

RESEARCH ARTICLE

Self-limiting paratransgenesis

Wei Huang¹, Sibao Wang², Marcelo Jacobs-Lorena^{1*}

1 Department of Molecular Microbiology and Immunology, Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, United States of America, **2** 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, China

* ljacob13@jhu.edu

Abstract

Presently, the principal tools to combat malaria are restricted to killing the parasite in infected people and killing the mosquito vector to thwart transmission. While successful, these approaches are losing effectiveness in view of parasite resistance to drugs and mosquito resistance to insecticides. Clearly, new approaches to fight this deadly disease need to be developed. Recently, one such approach—engineering mosquito resident bacteria to secrete anti-parasite compounds—has proven in the laboratory to be highly effective. However, implementation of this strategy requires approval from regulators as it involves introduction of recombinant bacteria into the field. A frequent argument by regulators is that if something unexpectedly goes wrong after release, there must be a recall mechanism. This report addresses this concern. Previously we have shown that a *Serratia* bacterium isolated from a mosquito ovary is able to spread through mosquito populations and is amenable to be engineered to secrete anti-plasmodial compounds. We have introduced a plasmid into this bacterium that carries a fluorescent protein gene and show that when cultured in the laboratory, the plasmid is completely lost in about 130 bacterial generations. Importantly, when these bacteria were introduced into mosquitoes, the bacteria were transmitted from one generation to the next, but the plasmid was lost after three mosquito generations, rendering the bacteria non-recombinant (wild type). Furthermore, no evidence was obtained for horizontal transfer of the plasmid to other bacteria either in culture or in the mosquito. Prior to release, it is imperative to demonstrate that the genes that thwart parasite development in the mosquito are safe to the environment. This report describes a methodology to safely achieve this goal, utilizing transient expression from a plasmid that is gradually lost, returning the bacterium to wild type status.

OPEN ACCESS

Citation: Huang W, Wang S, Jacobs-Lorena M (2020) Self-limiting paratransgenesis. PLoS Negl Trop Dis 14(8): e0008542. <https://doi.org/10.1371/journal.pntd.0008542>

Editor: José M. C. Ribeiro, National Institute of Allergy and Infectious Diseases, UNITED STATES

Received: March 13, 2020

Accepted: June 30, 2020

Published: August 18, 2020

Copyright: © 2020 Huang et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This work was supported by a grant from the National Institute of Allergy and Infectious Diseases (<https://www.niaid.nih.gov/>) grant AI 031478 to MJL and from the National Natural Science Foundation of China (http://www.nsf.gov.cn/english/site_1/index.html) grant 31830086 to SW. We thank the Johns Hopkins Malaria Research Institute mosquito core facility for help with mosquito rearing. Additional support was provided by the Johns Hopkins Malaria Research

Author summary

The intolerable burden of malaria demands the development of novel approaches to fight this deadly disease. Previously, the engineered symbiotic bacterium *Serratia* AS1 was shown in the laboratory to be able to spread through mosquito populations and render mosquitoes resistant to the parasite. However, a major concern of regulators for the release of such engineered bacteria into the field is that there is no option for “recall” in

Institute and the Bloomberg Philanthropies. Supply of human blood was supported by National Institutes of Health (<https://www.nih.gov/>) Grant RR00052. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

case something goes wrong. Here we show that *Serratia* AS1 loses plasmids as it replicates in mosquitoes and in culture, reverting to wild type. We also show that horizontal transfer of the plasmid from *Serratia* AS1 to other bacteria is extremely rare, as this was undetectable in the mosquito and in culture. Our results suggest that initial field trials can make use of this reversible system whereby released recombinant bacteria expressing anti-plasmodial compounds from a plasmid revert to wild type at a predictable rate.

