MOSQUITO CONTROL

Driving mosquito refractoriness to *Plasmodium falciparum* with engineered symbiotic bacteria

Sibao Wang,[1]* André L. A. Dos-Santos,[2]† Wei Huang,[2] Kun Connie Liu,[2]‡ Mohammad Ali Oshaghi,[2]§ Ge Wei,[1] Peter Agre,[2] Marcelo Jacobs-Lorena[2]*

The huge burden of malaria in developing countries urgently demands the development of novel approaches to fight this deadly disease. Although engineered symbiotic bacteria have been shown to render mosquitoes resistant to the parasite, the challenge remains to effectively introduce such bacteria into mosquito populations. We describe a *Serratia* bacterium strain (AS1) isolated from *Anopheles* ovaries that stably colonizes the mosquito midgut, female ovaries, and male accessory glands and spreads rapidly throughout mosquito populations. *Serratia* AS1 was genetically engineered for secretion of anti-*Plasmodium* effector proteins, and the recombinant strains inhibit development of *Plasmodium falciparum* in mosquitoes.

Malaria is endemic in more than 106 countries, with 212 million new cases and 429,000 global malaria deaths in 2015, mostly among young children in sub-Saharan Africa (*1*). Evidently, available tools are not sufficient for malaria control. Malaria is caused by *Plasmodium* parasites that are transmitted through the bite of infected female anopheline mosquitoes. The current mainstay approaches for controlling malaria are vector mosquito control and antimalarial drugs (*2*). With the increasing resistance of malaria parasites to drugs (*3*) and of mosquitoes to insecticides (*4*, *5*), new strategies to control the disease are urgently needed.

Interference with the mosquito's ability to support parasite development would hinder transmission. Malaria parasites suffer losses during their development in the mosquito vector, with the most severe bottleneck of *Plasmodium* development occurring in the lumen of the mosquito midgut (*6*). A promising approach is not to kill mosquitoes, but instead to convert them into an ineffective vector of malaria (*7*) by targeting the midgut stages (*8*).

Mosquito midguts carry a complex microbiome, as well as developing malaria parasites. Midgut bacterial populations increase 100- to 1000-fold after ingestion of a blood meal (*9*). Recombinant bacteria introduced into mosquito midguts are expected to be amplified as well, enhancing output of their products. An alternative strategy for malaria control is to genetically engineer symbiotic bacteria, rather than the mosquito itself, to produce anti-*Plasmodium* effector molecules (paratransgenesis). An early attempt to test this strategy for the control of malaria used laboratory *Escherichia coli* engineered to express proteins on the bacterial surface (*10*). Laboratory *E. coli*, however, does not survive well in the mosquito midgut, and the recombinant proteins remained attached to the bacteria, hindering the proteins from reaching their parasite targets. Recently, we engineered the natural symbiotic bacterium *Pantoea agglomerans* to secrete anti-*Plasmodium* effector molecules that inhibited parasite development (*11*). These studies left unresolved the challenge of introducing and propagating recombinant bacteria into vector populations in the field. Here we report on a different mosquito symbiont, *Serratia*, which rapidly disseminates through mosquito populations.

While working with ovary-associated bacteria from our laboratory colony of *Anopheles stephensi* mosquitoes, we fortuitously identified a previously unknown bacterial strain, named AS1, that efficiently colonizes mosquito ovaries (fig. S1). Phylogenetic analysis of the 16*S* ribosomal RNA (rRNA) gene sequence shows that the AS1 strain belongs to the genus *Serratia* and clusters with two strains of *Serratia marcescens marcescens* DSM30121 and *Serratia marcescens sakuensis* KRED (fig. S2). The 16*S* rRNA gene sequence of *Serratia* AS1 also shows 99% similarity to *Serratia* strains isolated from guts of other insects (table S1).

