosquito may ingest on the order of 10^3 to 10^4 gametocytes from an infected human that quickly differentiate into male and female gametes that mate to produce zygotes, which, in turn, differentiate into $\sim 10^2$ to 10^3 motile ookinetes. Ookinetes then migrate within the blood bolus until they reach the midgut epithelium, where they then traverse and differentiate into oocysts. Upon maturation, each oocyst releases thousands of sporozoites into the hemocoel, followed by invasion of the mosquito salivary glands. The transmission cycle is completed when the infected mosquito bites the next vertebrate host and delivers some of the sporozoites with the saliva (5). Clearly, a severe bottleneck occurs during the mosquito midgut stages of parasite development: even in areas of high transmission, mosquitoes typically carry five or fewer oocysts (5). This bottleneck makes the mosquito midgut a prime target for intervention (6, 7).

One option to interfere with parasite transmission is to genetically modify mosquitoes for midgut expression of “effector genes” that inhibit parasite development. Past evidence suggests that this strategy works successfully in the laboratory (8–11). However, one unresolved challenge is how to drive transgenes

into wild mosquito populations. Various genetic drive mechanisms have been proposed to accomplish this goal (12, 13), but these are technically very challenging, and it is not clear in what time frame they will succeed. An important additional challenge faced by genetic drive approaches, in general, is that anopheline vectors frequently occur in the field as reproductively isolated populations, thus posing a barrier for gene flow from one population to another.

An alternative strategy to deliver effector molecules is to engineer symbiotic bacteria from the mosquito midgut microbiome to produce the interfering proteins (paratransgenesis) (14). A key strategic consideration is that the mosquito microbiome (15, 16) resides in the same compartment where the most vulnerable stages of malaria parasite development occur (7). Moreover, bacteria numbers increase dramatically (hundreds- to thousands-fold) after ingestion of a blood meal (15), and output of anti-*Plasmodium* effector molecules from engineered bacteria can be expected to increase proportionally.

Paratransgenesis has shown promise for the control of other insect-borne disease (14). Chagas disease caused by the parasitic protozoan *Trypanosoma cruzi* is transmitted by the triatomid bug, *Rhodnius prolixus*. In proof-of-concept experiments, an obligate commensal Gram-positive bacterium was genetically modified to secrete cecropin A, a peptide that kills *T. cruzi*, making the bug refractory to the parasite (14). Our previous study using the rodent malaria parasite *Plasmodium berghei* and the *Anopheles stephensi* vector mosquito suggested that recombinant *Escherichia coli* expressing on their surface either a dimer of the salivary gland and midgut peptide 1 (SM1) or a modified phospholipase reduced oocyst formation (17). Yoshida et al. (18) also showed that recombinant *E. coli* expressing a single-chain immunotoxin significantly reduces *P. berghei* oocyst density in *A. stephensi* mosquitoes. One limitation of these earlier experiments is that they used *E. coli*, an attenuated laboratory bacterium that survives poorly in the mosquito gut (17). A second limitation was that the recombinant effector molecules either remained attached to the bacterial surface (17) or formed an insoluble inclusion body within the bacterial cells (18), thus preventing diffusion of the effector molecules to their parasite or mosquito midgut targets.

Here, we describe a substantially improved strategy to deliver effector molecules by engineering a natural symbiotic bacterium *Pantoea agglomerans* (previously known as *Enterobacter agglomerans*) to secrete antimalaria proteins in the mosquito midgut. We found that the development of the human parasite *Plasmodium*

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¹Present address: Neuroscience Program, University of Illinois at Urbana–Champaign, Urbana, IL 61801.

²To whom correspondence should be addressed. E-mail: mlorena@jhsph.edu.

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falciparum and the rodent parasite *P. berghei* in the mosquito was efficiently suppressed by up to 98%.