Introduction

While global malaria cases and deaths declined dramatically over the past decades, malaria incidence since 2014 has remained at similar levels [1]. Clearly, implementation of additional measures to contain this deadly disease are urgently needed. The mosquito microbiota shows great potential for fighting malaria parasite transmission by the mosquito, as the most vulnerable stages of parasite development occur in the mosquito gut, a compartment shared with the microbiota. Moreover, during parasite development within the blood bolus, the number of the surrounding bacteria increases by several hundred-fold [2]. We are exploring a strategy (paratransgenesis) that consists of engineering mosquito symbiotic bacteria to deliver anti-pathogen effector molecules [2–4]. We have shown that the *Serratia* AS1 bacterium strain isolated from *Anopheles* ovaries, can spread into mosquito populations and that this bacterium can be engineered to express anti-parasite genes [3]. Mosquitoes that carry these bacteria are largely refractory to the parasite. Translation of these findings to the field will require the approval of regulatory agencies. However, a major concern of regulators is that there should be an option for “recall” in case something goes wrong. This report addresses this concern.

Methods

Ethics statement

All animal experimental procedures were reviewed and approved by the Johns Hopkins University Animal Care and Use Committee (ACUC) under Protocol Number: M018H18.

Mosquito rearing. *Anopheles stephensi* (Dutch strain) [5] mosquitoes were maintained on 10% sterile sucrose at 27°C and 80 ± 5% relative humidity under a 14 h/10 h day-night cycle. Larvae were fed on cat food pellets and ground fish food supplement.

Bacterial culture and introduction into mosquitoes via sugar meal. *Escherichia coli* DH5 α (Invitrogen) was used for DNA cloning and plasmid amplification. *Serratia* AS1 and *E. coli* were cultured in LB broth or on agar plates at 28°C and 37°C, respectively. Bacteria were harvested by centrifugation (3,000xg, 10 min), washed twice in sterile phosphate buffered saline (PBS), and resuspended in 5% (wt/vol) sterile sucrose solution to obtain 10⁷ cells/ml. The bacterial suspension was added to sterile cotton pads and provided to two-day old mosquitoes for 24 h, then bacterial cotton pads were replaced with new sterile cotton pads containing 5% sucrose solution.

Bacterial genomic DNA isolation. Genomic *Serratia* AS1 DNA was extracted from pelleted cultures using a Qiagen DNeasy Blood and Tissue kit (cat. 69504).

***Serratia* AS1 sensitivity to antibiotics.** *Serratia* AS1 was cultured overnight at 200 rpm at 28°C and 10⁴ cells were spread on LB plates with or without ampicillin, kanamycin, chloramphenicol, streptomycin, apramycin, neomycin, tetracycline, rifampin, metronidazole, 5-fluorocytosine, spectinomycin and carbenicillin, and cultured for 24 h or 48 h at 28°C.

Construction of vectors. The apramycin cassette was amplified from the pDB47 plasmid [2] using primers ApraF and ApraR (S7 Table), PnptII-eGFP was amplified from the pUT-mini-km2-GFP [3] plasmid using primers GFPF and GFPR, with *NotI* recognition sites incorporated at the 5'- and 3'-ends. The PCR product was fused into the *NotI* site of the plasmid pUT-mini-Tn5-Cm [6] using the In-Fusion HD Cloning Kit, to generate pUT-mini-Apra-GFP. The *nptII* promoter was amplified from the pUT-mini-km2-GFP plasmid using primers pnptII1 and pnptII [2], mCherry was amplified from the mCherry-*Serratia* genome [3] using primers mCherry3 and mCherry4. The pnptII-mCherry fragment was amplified from the *nptII* promoter and mCherry fragments by fusion-PCR (Primer: pnptII1 and mCherry4). To construct pHL662-mCherry, pnptII-mCherry was PCR-amplified using primers mCherry5 and mCherry6, the PCR product was digested with *XmaI* and *HindIII* and cloned into the *XmaI* and *HindIII* sites of the plasmid pHL662 [7]. To construct punc-119c-mCherry, pnptII-mCherry was amplified using primers mCherry7 and mCherry8 and the PCR product was digested with *SpeI* and cloned into the *SpeI* site of the plasmid punc-119c [8].