A symbiotic bacterium with minimal fitness cost to mosquitoes is an important factor for the success of the paratransgenesis strategy. In comparisons with controls, we found that *Serratia* AS1 had little or no effect on the life span of adult *A. gambiae* and *A. stephensi* mosquitoes (fig. S3), nor did it influence blood-feeding behavior (fig. S4). Moreover, wild-type and recombinant *Serratia* strains expressing

[1]CAS Key Laboratory of Insect Developmental and Evolutionary Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China. [2]Department of Molecular Microbiology and Immunology, Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205, USA.
***Corresponding author. Email: sbwang@sibs.ac.cn (S.W.); mlorena@jhsph.edu (M.J.-L.)** †Present address: Instituto de Bioquímica Médica Leopoldo De Meis, Federal University of Rio de Janeiro, Rio de Janeiro, Rio de Janeiro 21941-902, Brazil. ‡Present address: Office of Regulatory Affairs, U.S. Food and Drug Administration, Pacific Regional Laboratory Northwest, 22201 23rd Drive Southeast, Bothell, WA 98021, USA. §Present address: Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Post Office Box 14155-6446, Tehran, Iran.

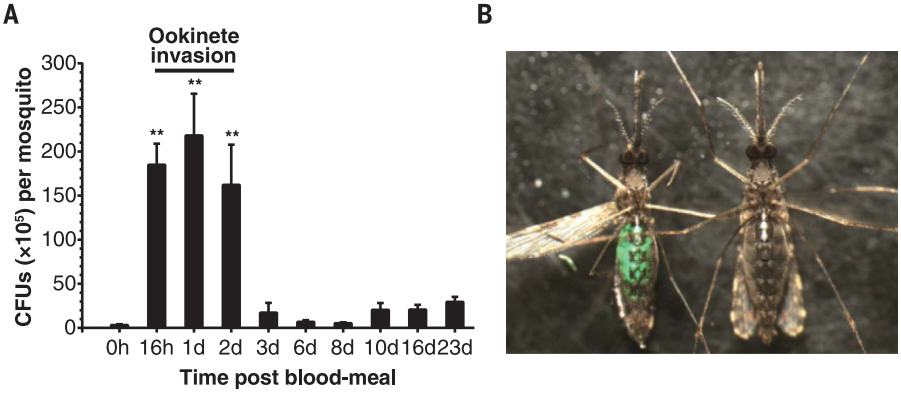


Fig. 1. *Serratia* AS1 bacteria stably colonize the midgut and rapidly proliferate after a blood meal. *Serratia* AS1 tagged with eGFP (AS1-GFP) was fed to 3-day-old *A. stephensi* mosquitoes in a sugar meal for 24 hours, then mosquitoes were allowed to feed on a blood meal. (**A**) Population dynamics of AS1-GFP. Fluorescent bacteria colony-forming units (CFUs) were determined by plating serially diluted homogenates of midguts on Luria-Bertani (LB) agar plates containing 100 μg/ml of kanamycin. Data were pooled from three biological replicates (shown are means ± SEM). $^{**}P < 0.01$ (Student's *t* test). The maximum bacteria number is reached when *Plasmodium* ookinetes would be invading the midgut if the blood were infected with the parasite. h, hours; d, days. (**B**) AS1-GFP in the midgut of a female at 24 hours after blood ingestion (left). On the right is a control mosquito.

antimalarial effectors (single or mixed) had no obvious negative impact on mosquito fecundity and fertility (fig. S5). These results imply that *Serratia* AS1 colonization and the anti-*Plasmodium* products pose little or no fitness cost to mosquitoes.

To measure *Serratia* AS1 colonization and persistence in mosquitoes, we integrated a fluorescent protein gene coding for enhanced green fluorescent protein (eGFP) or mCherry into the chromosome of the bacterium (AS1-GFP and AS1-mCherry). AS1-GFP bacteria were fed to adult mosquitoes in a sugar meal, and the ability of *Serratia* AS1 to colonize mosquito midguts was assessed at different times after feeding. AS1-GFP bacteria efficiently populated the mosquito midgut, even though these mosquitoes had an established microbiota. AS1-GFP bacterial numbers increased by more than 200-fold 24 hours after a blood meal (Fig. 1).