Results

Administration of Transgenic *P. agglomerans* via Sugar Meals and Rapid Proliferation After a Blood Meal. *P. agglomerans* is a natural symbiotic bacterium whose occurrence is widespread in anopheline mosquitoes (15–17). Our strain was originally isolated from midguts of an *Anopheles gambiae* laboratory colony and subsequently further selected for improved adaptation to the *A. gambiae* and *A. stephensi* midgut environments (17). To examine recombinant *P. agglomerans* colonization and survival in the mosquito midgut, we transformed *P. agglomerans* with a highly stable plasmid pNptII:gfp (19) that expresses green fluorescent protein (GFP). We found the GFP-tagged bacteria were easily administered to mosquito midguts via sugar meals (Fig. S1). Midguts of female mosquitoes that had been fed GFP-tagged *P. agglomerans* became strongly fluorescent after ingestion of a blood meal (Fig. 1A and B), indicating rapid bacteria proliferation. To quantify the dynamics of bacteria numbers, mosquitoes that had been fed GFP-tagged *P. agglomerans* were dissected at different times after a blood meal and midgut homogenates were plated on selective kanamycin-containing plates. Bacteria numbers increased dramatically by more than 200-fold during the first 2 d after a blood meal (Fig. 1C). Thereafter, bacteria numbers decreased to about prefeeding levels, presumably because the majority was excreted together with the remnants of blood digestion.

Recombinant *P. agglomerans* Strains Efficiently Secrete Anti-*Plasmodium* Effector Molecules. As mentioned previously, for maximum effectiveness of intervention, it is crucial that the effector molecules be secreted from the engineered bacteria to allow diffusion to the

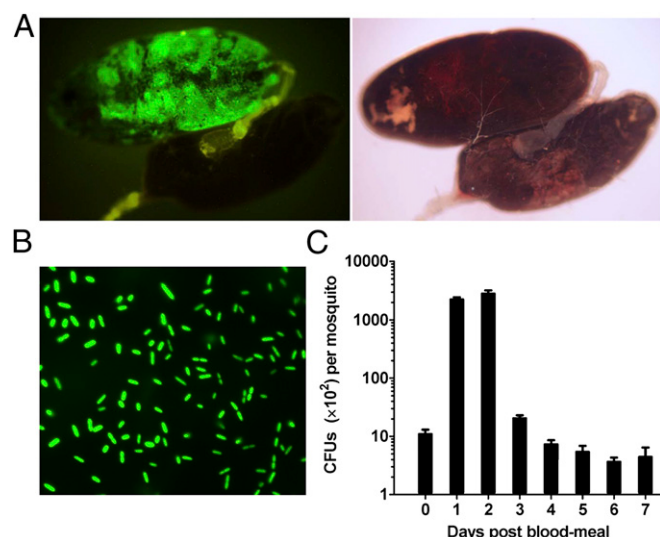


Fig. 1. Transgenic *P. agglomerans* rapidly proliferate in the midgut after a blood meal. GFP-tagged *P. agglomerans* were administered to 2-d-old *A. gambiae* via a sugar meal, and, 32 h later, the insects were fed on blood. (A) Visualization of GFP-tagged bacteria in the midgut at 24 h after a blood meal. The upper midgut is from a mosquito that had been fed GFP-tagged bacteria, and the lower midgut was from a control mosquito that did not feed on recombinant bacteria. A differential interference contrast image (Right) is paired with a fluorescent image (Left). (B) GFP-fluorescent bacteria recovered from a mosquito midgut. (C) Population dynamics of GFP-tagged *P. agglomerans* as a function of time after a blood meal. Fluorescent bacteria were fed to mosquitoes as described above, and midguts were dissected at the indicated times after a blood meal. Fluorescent bacteria colony-forming units (CFUs) were determined by plating serially diluted homogenates of midguts on LB/kanamycin agar plates. Data were pooled from three biological replicates.

parasite or mosquito midgut targets. We used the *E. coli* hemolysin (Hly)A system (20–22) to promote protein secretion from *P. agglomerans*. This type 1 secretion system requires three components to form a membrane pore (inner membrane components HlyB and HlyD and outer membrane component TolC). The HlyB–HlyD complex recognizes the C-terminal secretion signal of HlyA to guide direct export of HlyA–fusion proteins from the bacterial cytoplasm into the extracellular environment bypassing the periplasmic space (Fig. S24).