Measurement of plasmid loss rate. The pMyc Vec2 [9], punc-119c, pet-GFP [10], pHL662, SK(SK-YFP-ST1-A) [11], and pDB47 plasmids were separately electro-transformed into *Serratia* AS1. *Serratia* with or without the plasmid was cultured in liquid LB with or without antibiotic. The culture was started as 10^{-3} or 10^{-6} OD, and samples were tested for concentration and plasmid loss every 4 or 8 h (log phase) [12]. Generation = $\log_2(\text{Final concentration}/\text{initial concentration})$. Plasmid loss in the mosquito used *Serratia* that had apramycin-resistance (Apra^R) and GFP genes integrated into its genome. Two-day-old adult mosquitoes (50 males and 50 females) were allowed to feed for 24 h on a cotton pad moistened with 5% sterile sucrose solution containing 10^7 *Serratia* /ml carrying the punc-119c-mCherry or pHL662-mCherry plasmids. Larvae and adult mosquitoes of different stages were homogenized and plated on LB plates with or without apramycin for the determination of fluorescent colony numbers.

Plasmid copy number (PCN) determination. DNA of *Serratia* AS1 was extracted from pelleted cultures using a Qiagen DNeasy Blood and Tissue kit (cat. 69504). The PCN was calculated by dividing the copy number of the plasmid kanamycin gene by the single copy chromosomal gene LuxS (Accession number: AP019009.1) as control. Applied Biosystems Step One Plus Real-time PCR System was used for qPCR amplification and detection. Real-time qPCR reactions were performed in triplicate using 20 μ l mixtures. Each reaction contained 10 μ l of 2 \times SYBR Green PCR Master Mix (Toyobo, Kyoto, Japan), 1 μ l of template DNA, and 12.5 pmol of each primer. The PCR reaction was conducted for all amplicons with the following cycling conditions: 2 min at 95 $^{\circ}$ C, followed by 40 cycles of 95 $^{\circ}$ C for 15 s, 60 $^{\circ}$ C for 20 s, and 72 $^{\circ}$ C for 30 s. Upon completion of 40 cycles of PCR amplification, a dissociation step of ramping the temperature from 55 $^{\circ}$ C to 95 $^{\circ}$ C steady for 20 min was performed, while the fluorescence signal was continually monitored for melting curve analysis. We determined the cycle threshold (Ct) values after automatic adjustment of the baseline and manual adjustment of the threshold using Stepone software V2.3. The plasmid DNA standard curve was established according to the method of Lee et al [13]. Briefly, the standard curve includes a plot of the Ct values versus the log concentration of the plasmid DNA standard. For total DNA sample, the absolute quantity of both plasmid and chromosomal DNA were obtained by interpolating the Ct value against the standard curve.

Plasmid horizontal transfer in culture. *E. coli* was selected for its known ability to acquire plasmids and for use in conjugation positive controls. In addition, *P. agglomerans* was selected because it is one of the most common, abundant and well-studied mosquito symbionts. Donor and recipient cells were cultured separately overnight at 200 rpm at 28 $^{\circ}$ C (for *Serratia* AS1 and *P. agglomerans*) or 37 $^{\circ}$ C (for *E. coli*). We used two methods to measure the horizontal gene transfer.

For the first method (solid medium; Table 2), 10^{12} donor and recipient cells (ratio = 1:1) were mixed in 50 ml PBS, spread on ten 15 cm-LB agar plates and incubated for 6 h at 28 °C, after which all bacteria were collected from the plates with 100 ml LB. For the second method (static medium; S4 Table), 10^{12} donor and recipient cells (ratio = 1:1) were mixed in 200 ml LB culture and incubated for 6 h at 28 °C without shaking [14, 15]. For both methods, the entire culture was mixed with soft agar containing kanamycin and apramycin (or kanamycin and ampicillin for the *E. coli* controls), and plated on 15 cm-LB agar plates (20 ml each plate) (S1 Fig).

Horizontal transfer in mosquitoes. Two-day old mosquitoes were fed on *Serratia* (with chromosomally integrated apramycin and GFP genes) carrying the punc-119c-mcherry or pHL662-mcherry plasmids. Larvae or adult mosquito midguts of different stages were homogenized and spread on kanamycin LB plates for counting of fluorescent colonies. Horizontal transfer rate = mCherry CFUs/Total CFUs.

Statistical analyses. Multiple-sample comparisons were analyzed using the non-parametric Kruskal-Wallis test and medians were compared using Dunn's test. Other statistical significance was calculated using Student's t-test for unpaired comparisons between two treatments. A value of $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software).

