



The Strategy of Paratransgenesis for the Control of Malaria Transmission

Aminata Fofana^{1*}, Rakiswendé Serge Yerbanga^{1,2}, Etienne Bilgo¹,
Georges Anicet Ouedraogo³, Mathilde Gendrin^{4,5†} and Jean-Bosco Ouedraogo^{1,2†}

¹ Medical Biology/Public Health, Institut de Recherche en Sciences de la Santé (IRSS), Bobo-Dioulasso, Burkina Faso,

² Institut des Sciences et Techniques (INSTech), Bobo Dioulasso, Burkina Faso, ³ Medical Biology/Public Health, Laboratoire de Recherche et d'Enseignement en Santé et Biotechnologie Animale (LARESBA), Université Nazi Boni, Bobo-Dioulasso, Burkina Faso, ⁴ Institut Pasteur de la Guyane, Microbiota of Insect Vectors Group, Cayenne, French Guiana, ⁵ Institut Pasteur, Université Paris Cité, Parasites and Insect Vectors Department, Paris, France

Insect-borne diseases are responsible for important burdens on health worldwide particularly in Africa. Malaria alone causes close to half a million deaths every year, mostly in developing, tropical and subtropical countries, with 94% of the global deaths in 2019 occurring in the WHO African region. With several decades, vector control measures have been fundamental to fight against malaria. Considering the spread of resistance to insecticides in mosquitoes and to drugs in parasites, the need for novel strategies to inhibit the transmission of the disease is pressing. In recent years, several studies have focused on the interaction of malaria parasites, bacteria and their insect vectors. Their findings suggested that the microbiota of mosquitoes could be used to block *Plasmodium* transmission. A strategy, termed paratransgenesis, aims to interfere with the development of malaria parasites within their vectors through genetically-modified microbes, which produce antimalarial effectors inside the insect host. Here we review the progress of the paratransgenesis approach. We provide a historical perspective and then focus on the choice of microbial strains and on genetic engineering strategies. We finally describe the different steps from laboratory design to field implementation to fight against malaria.

Keywords: mosquitoes, bacteria, *Plasmodium*, paratransgenesis strategy, malaria transmission

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*Correspondence:

Aminata Fofana
aminatafofana920@gmail.com

†These authors have contributed
equally to this work

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INTRODUCTION

Insect-Borne Diseases (IBD) are responsible for significant human morbidity and mortality in the world. Most of the pathogens are transmitted by arthropods vectors through blood transmission (1). Some of these pathogens, which cause devastating infectious diseases such as malaria, dengue, filariasis, yellow fever, as well as zoonotic arboviruses, such as West Nile Virus (WNV), Saint Louis Encephalitis Virus (SLEV) and Eastern Equine Encephalitis Virus (EEE), are transmitted by mosquitoes (2). After the scaling up of vector control interventions using insecticides, efforts are now limited by the development of defense mechanisms by which insects can resist to toxic substances (3). We are also witnessing a spread of some vector-borne diseases due to the geographic expansion of mosquitoes which can adapt to different environments (4). Among these diseases, malaria remains a severe burden in developing countries causing 602,000 deaths in 2020 (5). The fight against this mosquito-borne disease using chemicals generated problems of resistance in

parasites and insects, so the development of additional control measures is imperative to reduce the transmission. One of the approaches under intense study to fight against IBD, referred to as paratransgenesis, relies on the genetic modification of a microbiota strain to express and deliver anti-pathogen effector molecules to reduce vectorial transmission. In this approach, genetically-engineered microbiota strains would be used to stop the development of causative agent of the disease and spread in natural mosquito populations to interfere with pathogen transmission (6). However, current challenges remain before any implementation of this strategy. This review provides an overview on historical and current work on paratransgenesis strategy to limit the burden of vector-borne diseases, with a special focus on malaria transmission. After providing a historical perspective, we focus on different requirements to consider when developing a paratransgenic approach, dealing with choice of an appropriate microbe and with the design of the paratransgenesis strategy. Finally, we discuss about the required steps on the way from laboratory to implementation.