We used the HlyA secretion system to test the following anti-*Plasmodium* effector molecules (Table S1): (i) two copies of the 12-aa SM1 peptide [(SM1)₂], which binds to the luminal surface of the mosquito midgut and blocks ookinete midgut invasion (23); (ii) a mutant phospholipase mPLA2 that inhibits ookinete invasion, probably by modifying the properties of the midgut epithelial membrane (24); (iii) a single-chain immunotoxin (pbs21scFv-Shiva1), composed of a single-chain monoclonal antibody (scFv) targeting the *P. berghei* major ookinete surface protein pbs21 and linked to the lytic peptide Shiva1 (18); (iv) a chitinase propeptide (Pro) that inhibits the enzyme and blocks ookinete traversal of the mosquito peritrophic matrix (25); (v) a synthetic antiparasitic lytic peptide, Shiva1 (26); (vi) a scorpion (*Pandinus imperator*) antimalaria lytic peptide, scorpine, which has hybrid properties of the lytic peptides cecropin and defensin (27); (vii) four copies of *Plasmodium* enolase–plasminogen interaction peptide (EPIP) [(EPIP)₄] that inhibits mosquito midgut invasion by preventing plasminogen binding to the ookinete surface (28); and (viii) a fusion peptide composed of Pro and EPIP (Pro:EPIP). All genes were synthesized with the *P. agglomerans* preferred codon usage (Table S2) and cloned in frame with an E-tagged C-terminal secretion signal domain of HlyA in a high-copy expression vector (Figs. S2B and S3). The resulting plasmids were individually transformed into *P. agglomerans* together with the low-copy plasmid pVDL9.3 coding for HlyB and HlyD (20). Constitutive expression and secretion of a fusion protein of the predicted size by each recombinant *P. agglomerans* strain were verified by Western blot analysis (Fig. 2A). The smaller sized peptides [(EPIP)₄, Shiva1, and Pro:EPIP] were more abundant in the culture supernatant than the larger sized proteins (mPLA2 and pbs21scFv-Shiva1) (Fig. 2A). The recombinant *P. agglomerans* strain expressing (EPIP)₄ had the highest secretion level, appearing to be more efficient than the recombinant strain carrying the parental E-HlyA plasmid (Fig. 2A). In vivo secretion of anti-*Plasmodium* effector molecules in the mosquito midgut was confirmed by use of immunofluorescence assays that detected the binding of the bacteria-produced SM1 peptide to the midgut epithelial surface (Fig. 2B). Control midguts from mosquitoes fed on recombinant *P. agglomerans* expressing E-tagged HlyA alone (HlyA) had no fluorescence above background.

Recombinant *P. agglomerans* Strains Efficiently Impair *P. falciparum* Development in Mosquitoes. To assess the effectiveness with which recombinant bacteria interfere with the development of the human malaria parasite *P. falciparum* in mosquitoes, recombinant *P. agglomerans* were administered to mosquitoes via cotton pads soaked with bacteria suspended in 5% (wt/vol) sucrose solution. Thirty-two hours later, mosquitoes were provided a *P. falciparum*-infected blood meal. Success of parasite development was evaluated by counting oocyst numbers on day 8 after the infectious blood meal. Initial experiments were conducted to evaluate the inhibition potential of recombinant *P. agglomerans* secreting a chitinase propeptide (Pro) compared with administration of the synthetic propeptide mixed with the blood meal. The propeptide inhibits *Plasmodium* chitinase activity and, thus, prevents crossing of the peritrophic matrix, resulting in decreased number of ookinets that reach the midgut epithelium (25). As shown in Fig. S4, inhibition by *P. agglomerans* expressing the propeptide was effective, on the order of 59% inhibition in highly