In addition to the midgut, AS1-GFP bacteria were found in hemolymph and ovaries (Fig. 2A and fig. S6). The presence of bacteria in ovaries raised the possibility of transmission to progeny. Indeed, we found that the bacteria attached to laid eggs, primarily on the chorion ridges and floats (Fig. 2B). These bacteria propagated in the water and were ingested by the larvae that hatched from these eggs (fig. S9).

When fed to male mosquitoes, AS1 bacteria colonized their accessory glands (Fig. 2C). To test the hypothesis that AS1 can be sexually transmitted, we allowed *Serratia*-carrying males to mate with virgin females and found that the eGFP-tagged bacteria transferred to the females and colonized their spermatheca, midgut, and ovaries (fig. S7). These results indicate that AS1 is venereally transmitted from males to females. Although larval gut microbiota are eliminated during mosquito metamorphosis (*12*), AS1 continued (possibly in the hemolymph; fig. S8A) to rapidly proliferate in the midguts of adults that emerged from these larvae (fig. S8B).

To test how well this bacterium spreads through mosquito populations, we conducted laboratory cage experiments in which virgin female and male mosquitoes, previously fed with *Serratia* AS1-mCherry and AS1-GFP, respectively,

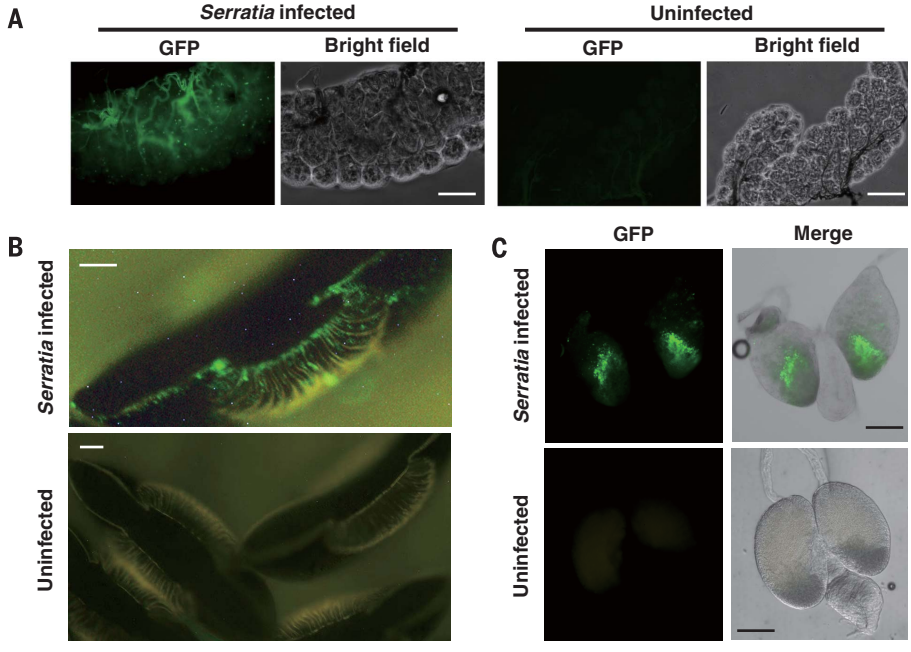


Fig. 2. *Serratia* AS1 colonizes the reproductive organs of *A. gambiae*. (**A**) *Serratia* AS1 colonization of female ovaries. AS1-GFP was fed to 2-day-old *A. gambiae* mosquitoes. Three days after females ingested blood, ovaries were dissected and visualized by fluorescent microscopy. Females not infected with AS1-GFP (right) were used as controls. (**B**) *Serratia* AS1 attaches to laid eggs. The egg in the upper image was laid by a mosquito that had been fed AS1-GFP and shows bacteria attachment to the chorion ridges. (**C**) *Serratia* AS1 colonization of male accessory glands. AS1-GFP was fed to newly emerged male mosquitoes. Three days later, the male reproductive organs were dissected and visualized by fluorescent microscopy. Scale bars, 100 μm.

