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Genetic approaches to interfere with malaria transmission by vector mosquitoes

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Abstract

Malaria remains one of the world's most devastating diseases, causing over one million deaths every year. The most vulnerable stages of *Plasmodium* development in the vector mosquito occur in the midgut lumen, making the midgut a prime target for intervention. Mosquito transgenesis and paratransgenesis are two novel strategies that aim at rendering the vector incapable of sustaining *Plasmodium* development. Mosquito transgenesis involves direct genetic engineering of the mosquito itself for delivery of anti-*Plasmodium* effector molecules. Conversely, paratransgenesis involves the genetic modification of mosquito symbionts for expression of anti-pathogen effector molecules. Here we consider both genetic manipulation strategies for rendering mosquitoes refractory to *Plasmodium* infection, and discuss challenges for the translation of laboratory findings to field applications.

Keywords

malaria; transgenesis; paratransgenesis; mosquitoes; effector molecules

Malaria control: present and future

Malaria is a major cause of global morbidity and mortality. Close to half of the world's population is at risk, about 300–500 million contract the disease annually and more than one million people die of malaria every year [1]. Clearly, the available means to fight the disease are insufficient. Unlike the two other major infectious diseases - AIDS and tuberculosis – *Plasmodium*, the causative agent of malaria, is absolutely dependent on completing a complex cycle in the vector *Anopheles* mosquito for transmission to occur [2]. Thus, eliminating the mosquito or interfering with its ability to support the parasite cycle will arrest malaria transmission. Current malaria control measures targeting the mosquito vector with insecticides have helped alleviate the malaria burden in many endemic areas [3]. However, the emergence and rapid spread of insecticide-resistant mosquitoes and of drug-resistant *Plasmodium* parasites combined with the lack of an effective vaccine severely

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undermine current control efforts [4]. Another rarely considered but equally important limitation is that insecticides leave intact the biological niche where mosquitoes reproduce. The mosquito populations that decline after insecticide use quickly rebound to pre-treatment levels when insecticide treatment stops or becomes ineffective.

Recently, the malERA consultative group stressed that malaria eradication cannot be achieved without introduction of novel control tools [5]. Transgenesis and paratransgenesis are two novel approaches for rendering mosquitoes refractory to *Plasmodium* infection. Here, we review recent advances on genetic approaches for interfering with the malaria parasite cycle in vector mosquitoes and consider the challenges in the translation of laboratory findings to field applications.

The malaria parasite cycle in the mosquito

Plasmodium development in the mosquito is complex and involves the completion of multiple developmental steps in the midgut (gametogenesis, fertilization, followed by zygote, ookinete and oocyst formation) and the crossing of two mosquito epithelia (midgut and salivary gland) (Figure 1a). While thousands of gametocytes are ingested when a female mosquito feeds on an infected individual, only about 10% successfully develop into ookinetes and about five of these succeed in invading the midgut epithelium to form sessile oocysts [6]. This is followed by a dramatic amplification of parasite numbers, when each oocyst releases thousands of sporozoites into the hemocoel after which they invade the mosquito salivary glands. The parasite is transmitted to the next individual through the bite of an infected mosquito [7]. The severe bottleneck in the mosquito gut (Figure 1b) makes this compartment a prime target for interfering with the parasite cycle in its vector [8, 9].

Genetic manipulation of mosquito vectorial competence

Since the mosquito is essential for parasite transmission, hindering the mosquito's ability to support parasite development will reduce or eliminate malaria transmission. One option to interfere with parasite transmission is to genetically modify the mosquito for midgut expression of "effector genes" whose products inhibit parasite development. This proof of concept was tested for the first time by genetically engineering *Anopheles stephensi* for midgut expression of the SM1 peptide. This peptide binds to a putative ookinete receptor on the luminal surface of the midgut epithelium and strongly inhibits ookinete midgut invasion [10]. The genetically modified mosquitoes were substantially impaired in their ability to transmit the parasite [11]. Subsequent reports from different laboratories making use of a variety of effector molecules reached similar conclusions [12–17]. Collectively, these studies constituted proof-of-concept that it is possible to reduce *Plasmodium* transmission via genetic modification of the vector mosquito. One crucial challenge for translating these findings to the field is to devise effective means to drive anti-malaria effector genes into wild mosquito populations. It is not sufficient to simply release large numbers of transgenic mosquitoes. An effective drive mechanism must be devised to give mosquitoes carrying effector genes a competitive advantage. Of several potential approaches that have been proposed, including the use of transposable elements or *Wolbachia*, two – MEDEA (maternal-effect dominant embryonic arrest) and HEG (homing endonuclease gene) – are particularly promising. The MEDEA drive system has shown promisein experiments using the *Drosophila* model system [18]. The approach is based on linking the effector gene to a maternal gene that is required for embryonic development and is inactivated in the ovary, and a second gene that rescues the defect by embryonic expression of the same gene. The technologies for transferring this approach to mosquitoes (e.g., identification of mosquito maternal effect genes and of mosquito embryonic promoters for the rescue constructs) have not yet been developed. The *Drosophila* experiments indicated that a high initial