HISTORICAL PERSPECTIVE

Paratransgenesis was investigated for the first time in the gut of the kissing bug *Rhodnius prolixus*, an insect belonging to the *Reduviidae* family and *Triatomidae* sub-family that transmits Chagas disease (7). The strategy consisted in manipulating the symbiotic bacterium *Rhodococcus rhodnii* to express the antimicrobial peptide Cecropin A in order to stop the development of *Trypanosoma cruzi* in the vector without any detected fitness cost for the host (8). The transmission and the stability of the transformed symbiont without antibiotic selection were also shown (9). These findings stimulated interest in the role of gut symbionts to reduce vector competence by genetic manipulation. The potential of paratransgenesis was subsequently tested in tsetse flies, vectors of sleeping sickness, to reduce the transmission of African animal trypanosomiasis. The common symbiotic bacterium *Sodalis*, colonizing the midgut, hemolymph and salivary glands of different species of these vectors (*Glossina morsitans*, *Glossina p. palpalis*, *Glossina austeni*, *Glossina brevipalpis*) was genetically modified to produce green fluorescent protein (GFP) and then inoculated in female flies. This resulted in the spread of the fluorescent bacterium in a cage population by vertical and transstadial transmission (10, 11). Subsequent studies identified antimicrobial peptide genes (*attacin*, *cecropin*, *dipteracin*) in tsetse flies (12–14) which can efficiently reduce vector competence, i.e. permissiveness of the vector to the transmitted parasite. Focusing on *Anopheles* mosquitoes, vectors of malaria, Bisi and Lampe engineered *Escherichia coli* and *Pantoea agglomerans* to express and secrete three anti-*Plasmodium* effectors using plasmids carrying secretion signals for the target molecules (15). Going further, Wang et al. engineered *P. agglomerans* to produce molecules that decreased by up to 98% the development of *Plasmodium falciparum* and *Plasmodium berghei* in the mosquito gut (16). To this aim, they used two types of strategies, the production of exogenous antimicrobial peptides which kill or suppress parasites

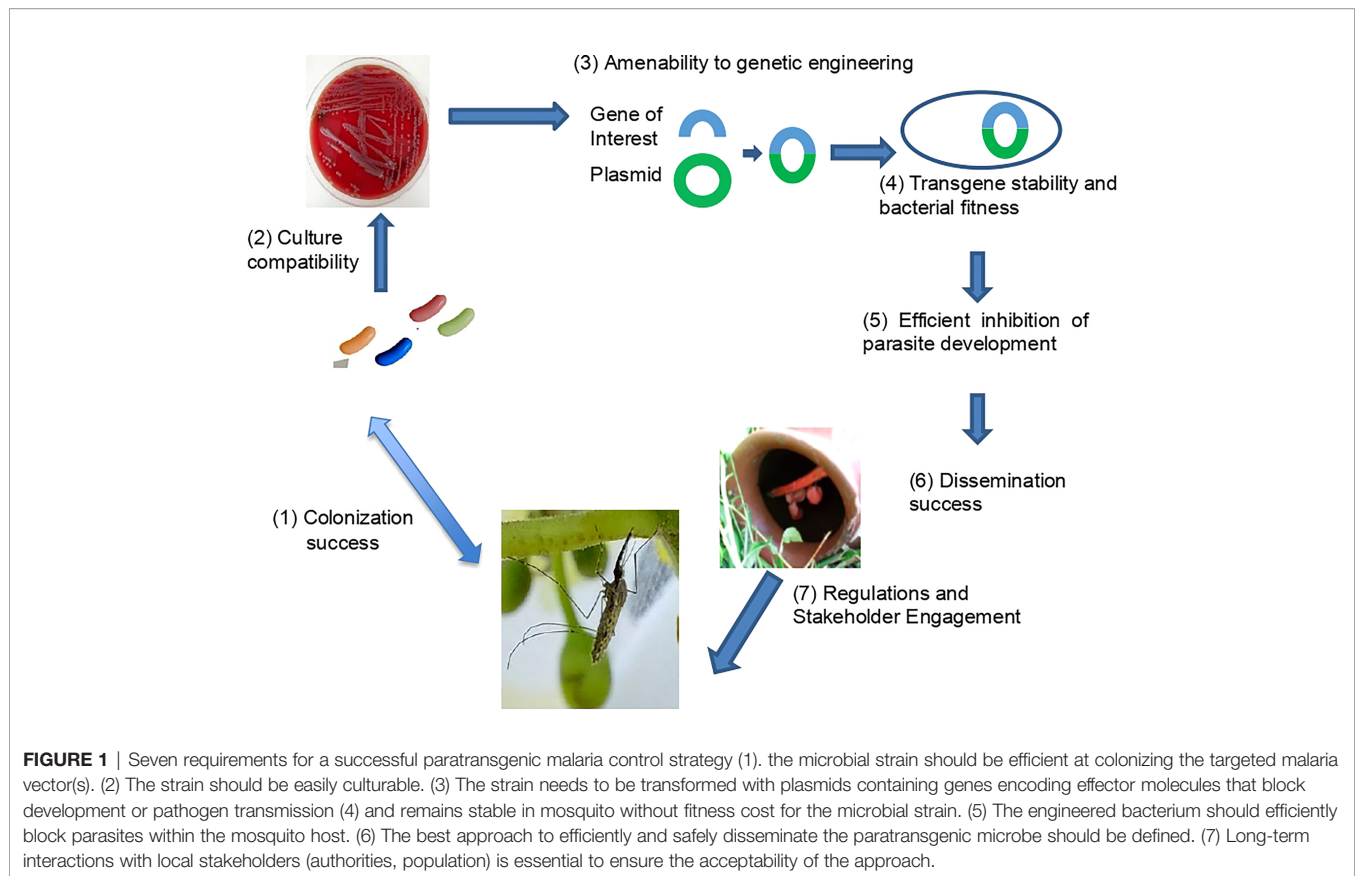
and of peptides that block key interactions between the malaria parasites and mosquitoes after binding to parasites or to epithelia. Since colonization of *P. agglomerans* in the mosquito may be a limiting factor, they subsequently focused on *Serratia AS1*, which efficiently colonizes mosquitoes and can be engineered to strongly inhibit *P. falciparum* (17). Current research focuses on methods to improve paratransgenesis efficiency, including a better control of transgene expression and ethical suitability of paratransgenic candidates (18, 19).