Results

We investigated whether upon replication, *Serratia* AS1 would lose an introduced plasmid, reverting to wild type. This *Serratia* AS1 is sensitive to apramycin, kanamycin, streptomycin, rifampin and spectinomycin (S1 Table). We tested six plasmids carrying different origins of replication plus an antibiotic-resistance gene (S2 Table). In culture, all plasmids were lost in about 130 generations (Fig 1A). There was a lag in loss of antibiotic resistance during the first ~100 generations because each bacterium carried ~15 or ~38 copies of the plasmid (S3 Table) and antibiotic resistance is lost only with the complete loss of the plasmid. There was a small but significant difference in the plasmid loss rate ($P < 0.01$): pHL662, SK(SK-YFP-ST1-A), and pDB47 plasmids were lost faster than the pMycVec2, punc-119c and pet-GFP plasmids (Fig 1A). We chose one plasmid from each group to investigate the loss rate when the bacteria are maintained through multiple mosquito generations (Fig 1B) [3]. To distinguish *Serratia* AS1 from the natural mosquito microbiome components, we stably integrated the GFP (green) and apramycin-resistance genes into its genome and introduced mCherry-tagged (red) plasmids into this bacterium. These bacterial colonies display both green and red fluorescence. However, if the plasmid gets lost, the colonies will display only green fluorescence. We found that the plasmids were completely lost in three mosquito generations, and pHL662-mCherry was lost faster than punc-119c-mCherry ($P < 0.05$) (Fig 1B).

An additional potential concern is whether the genetic material carried by the plasmids will horizontally transfer to neighboring bacteria. We fed mosquitoes with *Serratia* AS1 that had chromosomally-integrated a GFP gene (green) and that carry either the punc-119c-mcherry-kanR or the pHL662-mcherry-kanR plasmids (red) and followed these bacteria for three mosquito generations. Horizontal transfer to microbiome bacteria would result in red (but not green) fluorescent kanamycin-resistant bacteria, while the donor bacteria have green or both green and red fluorescence. A total of 648,860 (punc-119c-mCherry) and 861,250 (pHL662-mCherry) bacteria were assayed and no evidence for horizontal transfer was obtained (Table 1, S5 and S6 Tables). Because the number of bacteria that can be assayed in mosquito experiments is limited, we designed horizontal transfer experiments in culture that used a much larger bacteria number (10^{12}) (S1 Fig). No transfer was detected from *Serratia* AS1 to *Escherichia coli* or to *Pantoea agglomerans*, the latter being a common component of