introduction rate (~25%) was required which may constitute a limiting factor for field applications. More recently, another promising approach is being explored that makes use of HEG drive system [19–20]. This study showed that in cage experiments, the homing endonuclease gene *I-SceI* can rapidly spread among the transgenic *An. gambiae* progeny. An additional consideration for any gene drive approach is that there are on the order of 430 anopheline species, about 30–40 of which are natural vectors for human malaria [21] and very few of them have been shown to be amenable to genetic manipulation [22]. Moreover, anopheline vectors frequently exist as reproductively isolated populations (cryptic species) [23], thus preventing gene flow from one population to another. In addition, fitness load imposed by refractory gene(s), insertional mutagenesis and positional effects will have to be considered [15, 24, 25]. Once these issues are resolved, transgenesis could provide a powerful tool to combat malaria.

Anti-malarial effector genes

The identification of efficient anti-*Plasmodium* effector genes is an essential prerequisite for the generation of a refractory mosquito. Ideally, the effector molecules should interfere with parasite transmission without imposing a fitness cost to the mosquito. Based on their mode of action, the existing anti-*Plasmodium* effector molecules can be grouped into four classes (Table 2).

i. ***Parasite killing.*** This class includes peptides from the insect's innate immune system such as defensins [26], gambicin [27] and cecropins [28], and peptides from other sources that lyse parasites but do not affect the host insect, such as scorpine, a scorpion anti-malaria lytic peptide that has hybrid properties of the lytic peptides cecropin and defensin [29, 30], Hemolytic C-Type Lectin CEL-III [13], angiotensin II [31], magainins [32], synthetic anti-parasitic lytic peptides Shiva-1 and Shiva-3 [33] and gomesin [34].

ii. ***Interaction with parasites***: EPIP, a *Plasmodium* **E**nolase-**P**lasminogen **I**nteraction **P**eptide, is a peptide that inhibits mosquito midgut invasion by preventing plasminogen binding to the ookinete surface [35]. Other agents are single-chain monoclonal antibodies (scFvs) that bind to ookinete or sporozoite surface or secreted proteins. For instance, scFv 4B7 binds to *P. falciparum* ookinete surface protein Pfs25, 2A10 targets the *P. falciparum* circumsporozoite protein (CSP) [16, 36], anti-Pbs21 single chain antibody targets the *P. berghei* major ookinete surface protein Pbs21 [37], and scFv 1C3 binds a *P. falciparum* secreted enzyme chitinase 1 [16].

iii. ***Interaction with mosquito midgut or salivary gland epithelia:*** Examples of this class are SM1 – a 12-amino acid **S**alivary gland and **M**idgut peptide **1**, which binds to putative receptors on the luminal surface of the mosquito midgut and basal surface of the salivary gland epithelia, blocking ookinete and sporozoite invasion [10]; mPLA2 is a mutant phospholipase A2 that inhibits ookinete invasion, possibly by modifying the properties of the midgut epithelial membrane [12, 30, 38]; and a chitinase propeptide that inhibits this enzyme and in this way hinders ookinete traversal of the mosquito peritrophic matrix (PM) [39]. The PM is a chitin-based extracellular structure that surrounds the entire blood meal and must be crossed by the ookinete for reaching the mosquito midgut [40].

iv. ***Manipulation of mosquito immune system.*** Several laboratories have shown that alteration of mosquito immune-related genes can lead to decreased mosquito vectorial competence. Blood meal-induced expression of Akt, a key signaling component in the insulin signaling pathway renders the mosquito refractory to *Plasmodium* infection [15]. Overexpression of IMD pathway-mediated

transcription factor Rel2 renders the mosquito resistant to *Plasmodium* infection [14]. Manipulation of mosquito immune pathway using RNA interference or 'smart sprays' enhances mosquito anti-microbial response [41, 42].