REQUIREMENTS FOR EFFICIENT PARATRANSGENESIS

Choosing the Right Microbe An Efficient Colonizer in Field Mosquitoes

Several basic requirements for an effective implementation of paratransgenesis are described in **Figure 1**. The microbe chosen to deliver anti-parasite molecules should compete with other microbial species to survive in the mosquito midgut and in the environment. Since the first studies of the microbiota in the mosquito midgut over 50 years ago (20), research on mosquito microbiota composition has undergone dramatic development (21). A substantial bacterial growth is observed in the adult mosquito midgut after a blood meal (22), but bacterial diversity is also reduced at this stage (23). Moreover, the efficiency of transmission from mother to offspring, which varies extensively between microbes, needs to be assessed as it will have much consequences on the way a strain can be used.

Amongst bacteria frequently found in wild populations of mosquitoes, Straif et al. found that *P. agglomerans* was the dominant bacterium in the gut of different mosquito species in Mali and Kenya (24). This bacterial species has also been commonly associated to *Anopheles stephensi*, *Anopheles gambiae* and *Anopheles albimanus* (25). *Serratia* has been reported in a number of studies with a wide geographic distribution (26, 27). A novel strain of *Serratia* sp. (AS1) has been proposed as a paratransgenic candidate by Wang et al. (17) due to its efficient horizontal and vertical transmission. When introducing 5% of *Serratia*-colonized mosquitoes in a cage, they observed that the next generation is fully colonized. *Serratia* sp. AS1 colonizes the gut, hemolymph, ovaries and male accessory glands of mosquitoes without affecting their fitness. It can disseminate rapidly in a mosquito cage population through vertical, horizontal and transstadial transmission. This genus is ubiquitous and notably commonly found in diverse mosquito species (17). *Asaia* is another bacterial genus that is found frequently in field mosquitoes and able to colonize several tissues in different species of malaria vectors such as *An. stephensi* and *An. gambiae* (28). This bacterium colonizes the larval gut, salivary glands and reproductive organs of laboratory-reared *An. stephensi* and field *An. gambiae* (29–31). It disseminates in mosquito populations *via* transmission from male to female, from larvae to adult and from female to larval progeny (29, 31, 32).