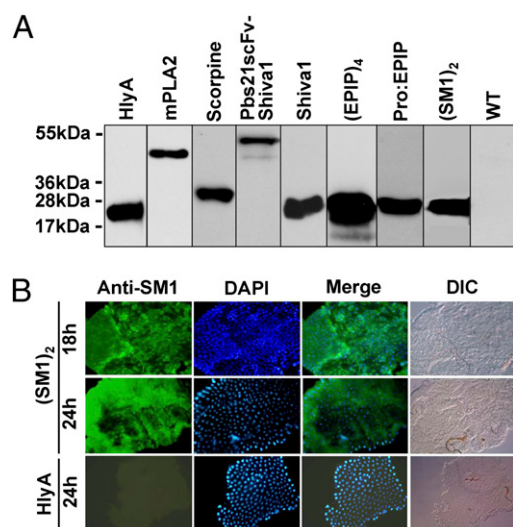


Fig. 2. The engineered *P. agglomerans* strains efficiently secrete anti-*Plasmodium* effector proteins. (A) Western blot analysis. Culture supernatants were concentrated using Amicon Ultra-4 Centrifugal Filter Units. Aliquots originating from the same volume of supernatant were fractionated by SDS/PAGE and subjected to Western blot analysis using a rabbit anti-E-tag antibody. The supernatant from nontransformed *P. agglomerans* (WT) served as a negative control. Images were taken using the same exposure time. (B) In vivo binding of the secreted SM1 peptide to the mosquito midgut epithelium. *A. gambiae* were fed on sugar solution containing recombinant *P. agglomerans* strains expressing either the (SM1)₂ or E-tagged HlyA alone (HlyA) and then fed on mouse blood. Midguts were dissected 18 or 24 h after blood meal, opened into a sheet, fixed, and incubated with an anti-SM1 antibody, followed by incubation with a fluorescent secondary antibody. Nuclei were stained with DAPI (blue). The differential interference contrast (DIC) images of the same field are shown to the right.

infected mosquitoes (also see comments concerning infection intensity under *Discussion*). Next, we evaluated the inhibitory capacity of the various recombinant *P. agglomerans* strains. As shown in Fig. 3, the recombinant *P. agglomerans* strain expressing E-tagged HlyA alone did not significantly inhibit oocyst formation compared with minus bacteria controls. Recombinant *P. agglomerans* expressing mPLA2, Pro:EPIP, or Shiva1 had a 85%, 87%, and 94% decrease in oocyst numbers, respectively. Recombinant *P. agglomerans* expressing scorpine or (EPIP)₄ had the highest inhibition (~98%) of oocyst formation (Fig. 3). Importantly, infection prevalence (percentage of mosquitoes that have one or more oocysts) was 90% in –Bact (control) mosquitoes and was reduced to 14% in mosquitoes carrying scorpine-secreting bacteria, representing an 84% transmission-blocking potential.

To evaluate the persistence of the inhibitory effect, we compared oocyst numbers in mosquitoes that were fed the same batch of infective blood meal at either 2 d (our standard procedure) or 4 d after bacterial administration. These experiments also evaluated the effect of mixing bacteria expressing two different effector proteins, [shiva1 + (EPIP)₄] and [scorpine + (EPIP)₄], on inhibition of *P. falciparum* infection. As shown in Fig. 4, inhibition of oocyst development was similar at both time points. Thus, the inhibitory effect of the bacteria lasts for at least 4 d after bacteria administration. Also, inhibition by a mixture of bacteria expressing two effectors (Fig. 4) was similar to that of bacteria expressing a single effector (Fig. 3). Possible reasons for this are considered under *Discussion*.

Recombinant *P. agglomerans* Strains Efficiently Impair *P. berghei* Development in Mosquitoes. We also evaluated the effectiveness of recombinant *P. agglomerans* strains to inhibit development of the rodent malaria parasite *P. berghei* in *A. stephensi* mosquitoes.

Mosquitoes harboring various recombinant bacterial strains were allowed to feed on the same *P. berghei*-infected mouse, followed by determination of oocyst numbers on day 14 postinfection. The recombinant *P. agglomerans* strain expressing (SM1)₂ reduced mean oocyst counts by 68%, an equal mixture of bacteria expressing pbs21scFv-shiva1 + (EPIP)₄ reduced oocyst counts by 79%, and a mixture of scorpine- and (EPIP)₄-expressing bacteria reduced oocyst counts by 83% (Fig. 5), despite relatively high infection rates (>60 oocysts per midgut; also see *Discussion*). No significant reduction was observed in the presence of the recombinant *P. agglomerans* strain expressing E-tagged HlyA alone (HlyA).

Recombinant *P. agglomerans* Strains Do Not Affect Mosquito Longevity.