We note that the identification of efficient effector molecules is as valuable to transgenesis as to paratransgenesis. A potential issue regarding anti-malaria effector molecules is that in the long run, parasites may develop resistance to their action in similar ways that they develop resistance to drugs that kill them in the human blood. For this reason, it will be important to combine multiple anti-*Plasmodium* effector proteins with different modes of action. In addition, the use of multiple effector genes will also maximize the effectiveness of interference with parasite development (additive or synergistic effects).

Genetic manipulation of mosquito pathogenic fungi and viruses

Insect fungal pathogens, *Metarhizium robertsii* and *Beauveria bassiana* are natural killer of insects including mosquitoes [43]. Several studies highlight the promising use of insect fungal pathogens for controlling adult malaria mosquitoes and reducing malaria transmission rates [44–49]. Recently, *M. robertsii* was genetically engineered to deliver anti-*Plasmodium* peptides or proteins into the mosquito hemocoel for killing sporozoites or preventing sporozoite invasion of mosquito salivary glands. The transgenic fungi significantly reduced sporozoite density in salivary glands [50], indicating that genetic modification of pathogenic fungi provides an enhanced tool to reduce malaria transmission.

Linear single-stranded DNA densoviruses were also found to infect several important vector mosquitoes (*Aedes aegypti* [51], *An. gambiae* [52], and *Culex pipiens* [53]), and to be able to be vertically transmitted [52, 54]. The densovirus AgDNV was found to efficiently infect *An. gambiae* larvae and to spread to the adult midgut, fat body and ovaries [52]. This virus was also found to be vertically transmitted to subsequent mosquito generations [52]. These properties suggest that densoviruses could be used to produce effector molecules in host mosquitoes [54]. However, the limited length of foreign DNA that these viruses can carry may become a limiting factor.

Mosquito symbionts and other associated organisms

Microbial associations with insects are ubiquitous and play an important role in shaping many aspects of insect digestive physiology, ecological adaptation and evolution. The gut microbiota is thought to be beneficial to the mosquito by providing nutritional supplements, tolerance to environmental perturbations and manipulation of host immune homeostasis [55]. Recently, the association between symbionts and their hosts has attracted increased attention from the perspective of their engineering to combat pathogens [56–60]. Many bacterial species have been identified from the midgut of field-collected anophelines, mostly Gram-negative proteobacteria and enterobacteria [61, 62]. The bacterial population structure in laboratory-reared adult mosquitoes was found to be similar to that of wild mosquitoes, suggesting that anopheline mosquitoes harbor their microbiome in a selective way [62]. A non-pathogenic bacterium, *Pantoea agglomerans*, was reported to be a dominant symbiotic bacterium in different mosquito species in Kenya and Mali [61], and also found in laboratory-reared *An. stephensi, An. gambiae* and *An. albimanus* mosquitoes [60, 63]. This bacterium is normally found on plant surfaces and blossoms [64–66], suggesting that flower nectar is a possible source of the mosquito microbiota in the field. Also, this property could potentially facilitate re-introduction of genetically-modified *P. agglomerans* into field mosquito populations.

The bacterial population in the mosquito gut increases by hundreds to thousands of times within 24 hours after a bloodmeal [30, 63]. The rapid proliferation of gut microbiota may

stimulate mosquito immune responses that limit the infection by malaria parasites [67, 68]. Reduction of the gut microbiota with antibiotics renders the mosquito more susceptible to *Plasmodium* infection. Conversely, co-infection of bacteria with *Plasmodium* gametocytes reduces the oocyst load in the mosquito midgut [67]. Recently, an *Enterobacter* bacterium strain Esp_Z, isolated from wild *An. arabiensis* mosquitoes in Zambia was found to significantly inhibit *P. falciparum* infection after co -feeding *An. gambiae* with bacteria and infectious blood. Reactive oxygen species (ROS) produced by this bacterium seems to mediate parasite killing in the midgut lumen prior to mosquito midgut invasion [69]. A recent study revealed a positive correlation between the abundance of commensal *Enterobacteriaceae* and *Plasmodium* infection in *An. gambiae* mosquito midgut, and suggested that *Enterobacteriaceae* might play a positive protective role in the natural infection of *P. falciparum* [70].

The acetic acid bacterium *Asaia sp*. was identified as a stable symbiont in laboratory *An. stephensi* colonies and in wild *An. gambiae* populations. *Asaia sp.* was also observed in several mosquito organs, including salivary glands and ovaries [58]. Importantly, *Asaia sp*. appears to be vertically transmitted from female to larval progeny, venereally transmitted from male to female during mating, and transstadially transmitted from larva to adult [58, 71, 72]. These features favor dissemination and should be helpful when considering potential introduction of genetically-modified bacteria into mosquito populations in the field.