Compatibility to Genetic Engineering

Another important point is the amenability of the strain to genetic engineering. Hence, the first proof-of-principle for such symbiotic control has been provided using *E. coli* (33). Recombinant *E. coli* was first shown to significantly reduce *P. berghei* infection into *Anopheles* mosquitoes when expressing a single chain immunotoxin transgene. Six years later, *E. coli* was transformed to display SM1 (Salivary gland and Midgut peptide 1) and phospholipase-A(2) (PLA2), two anti-*Plasmodium* effector molecules, in its outer membrane (34). In an attempt to transfer such an approach to bacteria that naturally colonize mosquitoes, some studies then focused on *P. agglomerans*, which offers transgenesis possibility (15, 16). These studies were highly successful but *Pantoea* was found to have a limited colonization ability in mosquitoes. *Serratia* and *Asaia* were also found to be amenable to genetic manipulation leading to production of antimicrobial peptides (19, 25, 29).

Some yeasts and fungi can also be genetically engineered and/or may combine an entomopathogenic effect with an antiplasmodial impact. For instance, *Metarhizium anisopliae* an entomopathogenic fungus is known as killer of adult *An. gambiae* mosquitoes (35). It can be modified to express SM1 and scorpine, which were found to inhibit the development of *Plasmodium* reducing the sporozoite load (i.e., infection in the salivary glands) by 71-90% (36).

Natural Impact on *Plasmodium*

Several non-engineered strains have been found to naturally block malaria transmission. Starting with such an antimalarial strain can be seen as an advantage as it may increase the efficiency of transmission blocking and have a natural long-lasting impact in case the transgene is lost. *Enterobacter* EspZ, isolated in wild mosquitoes in Zambia, interferes with parasite development before midgut epithelium invasion. It renders mosquitoes refractory to *P. falciparum* infection by generating Reactive Oxygen Species (ROS) (37). Some strains of *Serratia* have also been found to naturally inhibit *Plasmodium* in mosquitoes (38). *Serratia marcescens* Y1 and *Serratia ureilytica* isolated from *Anopheles sinensis* interfere in the gut immunity to block the development of *P. berghei*, *P. vivax* and/or *P. falciparum* (26, 39). *Chromobacterium* sp. Panama isolated in the midgut of *Ae. aegypti* was also found to have a natural anti-*Plasmodium* activity in *An. gambiae* by secreting romidepsin, a histone diacetylase inhibitor (40). The bacterium is reported to activate the immune response as well as to reduce the larval and adult stages survivorship (41). The endosymbiont *Wolbachia* has been detected in several mosquito species in Africa, in a minority of individuals (42, 43). In *An. gambiae* and *An. coluzzii*, it has been found to negatively impact the late stages of *Plasmodium* development (44, 45).

Considering non-bacterial candidates, the yeast *Wickerhamomyces anomalus* stably colonizes the *An. stephensi*

midgut reproductive organs. Some of its strains reduce the transmission of rodent malaria by inhibiting the *P. berghei* development (46). As it is recognized as a safe biocontrol agent, it has been proposed as an interesting “symbiotic-control tool” against transmission of malaria (47). Also related to fungi, *Microsporidia* MB naturally impairs *P. falciparum* development in *An. gambiae s.l.*, yet this fungus is not currently amenable to culturing and genetic engineering (48).

Safe to the Environment

The implementation of paratransgenesis strategies requires to control the spread of the engineered microbiota strain specifically in the targeted natural mosquito populations or to ensure that a potential spread to the environment would not cause any damage (Table 1). This may be achieved using vertically-transmitted endosymbionts to prevent colonization of non-target species and spread to the environment. For instance, *Wolbachia* is a vertically-transmitted bacterium, transferred within the egg from mother to offspring. This obligate intracellular bacteria is detected in 40 to 69% terrestrial arthropods species including insects, mites and isopods (64–66). It is notably found in some mosquitoes, including *Culex quinquefasciatus* and *Aedes albopictus* (67) and used in *Aedes aegypti* to inhibit transmission of arthropod-borne viruses, notably dengue, as it naturally reduces vector competence (68). The release of *Wolbachia*-infected mosquitoes has already been shown efficient to colonize mosquito populations with this bacterium and to efficiently limit dengue in several countries (69). Hence, its potential in *Anopheles* is also investigated with much interest to decrease the malaria burden. It is found in *Anopheles*, but at a lower rate than in some *Aedes* and *Culex* species (42, 43) and with less efficient transmission over generations due to competition with *Asaia* (70, 71). As it has a natural negative impact on *Plasmodium* and is not yet amenable to culturing and/or genetic engineering, its use in *Anopheles* may first be developed as a natural blocker of transmission rather than as a paratransgenic tool. A putative plasmid encompassing 14 genes was however identified recently in *Wolbachia* in *Culex* mosquitoes, which may open additional possibilities for its genetic manipulation (72).