A recombinant bacterium with minimal fitness cost to mosquitoes is also an important factor for the success of future field applications. To examine the impact of recombinant *P. agglomerans* strains expressing anti-*Plasmodium* molecules on mosquito lifespan, mosquitoes were fed on sugar alone or on various recombinant *P. agglomerans* and, 32 h later, were allowed to feed on a noninfected mouse. Thereafter, mortality was monitored twice daily. No significant differences in mosquito lifespan was detected among any of the mosquito groups (Fig. 6), suggesting these recombinant anti-*Plasmodium* products pose no obvious negative impact on mosquito fitness in laboratory conditions.

Discussion

Although current malaria control strategies targeting the mosquito vector and the parasite have helped alleviate the malaria

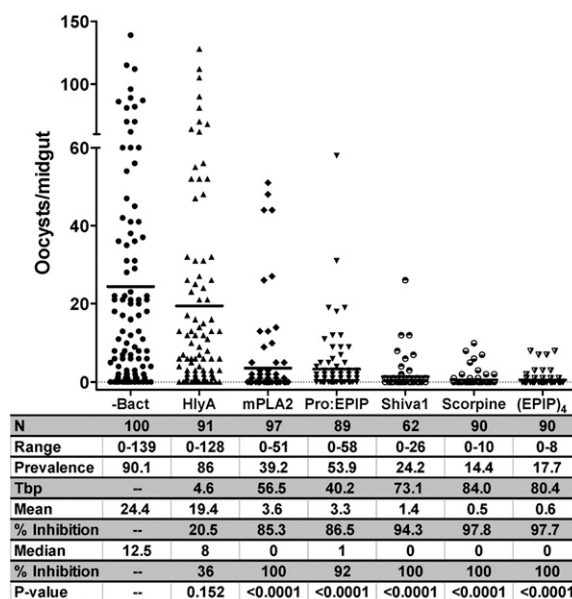


Fig. 3. Inhibition of *P. falciparum* development in vector mosquitoes by recombinant *P. agglomerans* strains. *A. gambiae* mosquitoes were fed on 5% (wt/vol) sugar solution supplemented with either PBS (–Bact) or recombinant *P. agglomerans* strains and 32 h later fed on a *P. falciparum*-infected blood meal. Oocyst numbers were determined 8 d after blood meal. Each dot represents the oocyst number of an individual midgut and horizontal lines indicate mean values. Data were pooled from four biological replicates. Recombinant *P. agglomerans* strains were engineered to express the E-tagged HlyA leader peptide alone (HlyA) or mPLA2, Pro:EPIP, Shiva1, scorpine, and (EPIP)₄ (Table S1), fused to HlyA (Fig. S3), as indicated. Inhibition, inhibition of oocyst formation relative to the –Bact control; Mean, mean oocyst number per midgut; Median, median oocyst number per midgut; N, number of mosquitoes analyzed; Prevalence, percentage of mosquitoes carrying at least one oocyst; Range, range of oocyst numbers per midgut; Tbp, transmission-blocking potential: 100 – {(prevalence of mosquitoes fed with recombinant *P. agglomerans*)/[prevalence of control (–Bact) mosquitoes] × 100}.