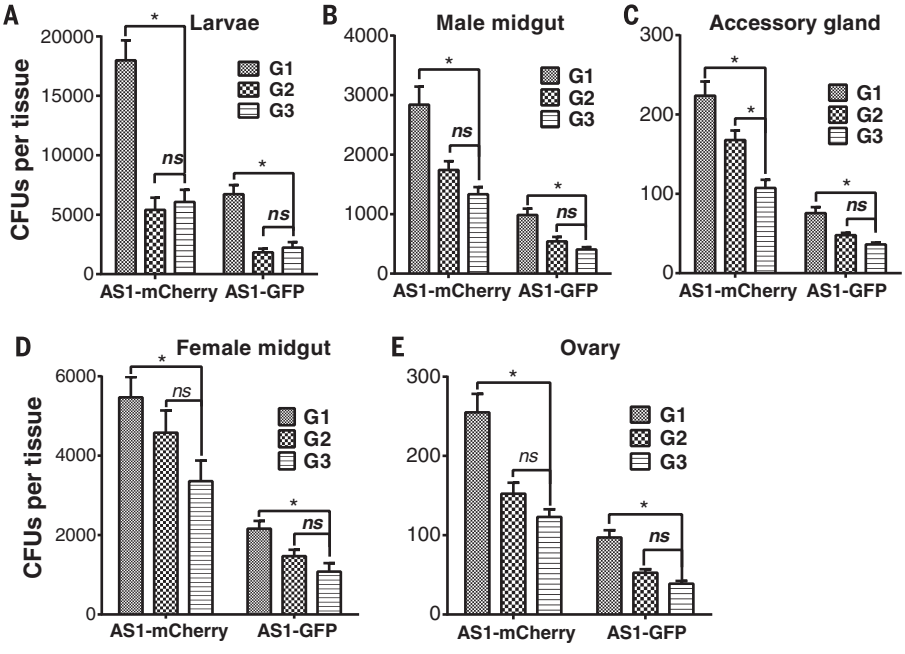
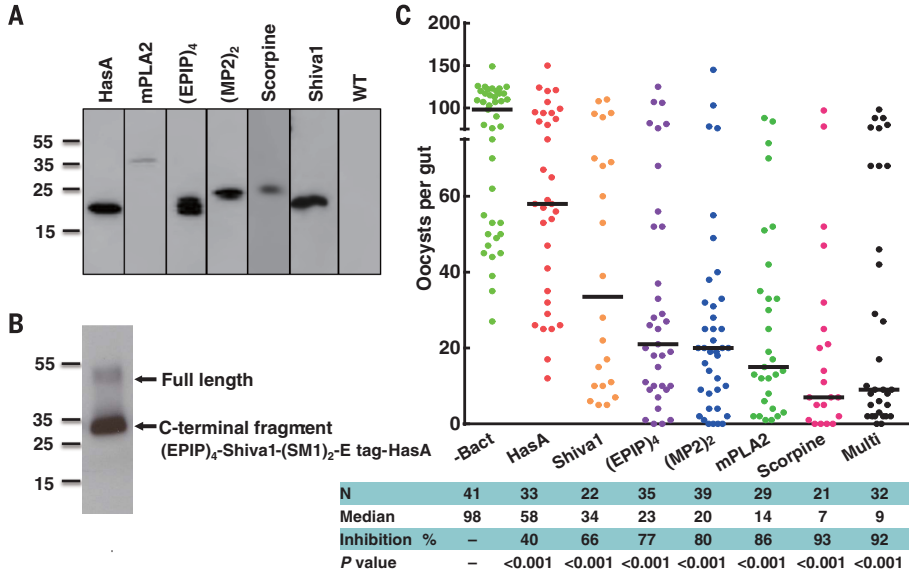