Paratransgenesis candidates may also be chosen because they are already ubiquitous in the environment, hence unlikely to cause any disturbance if used as paratransgenic transmission-blockers. For instance, *P. agglomerans* is found in nature, particular in surface of plants and flowers (73, 74), suggesting that its spread in natural mosquito populations may be envisaged using sugar baits (discussed below). It was described as a bacterium associated to wild and laboratory-reared mosquito species (22, 24, 34).

The impact of bacterial candidates on transmission of other pathogens should also be investigated. Notably, several *Serratia* species have been found to increase vector competence of *Ae. aegypti* to dengue and other arboviruses, notably via production of an enzyme which disturbs the gut mucus (75, 76). Thus, any *Serratia* paratransgenesis candidate will need to be specifically checked for such activity.

Strategies of Genetic Engineering Transgene

As parasite numbers drastically decrease in the early stages of infection in the mosquito gut lumen (77), paratransgenesis may be used to bolster this reduction to reach complete elimination. To this aim, one of the important requirements is to identify and produce efficient effector molecules to block parasite development or kill it; doing so without impairing vector fitness or impairing its microbiota and surrounding organisms would avoid any selective pressure in these organisms (25, 78). Several antiplasmodial transgenes have been described and categorized to different classes according to their mechanism of action by Wang and Jacobs-Lorena (25).

Firstly, Scorpine, Gambicin, Hemolytic C-Type Lectin CEL-III, angiotensin II, magainins, synthetic anti-parasitic lytic peptides Shiva 1, Shiva 3 and gomesin have been mentioned to have lysis activities on *P. falciparum* and *P. berghei* (25). The use of paratransgenic antimicrobial peptides may however affect the mosquito microbiota as well as the paratransgenic bacterium itself in addition to *Plasmodium*. This may lead to indirect effects, such as a reduced microbiota load or the loss of the transgene, and thus interfere with the efficiency of the approach.

Secondly, some effector peptides block parasite development by inhibiting ookinetes or sporozoites bind to midgut or salivary gland epithelium surfaces. For example, EPIP (*Plasmodium* Enolase-Plasminogen interaction peptide) inhibits midgut invasion by preventing plasminogen binding to the ookinete surface (79). In 2012, Wang et al. used a combination of the antimicrobial peptide scorpine and of a peptide composed of 4 EPIPs to efficiently inhibit ookinete development in the mosquito gut (16). Anti-sporozoite or anti-ookinete single-chain monoclonal antibodies scFvs (Single Chain Variable Fragment) were also found to inhibit *Plasmodium* development in the mosquito, especially when combined with antimicrobial peptides (80, 81) suggesting that such transgenes may also be of interest for paratransgenesis purposes. Other proposed peptides include the 12-amino acid peptide SM1, 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 (82), and phospholipase A2 (PLA2) that inhibits ookinete invasion, possibly by modifying the properties of the midgut epithelial membrane. *E. coli* has been engineered to inhibit the malaria parasite development by displaying SM1 and PLA2 on the bacterial surface, leading to a significant yet modest inhibition of *P. berghei* development (34).

Thirdly, manipulation of mosquito immunity may be an alternative strategy. Blood meal-induced expression of Akt, a key signaling component in the insulin signaling pathway, in transgenic mosquitoes renders the mosquito refractory to *Plasmodium* infection. Over expression of the IMD pathway-mediated transcription factor Rel2 renders the mosquito resistant to *Plasmodium* infection (83). Similarly, one can imagine the use of such microbes which would induce a basal immune response without too much cost for their own fitness. However, this kind of strategy has not yet been developed, as far as we know.

TABLE 1 | Paratransgenic candidates for microbiota-mediated control of malaria.

