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Symbiont Localization and Nature of Effector Molecules Generated in Malaria Vector-symbiont Relationships

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Authors' contributions

This work was carried out in collaboration between both authors. Author MCS did the literature searches and organization. Author SDI did the manuscript write-up. Both authors read and approved the final manuscript.

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ABSTRACT

The field of mosquito vector symbiosis is largely unexplored and yet it is likely that in the near future it will provide valuable opportunities for malaria control. Symbiont based malaria control approaches are gaining acceptance worldwide and there is need for detailed review to open up new research frontiers. Malaria vector symbionts localize in different parts of Anopheles mosquitoes and express proteins as effector molecules some with anti-Plasmodial effects. The types of effector molecules, their mode of action and site of action need to be elucidated. Microbial symbiotic species diversity and preferred locations in the malaria vector have not been adequately studied to understand the mode of transmission among vector species and from generation to generation. This is necessary for a better understanding of the behaviour and biology of symbionts before designing and executing symbiotic control strategies. The review highlights important developments in the dynamics of transmission of symbionts in malaria vector populations. The review forms a useful guide in the search and deployment of paratransgenic mosquitoes in

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symbiotic control of malaria. Aspects that require further elucidation by innovative research and new opportunities to exploit in malaria control are highlighted.

Keywords: Microbial symbionts; anti-Plasmodial effector molecules; malaria vectors; paratransgenesis; symbiotic control.

1. INTRODUCTION

Mosquitoes are considered the most dangerous insects in the world because they act as vectors of many devastating human diseases. Of all the known mosquito-borne diseases, malaria is undoubtedly one disease with the greatest detrimental impact on man [1]. There are about 3500 to 4000 mosquito species and those that transmit malaria belong to the genus Anopheles. Human malaria is transmitted only by *Anopheles* species and the major route of transmission to humans is via blood feeding of female *Anopheles* mosquitoes. Approximately 40 *Anopheles* species (principally *Anopheles gambiae* and *Anopheles funestus*) are able to transmit the parasites *Plasmodium* spp well enough to cause significant malaria illness and deaths worldwide [2].

Understanding the biology and behaviour of *Anopheles* mosquitoes help understand how malaria is transmitted and can aid in designing appropriate control strategies. Factors that affect a mosquito's ability to transmit malaria include its innate susceptibility to *Plasmodium*, its host choice and its longevity. Recently, microbial symbiotic relationships with known malaria vectors are reported to have an effect on vectorial capacity of Anopheles malaria vectors [3] hence important in control programmes. Malaria control programmes are beginning to consider microbial symbionts in addition to the susceptibility of malaria vectors to insecticides and the preferred feeding and resting location of adult mosquitoes. In this regard, more attention is focused on a relatively new concept 'Symbiotic Control' [SC] that targets manipulation of mosquito-symbiotic relationships as tool to control malaria [4].

Symbiotic relationships in insects are widely reported in literature [5]. Insect vector-symbiotic relationships are important for the evolutionary success of disease vectors and their wide distribution throughout the world. Some of the relationships are responsible for the great ability of insect disease vectors to adapt to different habitats. Among the factors that have significantly contributed to adaptability in malaria vectors is the mosquito gut microbiota [4].

Malaria vectors harbour various microorganisms as symbionts in what is known as malaria vector-symbiotic relationships [6]. The symbionts have elicited a lot of interest among biomedical scientists because they are beneficial to the host insects in many ways, including dietary supplementation, tolerance to environmental perturbations and maintenance and/or enhancement of host immune system homeostasis. Symbiont-mediated vector resistance to chemical insecticides has been reported [7] as well as defence against pathogens [8].

Microorganisms commonly involved in insect vector symbiotic relationships include various species of bacteria, fungi and viruses. They colonize different organs in malaria mosquito vectors mainly the mid-gut and to lesser extent salivary and reproductive organs either within or outside tissue cells [9].

Malaria vector-symbiotic relationships are associated with release of effector molecules within the host insect. The molecules generated through the natural association or by transgenesis are valuable agents that are reported to express anti-pathogen properties within the host. In some cases they may also negatively affect the life-span and vectorial capacity of the insect vector populations [4]. Some insect vector-symbiont relationships have been associated with negative insect behaviour and physiological changes [10] and emergence of insecticide resistance [7]. It is in this regard that effector molecules form partly the basis of SC, a new multifaceted approach that uses manipulation of symbiotic microorganisms to control insect disease vectors. The goal of SC is to reduce vector competence and subsequently reduce disease transmission to manageable levels. SC is promising and has generated a lot of interest amongst biomedical scientists worldwide.