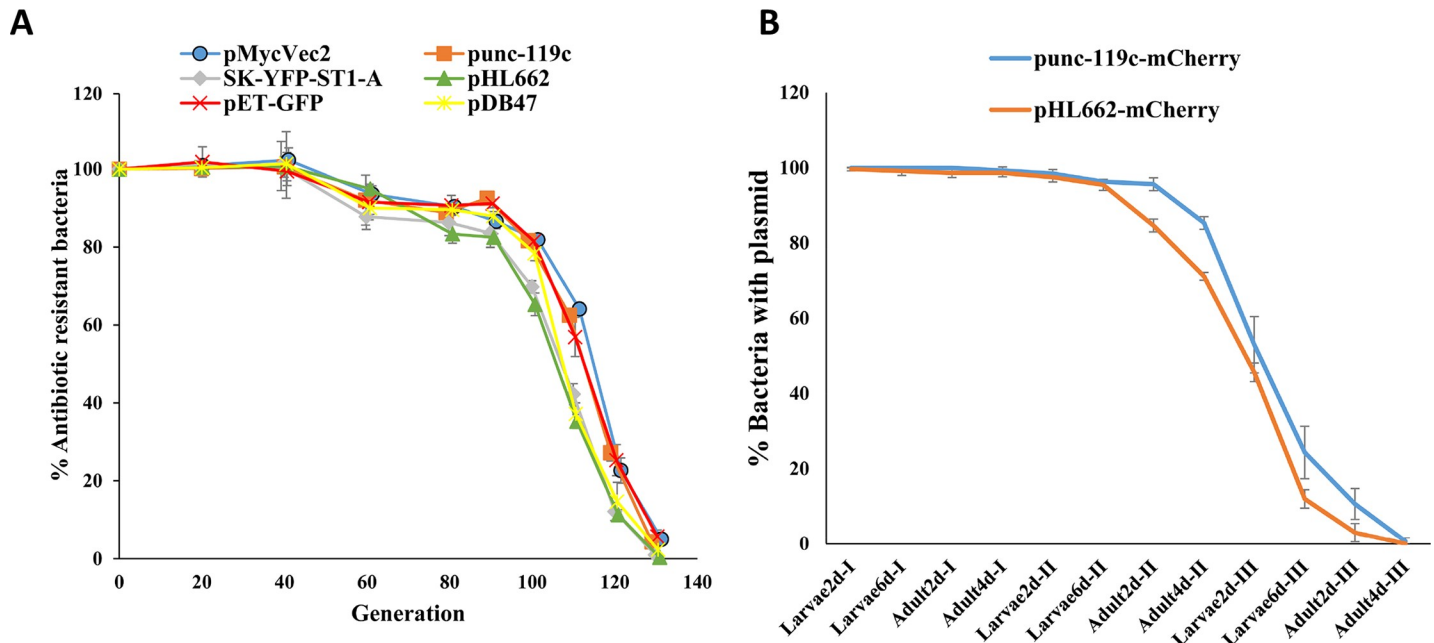


Fig 1. Measurement of plasmid loss rate. **A**, *Serratia* AS1 carrying plasmids encoding antibiotic resistance (S2 Table) were grown in liquid culture. Samples were taken every 4 or 8 h and colony forming units (CFU) were determined on LB plates with and without antibiotics, to measure bacteria growth and plasmid loss events. Data from three independent experiments were pooled. **B**, Chromosomally GFP-labelled (green) and apramycin-resistant *Serratia* AS1 carrying the indicated mCherry-tagged (red) plasmid were fed to 100 mosquitoes (50 males and 50 females) and these mosquitoes were propagated through three generations (I–III). Larvae (2- and 6-day old) and adults (2- and 4-day old) were homogenized and plated on apramycin plates to determine what percent of the green-fluorescent bacteria carry the mCherry-tagged plasmid. Data from three independent experiments were pooled.

<https://doi.org/10.1371/journal.pntd.0008542.g001>

mosquito microbiomes (Table 2 and S4 Table). In a positive control, the transfer rate from *E. coli* S17-1 with an F⁺ plasmid to *Serratia* AS1 was 4.3×10^{-6} (solid plate; Table 2) or 4.3×10^{-6} (static medium; S4 Table). In short, horizontal transfer from *Serratia* AS1 to bacteria from the mosquito microbiome or to bacteria in culture was undetectable.

Discussion

The low genetic stability and ease of manipulation of plasmid-based overexpression are advantageous features for initial field experiments, as they minimize environmental risk. Bacteria that do not inherit a plasmid may have a fitness advantage in that they multiply faster. In addition, plasmid loss rate as bacteria multiply depends on plasmid rate of replication that in turn determines Plasmid Copy Number per bacterium (PCN) [16,17]. In support of this assertion, plasmid pHL662 that has a PCN 14.6 is lost faster (Fig 1) than plasmid punc-119c that has a PCN 37.8 (S3 Table). Therefore, the half-life of recombinant gene expression after bacteria release may be adjusted to some extent.

Whereas *Serratia* AS1 loses the plasmid relatively rapidly during logarithmic growth in culture (about 130 generations or 40~45 hours), the dynamics in the mosquito are very different.

Table 1. Horizontal transfer in mosquitoes.

Plasmid	LB (total bacteria number)	LB/Kan GFP ⁺ & mCherry ⁺ (donor bacteria number)	LB/Kan mCherry ⁺ -only (recipient bacteria number)
punc-119c-mCherry	648,860	18,419	0
pHL662-mCherry	861,250	15,357	0

In vivo horizontal transfer of the punc-119c-mcherry and pHL662-mcherry plasmids from *Serratia* AS1 to the mosquito microbiome. *Serratia* carrying the plasmid were fed to mosquitoes (50 males and 50 females) and maintained for three generations (S5 and S6 Tables). No evidence of plasmid transfer was detected.

<https://doi.org/10.1371/journal.pntd.0008542.t001>

Table 2. Horizontal transfer in culture (solid plate).