The intracellular endosymbiotic bacterium *Wolbachia* can infect a large number of insects and other arthropod species, and may play key roles in modulating pathogen infection and transmission in several insect species [73, 74]. Recent studies showed that *Wolbachia* infection reduces pathogen levels in multiple mosquito species [75–77]. Somatic *Wolbachia* infections of *Anopheles* can also significantly inhibit *Plasmodium* oocyst formation through activation of the mosquito immune system [78]. However, *Wolbachia*-infected anopheline mosquitoes have not been found in nature and stable mosquito infections have not yet been reported [79].

Several yeasts such as *Candida, Pichia, Wickerhamomyces anomalus* were found in the guts of *Anopheles* mosquitoes [80–82]. Moreover, *W. anomalus* was also identified in the reproductive organs of malaria vectors *An. gambiae* and *An. stephensi* [81, 82], raising the possibility of use of symbiotic yeasts for delivery of anti-malaria effector proteins to anopheline vectors.

Paratransgenesis

Paratransgenesis refers to the genetic engineering of a microorganism associated with its insect host, as opposed to genetic modification of the insect itself. At the heart of the paratransgenesis strategy for malaria control is the fact that the mosquito microbiota and *Plasmodium* share the same compartment – the midgut – where the most vulnerable stage of the parasite development occurs (Figure 1b). These considerations suggest an alternate approach to interfere with malaria transmission by genetically engineering midgut symbiotic microorganisms to deliver anti-*Plasmodium* effector molecules [83]. Paratransgenesis has a number of attractive features [84]. i) As for most higher organisms, the mosquito carries a significant microbiota in its midgut; ii)A severe bottleneck of *Plasmodium* development occurs in the mosquito midgut lumen, making this compartment a prime target for intervention [8, 9]; iii) The developing parasite and the microbiota share the same midgut compartment, directly exposing parasites to molecules produced by engineered symbiotic bacteria; iv) the midgut bacterial population increases dramatically, by 100-to 1000 -fold, after ingestion of a blood meal [7], correspondingly increasing the output of effector

molecules produced by recombinant bacteria (Figure 2 a–b). The basic requirements for a paratransgenesis approach are listed in Table 1.

Paratransgenesis has already been proposed as a feasible means to control other insect borne diseases. The parasitic protozoan *Trypanosoma cruzi*, the causative agent of Chagas disease, is transmitted by the triatomid bug *Rhodnius prolixus* [56]. The *Rhodnius* obligate gram-positive bacterium *Rhodococcus rhodnii* was genetically engineered to produce the antimicrobial peptide cecropin A, and fed to naïve *R. prolixus* nymphs. Durvasula and collaborators [56] showed that expression of the anti -parasite peptide by the genetically modified symbionts significantly reduces *T. cruzi's* ability to survive in the bug.

Fighting malaria with paratransgenesis

Early reports on the use of paratransgenesis to fight malaria were based on the recombinant laboratory bacterium *E. coli* expressing a single-chain immunotoxin [85]. In another study, a dimer of the SM1 peptide or a modified phospholipase A2 [60] was used, resulting in a significant decrease of *P. berghei* oocyst numbersin *An. stephensi*. However, inhibition of parasite development was modest for two main reasons: i) the *E. coli* bacterium used for these studies were attenuated laboratory strains that survived poorly in the mosquito midgut, and ii) the recombinant anti-*Plasmodium* effector proteins either formed insoluble inclusion bodies [37] or were attached to the bacteria surface [60]. In either case, the effector molecules could not diffuse to their intended parasite or mosquito midgut targets.