This work reviews current knowledge and developments in *Anopheles* vector-symbiont relationships with particular interest on the localization of symbionts (bacteria, fungi, viruses) in the malaria insect vector and the nature of

molecules generated in malaria vectors. Aspects that require further elucidation by innovative research and new opportunities for exploiting anti-Plasmodial effector molecules for malaria control are highlighted.

2. MICROBIAL SYMBIONT LOCALIZATION IN MALARIA VECTORS

Microbial symbionts in malaria vectors are classified into bacteria, fungi and viruses. They locate either extra- or intracellular in various body cells, tissues and organs (Table 1). Bacteria colonize different organs in mosquitoes, mainly the mid-gut and to a lesser extent salivary glands and reproductive organs [9,11]. Insect salivary glands, ovaries and hemolymph are also known to be key organs for virus or parasite replication, but surprisingly the bacterial content of these organs in mosquitoes has not been fully characterized.

The insect gut is an important organ for nutrition and is considered as being immune-competent [12]. The gut forms an interface with the external environment and provides essential resources and space favourable to the multiplication of microorganisms ingested including the malaria parasites. Active gut bacteria contribute to mosquito digestion through the release of lytic enzymes and anti-Plasmodial effector molecules.

3. ANTI-PLASMODIAL SYMBIONT EFFECTOR MOLECULES

3.1 Bacterial Symbionts

The most prevalent microbial symbionts in malaria vectors are bacterial species from different genera that express a variety of anti-plasmodial effector molecules. These include species in the genera: *Pantoea; Asaia; Wolbachia*.

A bacterial symbiont *Pantoea agglomerans* of *Anopheles* mosquitoes has been engineered and reported to express and secrete anti-Plasmodium effector proteins [3]. The successful expression and secretion of anti-malaria molecules suggests that *P. agglomerans* is a potentially useful tool for malaria paratransgenic control. The anti-Plasmodium effector molecules produced are reported to inhibited development of the human malaria parasite *Plasmodium falciparum* and rodent malaria parasite *P. berghei* by up to 98% *in vivo*. It is thought that *P. agglomerans* induces the expression of peptides and many other antimalarial effector proteins known to interfere with the development of the parasite in mosquito mid-gut. The mechanism involves production of a DNA-protein that is imported to host nucleus targeting specific genes. The targeted genes express proteins that interfere with development of malaria parasite gametocytes in vector mid-gut. This argument is supported by studies carried on an engineered *Pantoea agglomerans*, which confirmed expression and secretion of anti-Plasmodium effector proteins. Several structurally distinct antimalarial effector proteins produced by species *P. agglomerans* and that are potential candidates for paratransgenesis are reported. The two most common are the salivary and midgut peptide 1 (SM1) and phospholipase A2 (PLA2) [3]. SM1 (8 kDa) is structurally similar to an epitope of the *Plasmodium* TRAP protein, a target used by the parasite to invade mosquito salivary glands and mid-gut epithelium [15]. PLA2 (21 kDa) binds to the asexual-stage surface protein of *Plasmodium berghei* and is reported to block oocyst development from gametocytes and ookinete formation in the mosquito mid-gut. A variant of PLA2 (23 kDa) is believed to intercalate in the mosquito midgut lining, preventing *Plasmodium* from migrating to the salivary glands hence halts development of sporozoites. It is therefore evident that effector mechanisms involve protein molecules that inhibit malaria parasite growth and development in the malaria vectors [16,17] possibly by activating immune genes with anti-Plasmodium effects [8].

The bacterium *Asaia*, has peculiar symbiotic relationship with malaria vector mosquitoes. This peculiarity relates to its unique characteristics: first of all, *Asaia* localizes in the gut, in the salivary glands and in the reproductive organs of vector mosquitoes of both sexes [13,14,18]. Secondly, *Asaia* are found in all the developmental stages of most malaria vectors [13] and uses different routes of transmission within and between malaria vector populations. It can be vertically transmitted to the progeny by maternal, paternal and trans-stadial routes and horizontally transmitted among individuals by mating and co-feeding [13-14,18,19].