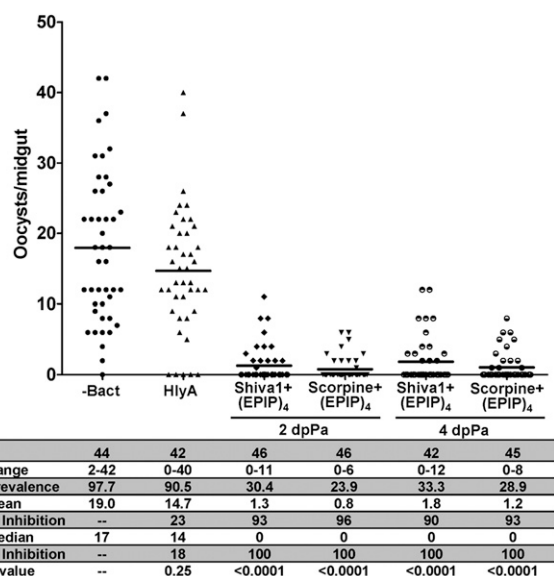


Fig. 4. Comparison of *P. falciparum* development between mosquitoes that fed on an infectious blood meal at either 2 or 4 d after administration of recombinant *P. agglomerans*. *A. gambiae* females were fed on 5% (wt/vol) sugar solution supplemented with either PBS (–Bact) or recombinant *P. agglomerans* strains as follows: HlyA, *P. agglomerans* expressing the E-tagged HlyA leader peptide alone; Shiva1 + (EPIP)₄ or scorpine + (EPIP)₄ (Table S1), a mixture of the two recombinant bacteria in equal numbers. Two days (2 dpPa) or 4 d (4 dpPa) after administration of bacteria, mosquitoes were fed on a *P. falciparum*-infected blood meal. Oocyst numbers were determined 8 d after the blood meal. Each dot represents the oocyst number of an individual midgut and horizontal lines indicate mean values. Data were pooled from three biological replicates. % Inhibition, inhibition of oocyst formation relative to the –Bact control; Mean, mean oocyst number per midgut; Median, median oocyst number per midgut; N, number of mosquitoes analyzed; Prevalence, percentage of mosquitoes carrying at least one oocyst; Range, range of oocyst numbers per midgut.

burden in many endemic areas (29), the emergence and rapid spreading of insecticide-resistant mosquitoes and drug-resistant parasites undermine such efforts (3). The Malaria Eradication Research Agenda (malERA) consultative group recently noted that malaria eradication will not be achieved without help of novel control tools (30). Here, we report on an alternative strategy to deliver antimalaria effector molecules to the mosquito midgut via engineered symbiotic bacteria.

The mosquito midgut serves as a prime target for blocking parasite transmission with engineered bacteria for two main reasons: (i) the most vulnerable part of the *Plasmodium* cycle in the mosquito and a bottleneck in parasite numbers occur in the mosquito midgut lumen (5); and (ii) the midgut compartment is shared between *Plasmodium* and the mosquito microbiome, directly exposing parasites to compounds secreted by resident bacteria. Furthermore, the number of bacteria in the mosquito midgut increases dramatically (by two to three orders of magnitude) after ingestion of a blood meal, consequently increasing the output of effector molecules produced by recombinant bacteria. The drop in recombinant bacteria number at 3 d after the blood meal (Fig. 1C) is of no consequence to the effectiveness of intervention because most of ookinete invasion of the mosquito midgut occurs at 24 to 36 h after the blood meal.

Symbiotic bacteria are thought to be beneficial to their insect hosts by providing nutritional supplements, tolerance to environmental perturbations, and manipulation of host immune homeostasis (31). In recent years, the relationship between symbionts and their hosts has attracted increasing attention from the perspective of engineering symbionts to combat pathogens

(14, 17, 32). Several bacterial species have been identified from the midgut of field-collected anophelines, mostly Gram-negative proteobacteria and enterobacteria (16, 33). A recent study found that the bacterial population structure of laboratory-reared adult mosquitoes is similar to that of field mosquitoes, suggesting that the mosquito gut harbors its microbiome in a selective way (33). A nonpathogenic bacterium *P. agglomerans* was reported as a dominant symbiotic bacterium in different mosquito species in Kenya and Mali (16) and also found in laboratory-reared *A. stephensi*, *A. gambiae*, and *Anopheles albimanus* mosquitoes (15, 17). It also occurs in the desert locust *Schistocerca gregaria* (34) and the honey bee *Apis mellifera* (35). A possible source of *P. agglomerans* for mosquitoes in the field is flower nectar, given that this bacterium was found on plant surfaces and blossoms (36–38). These considerations are relevant to future efforts to introduce recombinant bacteria into field mosquito populations. Importantly, *P. agglomerans* strains have been used as microbial control agents against the plant disease fire blight in the United States (37). These facts make *P. agglomerans* an excellent candidate for delivery of antimalarials to mosquitoes in the field.

Our data demonstrate that five potent anti-*Plasmodium* effector molecules secreted by *P. agglomerans* are able to efficiently inhibit *P. falciparum* development during early sporogony. The availability of multiple effector proteins, each acting by a different mechanism, greatly reduces the probability of selection of resistant parasites. Initial experiments indicated that mixing bacteria that secrete different effector molecules had no additive effect on inhibition of *Plasmodium* development (Figs. 4 and 5). This may be because the number of bacteria expressing each effector is cut in half in the mixture fed to mosquitoes.