Some of these shortcomings were addressed in recent studies with *P. agglomerans*, a bacterium commonly found in field vector mosquitoes in Africa [61] as well as in laboratory-reared *An. stephensi, An. gambiae* and *An. albimanus* [60, 63]. *P. agglomerans* easily grows in culture and can be engineered to secrete anti-*Plasmodium* proteins using the HlyA system [30, 86]. The engineered bacteria were tested for their ability to thwart *Plasmodium* development in the mosquito as follows. Recombinant bacteria were fed to mosquitoes via cotton balls soaked with a bacteria suspension in sugar solution. One day later, these mosquitoes were fed on a *Plasmodium*-infected blood meal. Control mosquitoes were fed bacteria transformed with the HlyA parental plasmid not fused to an effector protein. One or two weeks after the infectious blood meal, success of parasite development was measured by counting the number of oocysts per gut (only ookinetes that successfully cross the mosquito midgut epithelium can form oocysts). These experiments showed that recombinant bacteria secreting anti-malaria effector proteins strongly inhibit *Plasmodium* development in mosquitoes, as compared with mosquitoes fed control bacteria (Figure 2c). Inhibition varied from 85% for mPLA2 to 98% for scorpine and $(EPIP)_4$ without any detectable fitness cost to the transgenic bacteria [87] and or to the host mosquitoes [30]. Considering that a mosquito that produces one oocyst is as infective to a human host as a mosquito producing a large number of oocysts, a more important measure of transmission blocking potential is to compare the number of infected mosquitoes carrying one or more oocysts (infected mosquitoes) with the number of non-infected mosquitoes. In the experiments described above, the percentage of infected mosquitoes dropped from 90% in controls to 14~18% in mosquitoes carrying scorpine- or $(EPIP)_4$-expressing bacteria [30]. This strong reduction in the proportion of infected mosquitoes should translate into important reduction of transmission in the field. Moreover, the use of multiple effector molecules, each acting by a different mechanism, should greatly reduce the probability of selecting resistant parasites. The inhibition of parasite development was equivalent when using an African mosquito (*An. gambiae*) and an Asian mosquito (*An. stephensi*). Also, inhibition of *P. berghei* (a rodent parasite) and *P. falciparum* (a human parasite) was equivalent, suggesting that this approach may also work for other human parasites, such as *P. vivax*. Thus, the paratransgenesis strategy may well turn out to be "universal", being

effective for multiple mosquito and parasite species. These promising laboratory results will next need to be translated to field applications. A major outstanding issue is how to efficiently introduce the engineered bacteria into wild mosquito populations. While some approaches have been proposed (Box 1), none have as yet been experimentally verified.

Box 1

Genetic manipulation of mosquito vectorial competence via transgenesis and paratransgenesis

Mosquito transgenesis has the advantage of having no off-target effects as transgene expression is restricted to the engineered mosquito. The anti-*Plasmodium* effector genes can be engineered to express in specific tissues (midgut, fat body and salivary glands), only in females and in a blood-induced manner. While it has been shown that a mostly refractory mosquito can be produced in the laboratory, challenges remain for translating these findings to field applications. A method to drive effector genes into mosquitoes in the field still needs to be devised. The MEDEA [18] and homing nuclease (HEG) [19] approaches are among the most promising ones. Additional issues that need to be considered are the multiplicity of anopheline vector species (each needs to be separately engineered), the reproductive barriers within a given species (cryptic species), mass production and sex selection of transgenic mosquitoes (females cannot be mass-released in the field), the large size of the constructs expressing multiple effector genes and the possible loss of transgene expression over time [99].

Paratransgenesis refers to an alternative approach for delivery of effector molecules via the genetic modification of mosquito symbionts. Advantages of paratransgenesis are the simplicity of genetic modification of bacteria, the ease of growing the genetically modified bacteria in large scale, the fact that it by passes genetic barriers of reproductively isolated mosquito populations and effectiveness does not appear to be in fluenced by mosquito species. However, many challenges lay ahead. A major challenge is to devise effective means to introduce engineered bacteria into field mosquito populations. This may be accomplished by placing around villages, bating stations (cotton balls soaked with sugar and bacteria placed in clay jar refuges) [100] using engineered symbiotic bacteria that are vertically and horizontally transmitted among mosquito populations [58]. However, no experimental evidence for the effectiveness of such approach is presently available. Moreover, for future use in the field, the effector genes need to be integrated into the bacterial genome to avoid gene loss and also to minimize the risk of horizontal transgene transfer.

For both the transgenic and the paratrasngenic approaches, a major challenge for ultimate implementation will be to obtain the required approval from regulatory agencies and from the local population.

Perspectives

Current insecticide-based vector control strategies such as insecticide-impregnated bed nets, as well as other population-suppression strategies (e.g., **S**terile-**I**nsect **T**echnique or SIT [88, 89], RIDL (**R**elease of **I**nsects carrying a **D**ominant **L**ethal) [90] have the disadvantage that they create an “empty ecological niche”. This is because the use of these approaches leaves the environment where mosquitoes thrive unchanged and consequently mosquito populations revert to original density as soon as treatments end or when mosquitoes become resistant to the insecticide. In other words, any population-suppression strategy needs to be implemented forever.