The symbiont, *Asaia* is known to induce the expression of antimicrobial peptides by an unknown mechanism(s). However, several studies have reported that engineered *Asaia* strains produce unique proteins that colonize the mid-gut, reproductive organs and salivary glands of recipient mosquitoes [13,14,18]. There is keen

Table 1. Symbiont localization and mode of transmission in malaria vectors

Microbial symbiont	Location in malaria vector	Mode of transmission
Asaia spp	Gut, salivary glands, male and female reproductive organs.	Vertical transmission [13], paternal transmission to progeny by way of venereal transfer from male to females during mating [14]
Wolbachia spp	Intracellular in head, muscles, Malpighian tubules, ovaries, testes and haemolyph.	Maternally transmitted.
Pantoea agglomerans *Wickerhamomyces anomalus*	Mid-gut of many mosquitoes Mid-gut and reproductive system in larva, pupa and adult males and females.	Vertical transmission Multiple transmission patterns suggested; specifically vertical transmission.
Metarhizium anisopliae	Insect cuticle	Vertical transmission; horizontal transfer (mediated through environment) between mosquitoes at larval, pupa and adult stages.
Densonucleosis viruses (DNVs)	Various tissues, mainly mid-gut, fat body and ovaries.	Vertical transmission.

interest to use bacterium *Asaia* to express anti-parasite molecules within the mosquito body so as to inhibit malaria parasite growth and transmission.

Another extensively studied bacterial mosquito symbiont is the alpha-proteobacterium *Wolbachia*. It is a common cytoplasmic symbiont of many insect species [20]. Wolbachia are maternally inherited bacteria known to initiate cytoplasmic incompatibility (CI) in vector mosquitoes. They use the mechanism to spread their progeny from one generation of mosquito to another thus enhancing their transmission [20,21]. These self-replicating microbial symbionts are attractive as vehicles applicable for delivery of genes that express effector molecules; that could lead to population genetic replacement, modulate malaria vector population age structure and ultimately reduce malaria transmission. However, the challenge is that *Wolbachia* natural infections are rare in mosquito species belonging to the genus *Anopheles* [22]. Attempts to culture the bacteria in *Anopheles* mosquito cells have been cited in literature [23] thus opening many opportunities to determine its possible application in malaria control.

The species *Wolbachia pipientis* (strain wMelPop) is reported to produce unidentified effector molecules that induce immune up-regulation in malaria vectors possibly by up-regulation of several specific immune genes by unknown mechanism(s). The up-regulated vector immune system has direct negative effect(s) on the development of malaria parasites in the vector [24]. Other strains of *Wolbachia* (wMelPop and wAlbB) are associated with somatic infections in *An. gambiae*. The strain wMelPop locates mainly in fat body, head, sensory organs and other tissues but not in mid-gut and ovaries of *Anopheles gambiae*. Both strains are reported to inhibit *P. falciparum* oocyst development in the mosquito mid-gut though the mechanism(s) involved is/are not known. But the fact that the strains cause somatic infections in malaria vectors suggests that the two could potentially be considered as part of a strategy to control malaria-transmitting mosquitoes [25].

Evidence is emerging suggesting that *Wolbachia* infection in *An. gambiae* initiate production of effector molecules that stimulate expression of several immune genes whose products significantly reduce the intensity of *Plasmodium* infection by inhibiting *Plasmodium* development [24]. The effector molecules are reported to manipulate the reproductive properties of the insect hosts by inducing parthenogenesis, male-killing, feminization in addition to cytoplasmic incompatibility [26,27].

3.2 Fungal Symbionts

The fungus *Wickerhamomyces anomalus* (yeast) is a symbiont of some mosquito vector species and localizes in the mid gut and reproductive organs of the host vector [28,29]. Several strains

of *W. anomalus* produce killer toxins with an antimicrobial effect on a wide spectrum of human pathogens [30,31]. However, the effect of the killer toxins against *Plasmodium* parasite needs verification. There is possibility that if found active against the malaria parasite, the strains could be used to control malaria parasite development by the release of this natural killer toxins within mosquito organs. The variety of killer toxins produced suggests that the symbiont may be a good candidate for the expression of effector molecules in the mid gut of mosquito malaria vectors.

The killer toxins (KT) produced by *W. anomalus* have been identified as a group of glycoproteins with a variable molecular weight and whose activity is characterized by a large range of optimal pH and temperatures [32]. The composition of KT is not clear but KT-derived 'killer' peptide (KP) has been characterized from aggregates on the surface of parasites within mosquito vectors and attempts are being made to produce strains of *W. anomalus* capable to express and deliver anti-Plasmodium peptides at target sites within malaria vector mosquitoes.