Perhaps more important than the inhibition of oocyst formation [up to 98% for scorpine and (EPIP)₄] are the data on

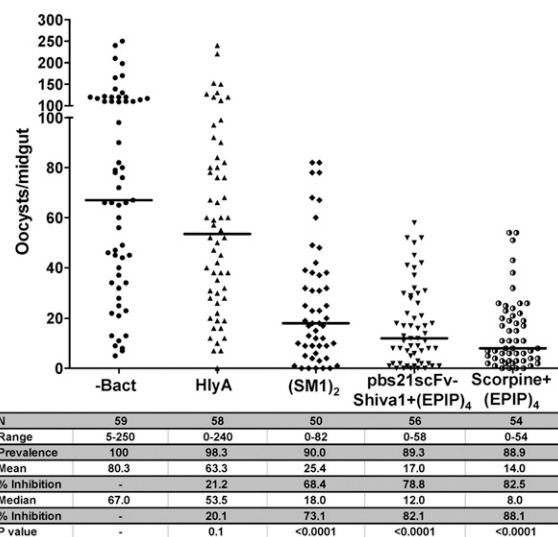


Fig. 5. Inhibition of *P. berghei* development in mosquitoes harboring recombinant *P. agglomerans* strains. *A. stephensi* females were fed on 5% (wt/vol) sugar solution supplemented with either PBS (–Bact) or recombinant *P. agglomerans* strains engineered to express E-tagged HlyA alone (HlyA), (SM1)₂, pbs21scFv-Shiva1/(EPIP)₄ (50–50 mixture) or scorpine/(EPIP)₄ (50–50 mixture) (Table S1 and Fig. S3) and 32 h later the five groups of mosquitoes were fed on the same *P. berghei*-infected mouse. *Plasmodium* infections were evaluated 14 d after infection. Each dot represents the number of oocysts in an individual midgut. Horizontal lines indicate mean values. Data pooled from three biological replicates. N, number of mosquitoes analyzed; Range, range of oocyst numbers per midgut; Prevalence, percentage of mosquitoes carrying at least one oocyst; Mean, mean oocyst number per midgut; Median, median oocyst number per midgut; Inhibition, inhibition of oocyst formation relative to the –Bact control.

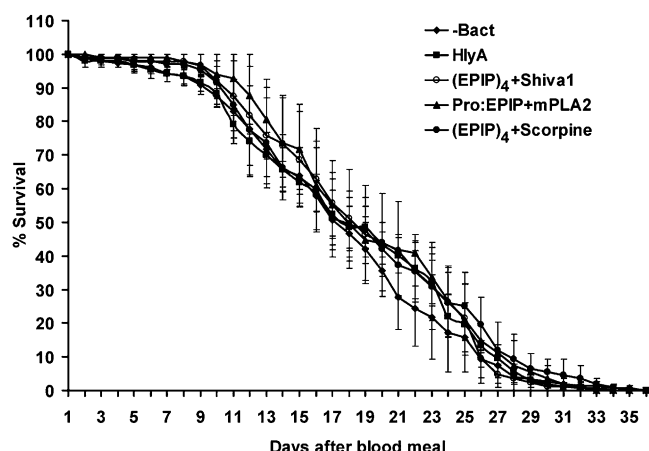


Fig. 6. Impact of recombinant *P. agglomerans* strains on lifespan of *Anopheles gambiae* females. *A. gambiae* mosquitoes were fed on 5% (wt/vol) sugar solution supplemented with either PBS (–Bact), or recombinant *P. agglomerans* strains as follows: HlyA, *P. agglomerans* expressing the E-tagged HlyA leader peptide alone; Shiva1 + (EPIP)₄ or scorpine + (EPIP)₄, a mixture of the two recombinant bacteria in equal numbers (Table S1 and Fig. S3). Thirty two hours later, mosquitoes were fed on a noninfected mouse, and mosquito survival was measured twice daily. Data were pooled from three independent experiments. There was no significant difference in survivorship among the groups.

prevalence. The percentage of *P. falciparum*-infected mosquitoes was 90% in controls and was reduced to 14–18% in mosquitoes carrying scorpine or (EPIP)₄ transgenic bacteria. This would represent an 80–84% reduction in the proportion of infected mosquitoes. The effectors appear to be similarly efficient in controlling a human and a rodent parasite, raising the possibility that they also will be effective in the control of other human parasites such as *Plasmodium vivax*.