Microbe	Mosquito host	Tissue localization	Function and Mechanism		Ref.
			Natural property	Engineered property	
Bacteria					
<i>E. coli</i>	<i>Anopheles</i>		Inhibition of <i>P. berghei</i> development	Expression of Single-Chain immunotoxins (SM1, PLA2)	(34, 49)
<i>P. agglomerans</i>	<i>Anopheles</i> , <i>Aedes</i>	Midgut, ovary	Inhibition of <i>P. berghei</i> and <i>P. falciparum</i> development	Efficient secretion of effector molecules	(15, 16)
<i>Asaia sp</i>	<i>An. stephensi</i> , <i>An. gambiae</i> , <i>Ae. aegypti</i>	Midgut, Salivary gland, ovary, eggs	Activation of antimicrobial peptide expression	Expression of Green Fluorescent Protein (GFP) or antiplasmodial peptides (<i>P. berghei</i>)	(28, 29, 50, 51)
<i>Enterobacter sp. Zambia (Esp_Z)</i>	<i>An. gambiae</i>		Production of reactive oxygen species with <i>in vitro</i> anti- <i>Plasmodium</i> activity	–	(37)
<i>Serratia marcescens</i> and <i>Serratia sp.</i>	<i>An. stephensi</i> , <i>An. gambiae</i> , <i>Ae. aegypti</i>	Midgut, salivary glands	Anti- <i>Plasmodium</i> effect, yet Enhancement of dengue virus infection	Produce of multiple antimalarial effector proteins with different killing mechanisms.	(17, 52, 53) (54, 55)
<i>Chromobacterium sp.</i>	<i>Ae. aegypti</i>	Midgut	Anti- <i>Plasmodium</i> and anti-dengue effects, Mosquitocidal activity	–	(41)
<i>Wolbachia (wMelPop and wAlbB)</i>	<i>An. stephensi</i> , <i>An. gambiae</i>		Inhibits <i>P. falciparum</i>	–	(56, 57)
Fungi					
<i>Wickerhamomyces anomalus</i>	<i>An. stephensi</i> , <i>Ae. aegypti</i>	Midgut, reproductive tract	Produces antimicrobial killer toxins	–	(58, 59)
<i>Metarhizium anisopliae</i>	<i>Anopheles</i> , <i>Aedes</i>	Penetrates cuticle, systemic infection		Expression of SM1 and scorpine in order to block <i>Plasmodium</i> transmission	(36, 60, 61)
<i>Beauveria bassiana</i>	<i>Anopheles</i> , <i>Ae. aegypti</i>	Penetrates cuticle, systemic infection	Indirectly decreases dengue infection by activating Toll/Jak-Stat pathway		(61–63)

Expression of the Transgene

When considering transgene expression, several issues need to be addressed. First, recombinant proteins may remain blocked into the bacterial cell, preventing them to reach their target. To address this, Wang et al. used the Type-1 secretion system HasA in *Serratia*, which allows the secretion of their unfolded transgenic polypeptide through both membranes in one step. Grogan et al. recently identified novel secretion signals in *Asaia* sp (84). Bacteria carrying two of these signals allowed more efficient excretion than the initial paratransgenesis strain. Even though the increased release of scorpine comes with a cost, reflected by a reduction in bacterial growth and colonization potential, strains carrying these signals have higher antiplasmodial activity against *P. berghei* than the initial strain. Secondly, the transgene needs to be expressed at a high level to reach efficiency. To this aim, Wang et al. cloned five effector genes, two of them present in several copies, as a single construct of in *Serratia*, which are secreted together and inhibit ookinete development in the mosquito (17). Thirdly, some transgenes are antimicrobials hence it is necessary to deal with potential fitness costs for the microbe. Shane et al. addressed this question by producing an *Asaia* strain expressing antiplasmodial effectors specifically after the blood meal in order to increase the fitness of their paratransgenic bacterium and thus to promote dissemination throughout large population of field mosquitoes (19).

Dealing With Transgene Stability

The stable introduction of an anti-parasitic gene at a sufficient frequency and efficacy into a population of vector mosquitoes remains challenging. The development of inducible transgenes contributes to address this problem by reducing fitness costs for bacteria (19). However, no transgene has yet been found to be stable in paratransgenic candidates. On the contrary, Huang et al. found that *Serratia* AS1, which efficiently spreads in *Anopheles* and inhibit *P. falciparum* in mosquitoes after genetic manipulation, loses the plasmid carrying the exogenous gene in three mosquito generations. They also did not detect any plasmid horizontal transfer from *Serratia* to other bacteria in the mosquito gut (18). They pointed out that this lack of stability may be an advantage in terms of safety: using such transient transgenes, which are lost after one to two months, would allow to reduce the burden of a seasonal outbreak without spreading transgenes in the long-term in a vector population, which would reduce parasite resistance selection risk or other non-foreseen problematics. Such an approach based on an unstable transgene may get authorities approval in a shorter term. This instability, together with the reported semi-isolation of mosquito populations from neighboring villages in terms of microbiota composition (85), may provide arguments to progress in investigating the feasibility of paratransgenesis in the field while reducing the risk of out-of-control transgene spread.