It is evident that the localization, the possibility to easily manipulate yeast and the chance to express effector molecules in a eukaryotic organism render *W. anomalus* a potentially good candidate for paratransgenesis. Furthermore, in many occasions both *W. anomalus* and bacterium *Asaia* locate in the mid-gut and reproductive organs of malaria vector mosquitoes in large numbers. Hence, there is possibility to use synergistically these two symbionts to release different effector molecules in malaria symbiotic control strategies.

Another fungus, *Metarhizium anisopliae*, is natural pathogen of mosquitoes. It is acquired at larval, pupa stages and transmitted vertically in mosquito vector populations [33]. A recent study has described the use of transgenic *Metarhizium anisopliae* fungus to inhibit malaria transmission by abolishing parasite development within the mosquito [29]. The fungus has a capability to infect mosquitoes through the cuticle by use of proteases and chitinases to solubilise cuticle [34]. Recombinant strains of *M. anisopliae* that express three effector protein molecules (SM1, a single chain antibody called PfNPNA-1 and an antimicrobial peptide called scorpine) have been reported [29]. These effector molecules target the sporozoite stage of the malaria parasite in the hemolymph and the salivary glands in *An. gambaie*. The molecules are reported to reduce sporozoite counts significantly especially when M. anisopliae strains expressing scorpine and [SM1] were used. These findings suggest that *Metarhizium*-mediated inhibition of *Plasmodium* development is a possible tool to inhibit malaria parasite development within the *An. gambiae* malaria vector.

3.3 Viral Symbionts

Viral symbionts locate in almost all tissues of insect hosts but organs mainly infected are the mid-gut, fat body and the ovaries. Virus species commonly associated with malaria vectors are densonucleosis viruses (DNVs) [35]. Viruses in this group can be genetically manipulated, are highly infectious and are transmitted to subsequent generations vertically in mosquito vectors. Cloned virus strain known as DNV (AgDNV) has been found capable to express gene(s) of interest in *An. gambiae* including an exogenous gene that generate enhanced green fluorescent protein (EGFP). In infected *An. gambiae*, the AgDNV virions generate EGFP detectable in various tissues such as mid gut, fat body and ovaries and are easily transmitted to subsequent mosquito vector generations. This is clear evidence that AgDNV could be used as part of a paratransgenic malaria control strategy by transduction of anti-Plasmodium effectors or insect-specific toxins in *Anopheles* mosquitoes. This is possible because DNV effect gene expression in mosquitoes that can generate effector molecules thought to be anti-Plasmodium peptides or insect-specific toxins in *Anopheles* mosquitoes [35].

4. CONCLUSION

Microbial symbionts in malaria vectors are classified into bacteria, fungi and viruses. They locate either extra- or intracellular in various body cells, tissues and organs. Many symbionts locate in the vector gut than any other part of the insect vector. It is important to note that most stages of *Plasmodium* development occur in the mosquito mid-gut. The mid-gut is therefore a compartment shared with symbiotic microorganisms and secretions of anti-malarial effector molecules in the mid-gut effectively inhibit development of the human parasite *Plasmodium falciparum* in the mosquito.

The mosquito mid-gut therefore serves as a prime target for blocking parasite transmission with effector molecules for two main reasons.

First because the most vulnerable part of the *Plasmodium* life cycle in the mosquito occur in the mosquito mid-gut lumen. Secondly, the vector mid-gut compartment is shared between the *Plasmodium* and the mosquito microbial symbionts, directly exposing parasites to effector molecules secreted by resident symbionts. Furthermore, the number of microbial symbionts in the mosquito vector mid-gut increases rapidly following ingestion of a blood meal. This consequently increases the output of effector molecules produced by symbionts which may be accompanied by devastating anti-Plasmodial effect. We therefore suggest that efforts to create recombinant symbionts should focus more on gut microbial symbionts.

Many symbionts of malaria vectors naturally produce various types of proteins as effector molecules but can also be cultivated outside the host, manipulated to express specific factors and reintroduced within he host to produce *in situ* effector molecules with inhibitory effect on *Plasmodium* development. The mode of action of some of the molecules is not clearly known but may modulate specific genes to express specific proteins and/or produce killer toxic substances that have negative effect on malaria parasite development in the vector. Whether the natural effector molecules identified to date have any effect(s) on non-target organisms other than *Plasmodium* species is not clearly known and require further research. Approaches to modify gut symbiont for *in-situ* expression of anti-pathogens effector molecules require large-scale studies to determine possible ecological parameters of paratransgenic mosquito in natural habitats and to evaluate potential risks like horizontal gene transfer from the modified bacteria to environmental microbes.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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