We note that in the field, mosquitoes carry a mean of five oocysts or fewer in their midguts (5). In some experiments, mosquitoes fed with blood containing high *P. falciparum* gametocytemia produced abnormally high oocyst numbers (mean >200 oocysts) (Fig. S5). However, even under these conditions, inhibition of parasite development was highly significant and in line with the results presented in Fig. 3, although the extent of inhibition was lower (up to 68% inhibition for mosquitoes carrying scorpine-secreting bacteria).

There are around 460 species of anopheline mosquitoes, over 100 of which are suitable vectors for human malaria (39). Very few of these have been genetically transformed (40). We found that the effector molecules investigated in this study are equally effective with *A. gambiae* (an African mosquito) and with *A. stephensi* (an Asian mosquito). The paratransgenesis strategy may well be “universal” as effectiveness does not appear to be influenced by mosquito species. Moreover, the fact that many major vector anopheline species exist as reproductively isolated populations (cryptic species) imposes an important technical barrier for the introduction of transgenes into mosquito populations in the field but does not affect the paratransgenesis strategy. It is important to note that paratransgenesis is compatible with current

mosquito control tools (insecticides, population suppression) and even with genetically modified mosquitoes. Should the technical barriers for transgene introgression into mosquito populations be solved, we envision the concomitant application of both strategies (genetically modified mosquitoes and paratransgenesis), because they can complement each other. However, more work lays ahead before this approach can be implemented in the field. One key issue is how to effectively introduce engineered bacteria into mosquitoes in the field. Our preliminary results suggest that this could be accomplished by placing baiting stations (cotton balls soaked with sugar and bacteria placed in clay jars) around villages where malaria is prevalent. Another important challenge is the resolution of regulatory, ethical, and social issues related to the release of genetically modified organisms.

Materials and Methods

Mosquito and Parasite Maintenance. We used two mosquitoes (*A. gambiae* Keele strain and *A. stephensi* Dutch strain) and two parasites *P. falciparum* strain NF54 and *P. berghei* ANKA 2.34. Mosquitoes and parasites were maintained as described in SI Text.

Bacterial Introduction into Mosquitoes via Sugar Meals. *P. agglomerans* were introduced into mosquitoes by feeding them with cotton pads soaked with a bacteria suspension (10^9 cells/mL) in 5% (wt/vol) sugar as described in SI Text.

Plasmid Construction and Protein Expression. The synthesized DNA encoding anti-*Plasmodium* effector molecules were optimized with the *P. agglomerans* preferred codon usage. Plasmid construction, bacterial genetic transformation, and verification of protein secretion using Western blot analysis were performed as described in SI Text.

***P. falciparum* Transmission-Blocking Assays.** Recombinant bacterial suspensions [1×10^9 cells/mL of 5% (wt/vol) sugar] soaked in cotton pads were fed to 2-d-old *A. gambiae* for 24 h, which were then starved for 8 h and allowed to feed for 30 min on a *P. falciparum* NF54 gametocyte-containing blood meal. Fully engorged mosquitoes were separated within 24 h. Success of parasite infection was evaluated by counting oocyst numbers at 8 d postinfection.

***P. berghei* Transmission-Blocking Assays.** Different groups of bacteria-fed mosquitoes were allowed to feed on the same *P. berghei*-infected mouse for 6 min and then maintained at 19 °C at 80% relative humidity. Success of parasite infection was evaluated by counting oocyst numbers at 14 d postinfection.

Immunofluorescence Assays. Recombinant *P. agglomerans* bacteria expressing (SM1)₂ were introduced to 2-d-old *A. gambiae* females via sugar meal (1×10^9 cells/mL) for 24 h. The mosquitoes were then allowed to feed on a noninfected mouse for 30 min. Midgut sheets were prepared at 18 and 24 h after blood feeding and fixed overnight in 4% (wt/vol) paraformaldehyde at 4 °C, washed in PBS, and blocked with 4% (wt/vol) BSA at 4 °C. The gut sheets were then probed with anti-SM1 peptide antibody (1:1,000), followed by Alexa Fluor-conjugated goat anti-rabbit IgG (Sigma; 1:1,000). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

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