Transgenesis and paratransgenesis are two novel promising means for interfering with *Plasmodium* development or infection of the vector mosquito through delivery of anti-*Plasmodium* effector molecules within the mosquito. Both are "population replacement" strategies that once implemented, should require much less follow-up effort than population-suppression strategies. The main properties of transgenesis and paratransgenesis are shown in Box 1. While many technical aspects have been successfully addressed, several major issues need to be resolved before transgenesis and paratransgenesis can be implemented in the field. One key issue for both approaches is to devise means to effectively drive transgene or the engineered bacteria into mosquito populations in the field. Other major topics that need to be addressed are the resolution of regulatory, ethical and the public acceptance issues relating to the release of genetically modified (GM)organisms in nature. While the GM subject is controversial, its resolution will ultimately rely on weighing risks against benefits. As these issues are considered, the benefit of saving lives should provide strong argument in its favor.

Transgenesis or paratransgenesis is not a cure-all solution for malaria control. Rather, both are envisioned as a complement to existing and future control measures. In this regard, transgenesis and paratransgenesis are compatible with each other (possibly additive) and with insecticides and population suppression approaches. Moreover, the diversity of effector proteins [91] make both approaches not unique to malaria but might also be extended to the control of other major mosquito-borne diseases, such as dengue and yellow fever.

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Highlights

- Mosquito gut is a prime target for interfering with *Plasmodium* cycle in its vector.
- Genetic approaches and challenges to block malaria transmission are considered.
- Transgenic mosquitoes are engineered to produce anti-*Plasmodium* molecules.
- Paratransgenesis uses engineered symbionts to deliver anti-*Plasmodium* molecules.
- Both approaches hold promise but field implementation issues remain to be resolved.

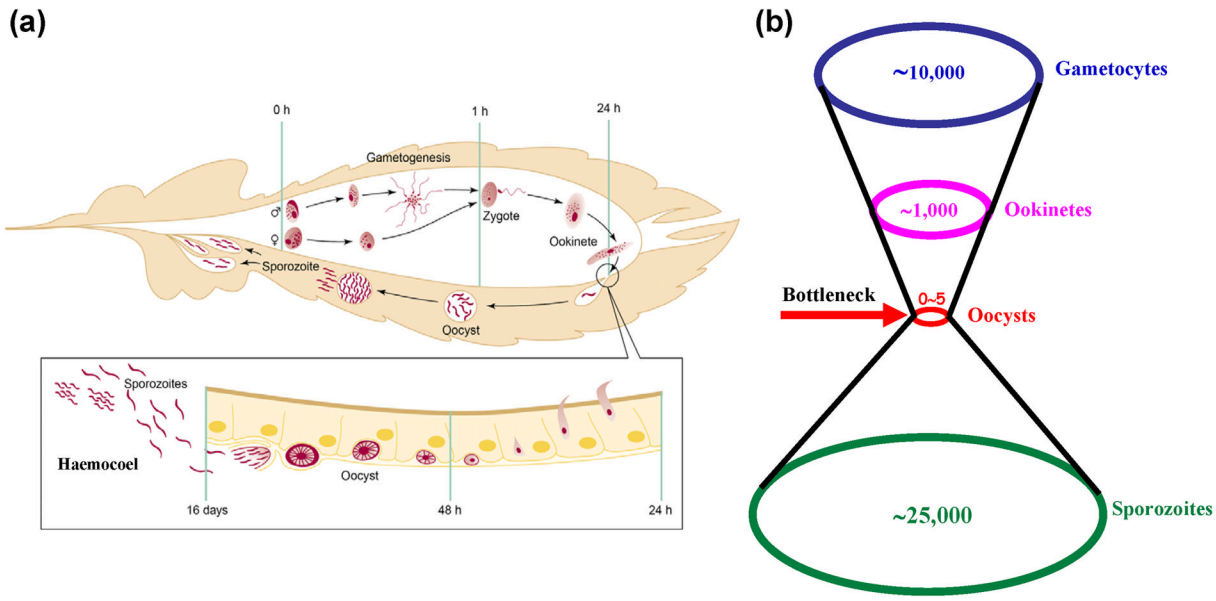

Figure 1. The malaria parasite cycle in the mosquito vector

(a) Life cycle of *Plasmodium* in the mosquito. The approximate developmental time at which each stage occurs in *Plasmodium berghei* (maintained at 20°C) is indicated. Transmission starts when the mosquito ingests an infected bloodmeal (0 h). Within minutes, gametocytes develop into gametes (the star-shaped figure illustrates exflagellation, which is the formation of male gametes) that fuse to form the zygote that differentiates into a motile ookinete. At 24 h, the ookinete invades the midgut epithelium and differentiates into an oocyst. About 2 weeks later, the oocyst ruptures, releasing thousands of sporozoites into the mosquito body cavity (hemocoel). Of all the tissues that sporozoites come in contact with, they can invade only the salivary gland. When the mosquito bites another vertebrate host, transmission is completed by release of sporozoites from the salivary glands (not shown). Reproduced with permission from Ref. [92]. (b) *Plasmodium* parasite numbers undergo a severe bottleneck during its development in the mosquito gut.