Thus, two alternative strategies with different advantages can be considered regarding transgene stability. On one hand, implementation of stable paratransgenesis may be performed once and reduce disease transmission for years, similar to the current use of non-engineered *Wolbachia* to limit dengue

transmission (57, 68, 71, 86, 87). This approach proves to be cost effective but requires a good control of the risks to the environment, it is thus restricted so far to non-genetically-engineered biocontrol agents. Alternatively, an unstable paratransgenesis approach, where the microbe and/or the transgene are lost after some generations, may be used to reduce environmental risks (18). In this case, implementation campaigns will be regularly required.

Avoiding Development of Antimicrobial Resistance

Prior to release genetically-modified bacteria to the environment, it is important to consider antibiotic resistance issues. Antibiotic-resistance elements are known to be efficiently spread across bacterial species, which has disastrous consequences for human health. When considering paratransgenesis, it is essential not to introduce any antibiotic-resistance marker used for genetic engineering and to avoid resistance to antimicrobial peptides.

First, plasmids and/or chromosomes need to be cleared from any antibiotic-resistance gene used for genetic engineering. To this aim, counter selection methods have been set up to kill bacteria under some specific nutritive conditions if they carry an adjacent gene to the resistance marker (88).

Secondly, the antiparasitic activity should be designed in a way to avoid the rise of microbial resistance. Considering resistance in *Plasmodium*, the combination of several antimicrobial peptides with inhibitors of parasite binding to the gut, as developed in *Serratia*, increases efficiency while also reducing the risk of developing resistance (17). Considering resistance in a wider microbial community due to the pressure of wide-spectrum antimicrobial peptides, the latter are thought to be safer than empirical antibiotics, with a low tendency to select for resistance (89). As they are thought to be key to deal with antibiotic resistance issues in the future, the resistance that they select for is under active study (90). Where resistance may still develop, it will be important to know about resistance mechanisms and their potential for horizontal transfer prior to any use in paratransgenesis. Inducible expression of the transgene in specific conditions, e.g. specifically in the blood-fed mosquito gut, may also reduce the selective pressure on the microbiota. Alternatively, the identification of any antimicrobial peptide with a narrow spectrum, active on parasites without harming the mosquito microbiota, would be interesting.

From the Laboratory to Implementation

Unlike vector control tools, the goal of paratransgenesis in malaria control is not to kill the mosquito, but to transform it in an ineffective vector. Similar to population replacement strategies, the ecological niche remains occupied by the same mosquito species, yet vectorial capacity is reduced. Hence, this type of approach is thought to have a reduced risk of ecological disturbance due to the spread of an initially low-abundant competitor species, compared approaches removing a mosquito population and thus leaving an empty niche. Population replacement strategies notably include the release of *Wolbachia*-carrying *Ae. aegypti* to inhibit dengue transmission (69, 91), or of genetically-engineering mosquitoes with reduced vector competence (92).

Prior to implementation, vector control using paratransgenesis requires in depth ecological studies. This involves different steps in contained environments, starting with as small and large cages in the laboratory, following up with semi-field trials and finally with field trials. Some recent studies focused on the ability of some paratransgenesis candidate strains to colonize mosquito populations. Mancini et al. used large cages simulating near-natural ecosystem conditions for an effective paratransgenesis set-up. Upon introduction of transgenic *Asaia* in large cages via colonized males or soaked cotton pads, they observed an efficient spread of transgenic bacteria in populations of *An. gambiae* or *An. stephensi* with both introduction methods, indicating a promising perspective to open field trials (32). It would be interesting to perform the same test with other paratransgenesis candidates to have a wider picture of their diffusion and transmission through mosquito populations. Moreover, before field trials, the efficacy and safety of transgenes and paratransgenic mosquito life-history traits (lifespan, mobility, mating success) would need to be tested in large outdoor settings such as 'Malaria Spheres' in order to expose mosquitoes to normal environmental conditions. A recent study indicated that the spread of engineered refractoriness to *Plasmodium* infection in a vector population depends on the dispersal and mating behavior of the introduced engineered specimen (93). In Burkina Faso, the recent semi-field study of Nignan et al. showed that colonized mosquitoes were more likely to mate together than with their field counterparts (94). As mating success is known to be affected by microbiota composition in laboratory conditions (95), such cross-mating assessment in semi-field settings will also be required regarding candidate paratransgenic approaches. Modifications in microbiota composition in the surrounding mosquito population will also need to be tested in semi-field conditions before environmental trials, to assess colonization success of the paratransgenic microbe and other secondary impacts on mosquito microbiota.