Donor cell (10 ¹²)	Carried plasmid	Recipient cell (10 ¹²)	Transfer rate
<i>Serratia</i> AS1	punc-119c	<i>Serratia</i> AS1/Apra+GFP	0
<i>Serratia</i> AS1	punc-119c	<i>P. agglomerans</i> /Apra+GFP	0
<i>Serratia</i> AS1	punc-119c	<i>E. coli</i> (DH5 α)/Apra+GFP	0
<i>Serratia</i> AS1	pHL662	<i>Serratia</i> AS1/Apra+GFP	0
<i>Serratia</i> AS1	pHL662	<i>P. agglomerans</i> /Apra+GFP	0
<i>Serratia</i> AS1	pHL662	<i>E. coli</i> (DH5 α)/Apra+GFP	0
<i>E. coli</i> (S17-1)	F ⁺	<i>Serratia</i> AS1	7.8 \times 10 ⁻⁶
<i>E. coli</i> (S17-1)	pHL662 (F ⁻)	<i>Serratia</i> AS1	0

Horizontal transfer of the indicated plasmid was performed using 10¹² donor and recipient bacteria each, incubated on solid plates for 6 h (Method 1; see [Methods](#)). Positive and negative control experiments were performed using *E. coli*(S17-1) carrying an F⁺ or the pHL662 (F⁻) plasmid, respectively. See also [S1 Fig](#) and [S4 Table](#). The punc-119c and pHL662 plasmids carry a kanamycin-resistance gene; the F⁺ plasmid carries an ampicillin-resistance gene; *Serratia* AS1 is naturally resistant to ampicillin. Transfer rate = transconjugants/recipients. Pooled data from two independent experiments.

<https://doi.org/10.1371/journal.pntd.0008542.t002>

Growth of bacteria in the mosquito is limited by nutrient conditions, oxygen levels and other micro-environment factors, amounting to a much slower replication. *Serratia* AS1 persists in mosquitoes for three generations (50~60 days). Importantly, about 50% of these bacteria carry the plasmid at the beginning of the F3 generation, implying that these bacteria express the anti-parasite effectors. Recall that the intent here is to obtain evidence for safety of the expressed effector molecules, not to implement a broad anti-malaria campaign.

The finding that no plasmid horizontal transfer was detected in mosquitoes has two caveats. One is that it is not known whether transfer may have occurred to non-culturable mosquito bacteria, as such an assay is not easy to implement. The other is that the total number of bacteria that can be tested in an *in vivo* experiment is relatively small. To address this caveat, we assayed horizontal transfer in culture from *Serratia* AS1 to *E. coli* and *P. agglomerans*, a ubiquitous component of the mosquito microbiome using very high bacteria numbers (10¹²) and conditions that favor bacteria-bacteria contact. Even under these conditions that strongly favor plasmid transfer, none was observed. Whereas the possibility of a very rare gene transfer in the mosquito cannot be excluded, such event should have minimal consequences as the genes encoded by the plasmid (fluorescent proteins, anti-plasmodial small proteins) would not confer fitness advantage to the recipient cell.

The approach proposed here is envisioned as a prelude for full implementation of the paratransgenesis strategy. Once environmental safety of the anti-*Plasmodium* compounds is corroborated, *Serratia* carrying effector genes stably integrated in their chromosome would be constructed. Introduction into the field can be accomplished by mixing the bacteria with attractive sugar baits (both male and female mosquitoes need sugar for survival [18]). Because the engineered *Serratia* AS1 is sexually transmitted from male to female mosquitoes and is passed from one generation to the next by attaching to eggs, it can efficiently spread into mosquito populations [3]. Paratransgenesis is compatible with other malaria-containment measures, including the use of insecticides and mosquito transgenesis.

Supporting information

S1 Table. *Serratia* AS1 sensitivity to antibiotics. *Serratia* AS1 was plated on LB plates with or without 50 μ g/ml ampicillin, 50 μ g/ml kanamycin, 34 μ g/ml chloramphenicol, 50 μ g/ml streptomycin, 80 μ g/ml apramycin, 10 μ g/ml tetracycline, 10 μ g/ml rifampin, 50 μ g/ml

metronidazole, 0.5 µg/ml 5-fluorocytosine or 50 µg/ml spectinomycin and cultured for 24 h or 48 h at 28 °C.