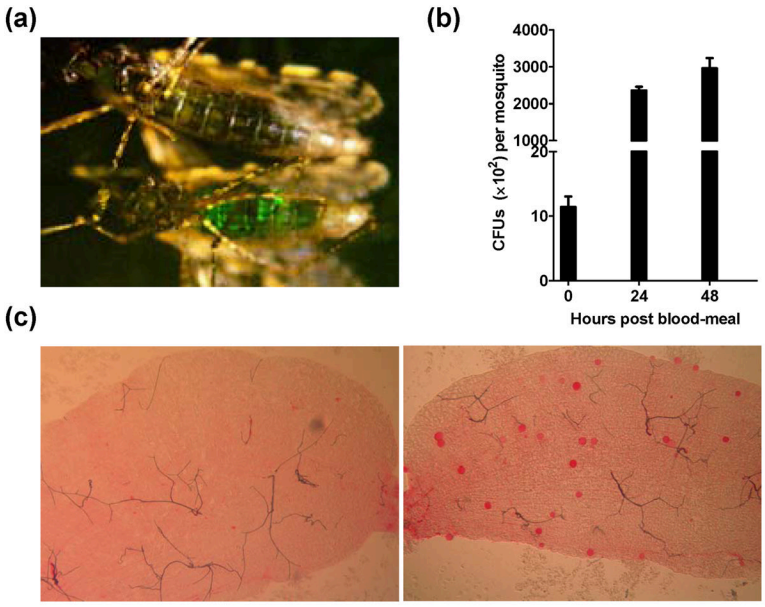

Figure 2. Engineered *Pantoea agglomerans* efficiently inhibits *Plasmodium falciparum* development in mosquitoes

(a) Visualization of GFP -tagged *P. agglomerans*in the mosquito midgut 24 h after a blood meal. GFP-expressing *P. agglomerans* were administered to 2 day-old *Anopheles gambiae* via a sugar meal. The upper mosquito fed on wild-type bacteria, and the lower mosquito fed GFP-tagged bacteria. **(b)** *P. agglomerans* rapidly proliferate in the midgut after a blood meal. The number of fluorescent bacteria colony-forming units (CFUs) was determined at each of the indicated times by plating serially diluted midgut homogenates on LB/kanamycin agar plates. **(c)** Inhibition of *P. falciparum* development in *An. gambiae* by recombinant *P. agglomerans* engineered to secrete scorpine, a potent antiplasmodial peptide. Wild type *P. agglomerans* was fed to one group of *An. gambiae* mosquitoes via a sugar meal while *P. agglomerans* engineered to secrete scorpine was fed to the other group of mosquitoes. After 32 h both groups of mosquitoes were fed on the same *P. falciparum*-infected blood meal. Midguts were dissected 8 d post infection and oocyst number per midgut was determined after staining with 0.1% (wt/vol) mercurochrome. Left panel: a midgut from a mosquito carrying scorpine-secreting *P. agglomerans*; Right panel: a midgut from a control mosquito carrying an equal number of wild type *P. agglomerans*. Note the strong reduction in oocyst numbers in the midgut from the mosquito fed scorpine-secreting bacteria.

Table 1

The basic requirements for paratransgenesis

i	Stable symbiotic relationship between microorganism and vector
ii	Symbiotic microorganism can be cultured *in vitro* and genetically manipulated
iii	Effector gene product should not impair symbiont and vector fitness
iv	Effector gene product should be secreted to assure interaction with the target pathogen
v	An efficient means of introducing the engineered symbiont into field must be devised