Fig. 3. *Serratia* AS1 bacteria efficiently spread throughout multiple mosquito generations. A total of 190 virgin females, 190 virgin males, 10 virgin females fed with AS1-mCherry, and 10 virgin males fed with AS1-GFP were added to a cage. After 3 days, these mosquitoes were fed blood and allowed to lay eggs. The resulting larvae were reared to adulthood following standard protocol. Bacterial load (CFUs) was determined by plating serial dilutions of tissue homogenates on LB agar plates containing 100 μg/ml of kanamycin and counting fluorescent colonies (fig. S9). (**A**) CFUs per fourth-instar larva gut. (**B**) CFUs per male midgut. (**C**) CFUs per male accessory gland. (**D**) CFUs per female midgut. (**E**) CFUs per female ovary. G1, G2, and G3 stand for first, second, and third generation, respectively. Values are means ± SEM from 10 to 15 mosquitoes in one experiment. The experiments were repeated three times with similar results. *$P < 0.05$; ns, not significant (Student's t test).



N	41	33	22	35	39	29	21	32
Median	98	58	34	23	20	14	7	9
Inhibition %	–	40	66	77	80	86	93	92
P value	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Fig. 4. Inhibition of *P. falciparum* infection of *A. gambiae* by recombinant *Serratia* strains that are engineered to secrete anti-*Plasmodium* effector molecules. (**A**) Western blot analysis of protein secretion. Culture supernatants were concentrated using Amicon Ultra-4 centrifugal filter units. Aliquots originating from equal supernatant volumes were analyzed by Western blotting using an antibody against E-tag. The culture supernatant of wild-type *Serratia* AS1 (WT) served as a negative control. HasA, secretion leader; Shiva1, a cecropin-like synthetic antimicrobial peptide; EPIP, enolase-plasminogen interaction peptide (lysine-rich enolase peptide); MP2, midgut peptide 2; mPLA2, inactive bee venom phospholipase A2; scorpine, scorpion *Pandinus imperator* venom antimicrobial peptide. (**B**) Western blot analysis of the multiprotein secretion encoded by five different effector genes (fig. S10). (**C**) Inhibition of *P. falciparum* infection of *A. gambiae* by recombinant *Serratia* strains. The recombinant bacteria were introduced into *A. gambiae* females in a sugar meal, except in the case of controls, which were fed no AS1 bacteria (−Bact). Multi, a fusion protein composed of MP2, scorpine, $(EPIP)_4$, Shiva1, and $(SM1)_2$ peptides (fig. S10). In the graph, circles represent the number of oocysts in individual midguts, and horizontal lines indicate the median number of oocysts per midgut (also listed in the table below). Data were pooled from two biological replicates. *N*, number of mosquitoes analyzed; inhibition %, percent inhibition of oocyst formation relative to the −Bact control. Statistical comparisons of oocyst intensities to those in the −Bact control were performed using the Mann-Whitney test.

were introduced into a cage at a proportion of 5%. All the progeny larvae and newly emerged adults carried both AS1-GFP and AS1-mCherry (fig. S9), indicating that *Serratia* AS1 bacteria can spread vertically, horizontally, and transstadially through a mosquito population and were maintained through a complete vector life cycle. Further cage experiments determined the efficiency of *Serratia* AS1 transmission from one generation to the next, starting with an initial introduction of 5%. We found that both AS1-mCherry originating from the introduced females and AS1-GFP originating from the introduced males were present in all larvae, adult midguts, and reproductive organs (ovary and accessory gland) for three consecutive generations (Fig. 3, A to E). These results indicate that *Serratia* AS1 originating from females and males can spread efficiently (from 5 to 100%) in one generation and then persist for subsequent multiple generations.