(DOCX)

S2 Table. Plasmids used in this study.

(DOCX)

S3 Table. Plasmid copy number in *Serratia* AS1. Plasmid copy number was determined by quantitative PCR as detailed in Methods. Pooled data from three independent experiments.

(DOCX)

S4 Table. Horizontal transfer in culture (in static medium). Horizontal transfer of the indicated plasmid was performed using 10^{12} donor and recipient bacteria each, incubated in liquid medium without agitation for 6 h (Method 2; see [Methods](#)). Positive and negative control experiments were performed using *E. coli*(S17-1) carrying an F⁺ or the pHL662 (F⁻) plasmid, respectively. See also [S1 Fig](#) and [Table 2](#). The punc-119c and pHL662 plasmids carry a kanamycin-resistance gene; the F⁺ plasmid carries an ampicillin-resistance gene; *Serratia* AS1 is naturally resistant to ampicillin. Transfer rate = transconjugants/recipients. Pooled data from three independent experiments.

(DOCX)

S5 Table. punc-119c-mCherry horizontal transfer in mosquitoes. Chromosomally GFP-labelled *Serratia* AS1 carrying the indicated mCherry-tagged plasmid were fed to mosquitoes and these mosquitoes were propagated through three generations (I–III). Larvae (2- and 6-day old) and adults (2- and 4-day old) were homogenized and plated on LB plates without (Total bacteria) and with kanamycin to determine percent fluorescent bacteria carrying the plasmid. The *Serratia* AS1 fed to the mosquitoes form green- and red-fluorescent kanamycin-resistant colonies, while transfer of the plasmid to bacteria from the mosquito microbiome would form red-fluorescent-only kanamycin-resistant colonies. No such colonies were found. Pooled data from three independent experiments.

(DOCX)

S6 Table. pHL662-mCherry horizontal transfer in mosquitoes. Chromosomally GFP-labelled *Serratia* AS1 carrying the indicated mCherry-tagged plasmid were fed to mosquitoes and these mosquitoes were propagated through three generations (I–III). Larvae (2- and 6-day old) and adults (2- and 4-day old) were homogenized and plated on LB plates without (Total bacteria) and with kanamycin to determine percent fluorescent bacteria carrying the plasmid. The *Serratia* AS1 fed to the mosquitoes form green- and red-fluorescent kanamycin-resistant colonies, while transfer of the plasmid to bacteria from the mosquito microbiome would form red-fluorescent-only kanamycin-resistant colonies. No such colonies were found. Pooled data from three independent experiments.

(DOCX)

S7 Table. Oligonucleotide primers used in this study.

(DOCX)

S1 Fig. Horizontal transfer in culture. Rate of transfer of plasmid between donor and recipient strains. Only bacteria that received the plasmid can grow on [apramycin + kanamycin] LB plates. (TIF)

Author Contributions

Conceptualization: Marcelo Jacobs-Lorena.

Data curation: Wei Huang.

Formal analysis: Wei Huang.

Funding acquisition: Marcelo Jacobs-Lorena.

Investigation: Wei Huang, Marcelo Jacobs-Lorena.

Methodology: Wei Huang, Marcelo Jacobs-Lorena.

Project administration: Marcelo Jacobs-Lorena.

Resources: Sibao Wang.

Supervision: Marcelo Jacobs-Lorena.

Writing – original draft: Wei Huang.

Writing – review & editing: Wei Huang, Sibao Wang, Marcelo Jacobs-Lorena.