Table 2

Anti-*Plasmodium* effector molecules

Effector	Properties	Target parasite	Parasite stage(s)	Function or mechanism	Refs
Parasite killing					
Scorpine	Scorpion *Pandinus imperator* venom peptide	*P. falciparum* *P. berghei*	Gametocyte to Ookinete	Cecropin and defensin-like lytic peptide.	[29]
Shiva1	Cecropin-like synthetic peptide	*P. berghei* *P. falciparum*	Gametocyte to Ookinete	Lyses parasites	[93]
Shiva-3	Cecropin-like synthetic peptide	*P. falciparum* *P. berghei*	Gametocyte to ookinete	Lyses parasites	[33]
Cec A	*An. gambiae* cecropin A	*P. berghei*	Ookinete	Lyse parasites.	[28]
Cec B	*An. gambiae* cecropin B	*P. falciparum*	Oocyst	Lyses parasites	[32]
DEF1 A	*An. gambiae* defensin A	*P. falciparum* *P. berghei*	Ookinete	Lyses parasites	[26]
Gambicin	*An. gambiae* antimicrobial peptide	*P. falciparum* *P. berghei*	Ookinete	Lyses parasites	[27]
Angiotensin II		*P. gallinaceum*	Sporozoite	Lyses parasites	[31]
Magainins	Peptides from the African clawed frog *Xenopus laevis* skin	*P. falciparum* *P. knowlesi* *P. cynomolgi*	All mosquito stages	Lyses parasites	[32]
Gomesin	A antimicrobial peptide from Spider	*P. falciparum* *P. berghei*	All mosquito stages	Lyses parasites	[34]
CEL-III	Hemolytic C-Type Lectin	*P. falciparum* *P. berghei*	Ookinete, oocyst	Lyses parasites	[13]
TP10	Wasp venom peptide	*P. falciparum*	Gametocyte to ookinete	Lyses the parasites.	[94]
AdDLP	*Anaeromyxobacter dehalogenans* defensin-like peptide	*P. berghei*	Ookinete	Lyses the parasites	[95]
Meucin-25	Scorpion *Mesobuthus eupeus* venom gland	*P.. berghei* *P. falciparum*	Gametocyte	Anti-microbial linear cationic peptide	[96]
Drosomycin	An inducible antifungal peptide initially isolated from the *Drosophila melanogaster* haemolymph	*P. berghei*	Ookinete	Lyses the parasites	[97]
Interaction with parasites					
EPIP	Enolase-Plasminogen Interaction Peptide	*P. falciparum* *P. berghei*	Ookinete	Inhibits mosquito midgut invasion by preventing plasminogen binding to the ookinete surface	[35]
Pro:EPIP	A fusion peptide composed of a chitinase propeptide (Pro) and EPIP	*P. falciparum* *P. berghei*	Ookinete	Blocks ookinete traversal of the mosquito peritrophic matrix and prevents plasminogen binding to the ookinete surface	[30]

Effector	Properties	Target parasite	Parasite stage(s)	Function or mechanism	Refs
Pbs21scFv-Shiva1	Single-chain immunotoxin	*P. falciparum* *P. berghei*	Gametocyte to Oocyst	A single-chain monoclonal antibody (scFv) targeting the major ookinete surface protein pbs21 and linked to the lytic peptide Shiva1.	[37]
scFv 4B7	A single-chain antibody	*P. falciparum*	Ookinete	Binds to *P. falciparum* ookinete surface protein Pfs25	[16, 36]
scFv 2A10	Single-chain antibody	*P. falciparum*	Ookinete	Targets the *P. falciparum* circumsporozoite protein (CSP)	[16, 36]
PfNPNA-1	Single-chain antibody	*P. falciparum*	Sporozoite	Recognizes the repeat region (Asn-Pro-Asn-Ala) of the *P. falciparum* surface circumsporozoite protein	[50]
scFv 1C3	Single-chain antibody	*P. falciparum*	Ookinete	Binds a *P. falciparum* secreted enzyme chitinase 1	[16]
Interaction with mosquito midgut or salivary gland epithelia					
SM1	Salivary gland and midgut peptide 1	*P. berghei* *P. falciparum*	Ookinete, sporozoite	Blocks ookinete invasion of the midgut epithelium or sporozoite invasion of the salivary gland epithelium.	[10]
mPLA2	Bee venom phospholipase	*P. falciparum* *P. berghei*	Ookinete	Inhibits ookinete midgut invasion, probably by modifying the properties of the midgut epithelial membrane	[12]
Pro	A chitinase propeptide	*P. falciparum* *P. berghei*	Ookinete	Inhibits the enzyme and blocks ookinete traversal of the mosquito peritrophic matrix	[39]
Pchtscfv	Single-chain antibody	*P. falciparum*	Ookinete	Inhibits the *P. falciparum* chitinase and blocks ookinete traversal of the mosquito peritrophic matrix	[98]
Manipulation of mosquito immune system					
Akt	A protein kinase	*P. falciparum* *P. berghei*	Ookinete	Akt boosts mosquito innate insulin immunity via signaling	[15]
Rel2	*Anopheles* IMD pathway transcription factor	*P. falciparum* *P. berghei*	Ookinete, sporozoite	Rel2 overexpression enhances mosquito IMD pathway	[14]