We used the *Serratia* HasA (heme-binding protein) exporting system (*13*) (fig. S10A) to test five potent anti-*Plasmodium* effector molecules (*11*). The MP2 (midgut peptide 2) dodecapeptide, identified from a phage display screen, binds tightly to the mosquito midgut and inhibits *P. falciparum* invasion with high efficiency (*14*). All genes were cloned in an expression vector in frame with an epitope (E)–tagged HasA and driven by a strong constitutive promoter (fig. S10B). Expression and secretion of individual fusion proteins by each recombinant *Serratia* strain were validated by Western blot analysis of bacteria culture supernatants. All showed high secretion levels (Fig. 4A). To obtain the maximum impact on parasite inhibition and minimize the probability of the development of parasite resistance, we also genetically engineered *Serratia* AS1 to simultaneously produce multiple antimalarial effector proteins with different killing mechanisms. The five effector genes were cloned in a single construct, $(MP2)_2$-*scorpine*-$(EPIP)_4$-*Shiva1*-$(SM2)_2$, under the control of a single promoter (Fig. 4B and fig. S10C).

Recombinant *Serratia* were fed to mosquitoes in sugar meals 48 hours before mosquitoes fed on a *P. falciparum*–infected blood meal. All recombinant strains strongly inhibit *P. falciparum* development (Fig. 4C). Strains expressing scorpine and multiple effector proteins were the most effective, reducing oocyst loads by 92 to 93% (Fig. 4C).

Past evidence suggests that genetic modification of mosquito vectors of malaria works efficiently in the laboratory (*15*–*18*). The challenge that remains is how to drive transgenes into wild mosquito populations. Progress has recently been made in the development of a genetic drive (*19*–*21*), but few of the ~30 to 40 known anopheline vector species are presently amenable to genetic manipulation (*22*). Moreover, anopheline vectors frequently occur as reproductively isolated populations (cryptic species) (*23*), thus preventing gene flow from one population to another.

Previously, we showed that anti-*Plasmodium* effector molecules secreted by the mosquito symbiont recombinant *Pantoea agglomerans* efficiently inhibit development of the human and rodent malaria parasites in different *Anopheles* species (*11*). This approach is equally effective with *A. gambiae* (an African mosquito) and *A. stephensi* (an Asian mosquito). The availability of multiple effector proteins with different mechanisms reduces the probability of selection of resistant parasites. However, effective means to introduce the engineered bacteria into wild mosquito populations have been lacking.

In this study, we identified a novel *Serratia* strain, AS1, that is transmitted efficiently both vertically and horizontally, in contrast to other bacterial symbionts tested (*24*, *25*). Moreover, AS1 can colonize diverse anopheline species from different continents and can be genetically manipulated for protein secretion. The engineered strains inhibited development of *P. falciparum* in mosquitoes. These antimalarial effectors (single or mixed) do not have an obvious negative impact on mosquito longevity (*11*) or fecundity and fertility (fig. S5). A close relative of *Serratia* AS1, *Serratia marcescens*, is widespread in nature (table S1). It is a dominant component of the microbiome of different wild mosquito species (*26*–*30*) and is commonly found in water, soil, and plant surfaces (*31*).

Serratia AS1 fed to adult mosquitoes crosses the midgut epithelium by an unknown mechanism and colonizes ovaries, where it adheres to eggs. How this bacterium evades the mosquito immune system is not known. Rapid spread of AS1 through wild populations could plausibly occur by dissemination in mosquito breeding water. It is important to note that eradication of malaria can only succeed by combining all available means, including insecticides, drugs, genetic modification, and possibly vaccines.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/357/6358/1399/suppl/DC1
Materials and Methods
Figs. S1 to S10
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