References

1. WHO. World malaria report. Geneva: World Health Organization. 2019: <https://www.who.int/publications/i/item/world-malaria-report-2019>.
2. Wang S, Ghosh AK, Bongio N, Stebbings KA, Lampe DJ, Jacobs-Lorena M. Fighting malaria with engineered symbiotic bacteria from vector mosquitoes. *Proc Natl Acad Sci USA*. 2012; 109:12734–39. <https://doi.org/10.1073/pnas.1204158109> PMID: 22802646
3. Wang S, Dos-Santos ALA, Huang W, Liu KC, Oshaghi MA, Wei G, et al. Driving mosquito refractoriness to *Plasmodium falciparum* with engineered symbiotic bacteria. *Science*. 2017; 357:1399–1402. <https://doi.org/10.1126/science.aan5478> PMID: 28963255
4. Shane J. L., Grogan C. L., Cwalina C., & Lampe D. J. Blood meal-induced inhibition of vector-borne disease by transgenic microbiota. *Nature communications*. 2018; 9(1), 1–10. <https://doi.org/10.1038/s41467-017-02088-w>
5. Jaramillo-Gutierrez G., Rodrigues J., Ndikuyeze G., Povelones M., Molina-Cruz A., Barillas-Mury C. Mosquito immune responses and compatibility between Plasmodium parasites and anopheline mosquitoes. *BMC microbiology*. 2009; 9(1): 154.
6. Friehs K. Plasmid copy number and plasmid stability. *Adv Biochem Eng Biotechnol*. 2004; 86:47–82. <https://doi.org/10.1007/b12440> PMID: 15088763
7. Lau BT, Malkus P, Paulsson J. New quantitative methods for measuring plasmid loss rates reveal unexpected stability. *Plasmid*. 2013; 70:353–61. <https://doi.org/10.1016/j.plasmid.2013.07.007> PMID: 24042048
8. de Lorenzo V, Herrero M, Jakubzik U, Timmis KN. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J Bacteriol*. 1990; 172:6568–72. <https://doi.org/10.1128/jb.172.11.6568-6572.1990> PMID: 2172217
9. Chen S, Larsson M, Robinson RC, Chen SL. Author Correction: Direct and convenient measurement of plasmid stability in lab and clinical isolates of *E. coli*. *Sci Rep*. 2018; 8:6056. <https://doi.org/10.1038/s41598-018-23595-w> PMID: 29643388
10. Adamson DN, Lim HN. Rapid and robust signaling in the CsrA cascade via RNA-protein interactions and feedback regulation. *Proc Natl Acad Sci USA*. 2013; 110:13120–25. <https://doi.org/10.1073/pnas.1308476110> PMID: 23878244
11. Ferguson AA, Fisher AL. Retrofitting ampicillin resistant vectors by recombination for use in generating *C. elegans* transgenic animals by bombardment. *Plasmid*. 2009; 62:140–5. <https://doi.org/10.1016/j.plasmid.2009.06.001> PMID: 19520111
12. Kaps I, Ehrh S, Seeber S, Schnappinger D, Martin C, Riley LW, et al. Energy transfer between fluorescent proteins using a co-expression system in *Mycobacterium smegmatis*. *Gene*. 2001; 278:115–24. [https://doi.org/10.1016/s0378-1119\(01\)00712-0](https://doi.org/10.1016/s0378-1119(01)00712-0) PMID: 11707328
13. Shis DL, Bennett MR. Library of synthetic transcriptional AND gates built with split T7 RNA polymerase mutants. *Proc Natl Acad Sci USA*. 2013; 110:5028–33. <https://doi.org/10.1073/pnas.1220157110> PMID: 23479654

14. Esvelt KM, Mali P, Braff JL, Moosburner M, Yaung SJ, Church GM. Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nat Methods*. 2013; 10:1116–21. <https://doi.org/10.1038/nmeth.2681> PMID: 24076762
15. Lee C, Kim J, Shin SG, Hwang S. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. *J Biotechnol*. 2006; 123:273–80. <https://doi.org/10.1016/j.jbiotec.2005.11.014> PMID: 16388869
16. Cafini F, Thi Le Thuy N, Roman F, Prieto J, Dubrac S, Msadek T, et al. Methodology for the Study of Horizontal Gene Transfer in *Staphylococcus aureus*. *J Vis Exp*. 2017; 121. <https://doi.org/10.3791/55087> PMID: 28362383
17. Cafini F, Nguyen le TT, Higashide M, Roman F, Prieto J, Morikawa K. Horizontal gene transmission of the *cf* gene to MRSA and *Enterococcus*: role of *Staphylococcus epidermidis* as a reservoir and alternative pathway for the spread of linezolid resistance. *J Antimicrob Chemother*. 2016; 71:587–92. <https://doi.org/10.1093/jac/dkv391> PMID: 26661395
18. Barredo E, DeGennaro M. Not Just from Blood: Mosquito Nutrient Acquisition from Nectar Sources. *Trends in Parasitology*, 2020.