In such set ups, Lovett et al. showed a successful dissemination of wild-type *Metarhizium pingshaense* fungi in *An. gambiae* and *An. coluzzii* mosquitoes in Burkina Faso (96). Such outdoor experiments allow to take into account highly variable and uncontrollable parameters which mosquitoes will face in the field, including temperature and relative humidity fluctuations that influence mosquito physiology, metabolism and survival (97). Highly variable factors including seasonality, diet, genetic identity, blood source, larval breeding site are also known to affect mosquito microbiota composition independently to mosquito species (98, 99). How such factors may affect the efficiency of a paratransgenesis approach has not yet been specifically assessed.

Going to field conditions, release of modified bacteria has not yet been performed but a recent study used cotton baits soaked with sugar, fruit cocktail or honey and placed in clay jars or in window entry traps around villages where malaria is prevalent to evaluate sugar-bait set ups for prospective bacterial release (100). Window entry traps were found to attract more mosquitoes but the proportion of sugar fed mosquitoes was higher in clay jars, and honey was the best attractant. Prior to any field study involving a paratransgenesis candidate, the spread of genetically engineered constructs will require to ensure the

acceptability of the chosen approach with the population. As this method may lead to some modification in the environment, interactions with local stakeholders will be required to balance such changes with the benefit for human health and the cost effectiveness of the approach. Guidance on such public engagement practices has been published, emphasizing that specific funding should be allocated to public engagement, which should start from early stages of the project and be active over many years, throughout the project. Such long-term interaction with mutual respect between different stakeholders and experts in diverse topics such as biology, history or politics, aims to promote honesty and transparency, and thus to reduce the risk of distrust (101, 102). Such experience is being built in countries including Burkina Faso, Mali and Uganda, where the first field trials of gene drive are expected in the next 5-10 years through the Target malaria project (103). This project identified key stakeholders, who will similarly need to be involved when considering the implementation of any other novel transmission-blocking approach such as paratransgenesis (103, 104).

CONCLUSION

This work reviewed recent progress in the development of paratransgenesis, a promising proposed strategy to reduce malaria transmission. We reviewed the arguments to consider when selecting paratransgenesis candidates and the genetic manipulation strategies under development. Vast efforts have also been spent to investigate bacterial spread in laboratory and semi-field mosquito populations. The stable introduction of modified microbes in field vector populations, the effect of antibiotics during mass-drug administration campaigns on transgenic bacteria in mosquitoes in endemic area and the risk of antimicrobial resistance selection against introduced transgenes are still questions to address before effective implementation of this approach. Despite these concerns, the approach presents advantages including a simple and fast genetic manipulation and potential for easy and low-cost introduction into mosquito populations. Moreover, the spread of the genetically-engineered bacteria within wild mosquito populations will not face the genetic reproduction barriers observed with genetically-modified mosquitoes. Regarding the regulatory and ethical requirements, accurate guidelines for field application on release of modified bacteria in the environment will need to be discussed in close collaboration with local authorities and representatives of the population. Altogether, paratransgenesis appears as an approach of interest for malaria control, compatible with other current and future control methods, from the use of insecticides to transgenic mosquitoes or transmission-blocking strategies.

AUTHOR CONTRIBUTIONS

Conceptualization, AF, MG, and RSY; Methodology, AF and M.G; Writing – Original Draft, AF and MG; Writing – Review and Editing, AF, EB, and MG; Supervision, MG, GO, RSY, and J-

BO. All authors contributed to the article and approved the submitted version.

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