Symbiotic control of mosquito borne disease

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It is well accepted that the symbiotic relationships insects have established with several microorganisms have had a key role in their evolutionary success. Bacterial symbiosis is also prevalent in insects that are efficient disease vectors, and numerous studies have sought to decrypt the basic mechanisms of the host–symbiont relationships and develop ways to control vector borne diseases. ‘Symbiotic control’, a new multifaceted approach that uses symbiotic microorganisms to control insect pests or reduce vector competence, seems particularly promising. Three such approaches currently at the cutting edge are: (1) the disruption of microbial symbionts required by insect pests; (2) the manipulation of symbionts that can express anti-pathogen molecules within the host; and (3) the introduction of endogenous microbes that affect life-span and vector capacity of the new hosts in insect populations. This work reviews current knowledge on microbial symbiosis in mosquitoes that holds promise for development of symbiotic control for mosquito borne diseases.

Keywords: Symbiotic control, Mosquito, Vector borne diseases

Introduction

Some of the most devastating infectious diseases, such as malaria, dengue, yellow fever, and filariasis, are transmitted by mosquitoes, and cause a dramatic health burden to people living in endemic regions of the world. Mosquitoes, like all insects, are an example of great evolutionary success, as witnessed by the large number of species that have been described (around 3500) and their wide distribution throughout the world, except for Antarctica, a sign of their great ability to adapt to very different types of habitats. Among factors that significantly contribute to this great adaptability, mosquito microbiota may play a critical role. Although early studies on microbiota associated with some mosquito species date to a few decades ago,^{1,2} and though bacteria, like some sero-varieties of *Bacillus thuringiensis*, have been proposed in the biological control of mosquito borne diseases (MBD),³ only recently has research in ‘mosquito-symbiosis’ expanded, with the goal of developing innovative MBD control strategies through Symbiotic Control (SC), a multifaceted approach that uses symbiotic microorganisms to control insect pests or reduce vector competence.

Recent works have examined (1) the roles of symbiotic microorganisms in specific biological traits of several insect hosts that should also be exploited in

mosquitoes and (2) the feasibility of SC in countering vector borne diseases.

In fact, a good deal of recent evidence clearly indicates that microbial symbionts, particularly bacteria, may influence specific traits of insect–host biology such as mating behaviour in *Drosophila paulistorum*,⁴ insecticide resistance in the *Riptortus pedestris* bean bug, and fitness, nutrition,⁵ defence, and reproduction in a significant number of insect species.^{6,7} All these particular associations between symbionts and insects open interesting perspectives for MBD control; the relationships between bacteria and mosquitoes merits focused investigation.

One of the best examples of the feasibility of using insect symbionts to control vector borne diseases has been achieved with the vector of Chagas disease.⁸ Chagas disease is caused by the parasitic protozoan *Trypanosoma cruzi*, transmitted by the so-called kissing bug, an insect belonging to the Reduviidae family (Triatominae subfamily). The kissing bug feeds throughout its entire developmental cycle on vertebrate blood; thus, it harbours symbiotic bacteria in the gut, *Rhodococcus rhodnii*, which produce nutrients, such as vitamins, which allow the insect to compensate for a based exclusively on blood feeding.

Researchers were able to cultivate populations of this symbiont, genetically modify them to produce anti-trypanosomal effector molecules, and place them back into the triatomina to express the anti-trypanosomal molecules in the insect gut. This

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procedure, known as paratransgenesis, has proven effective in generating insects incapable of transmitting the disease.⁹

Moreover, semi-field trials have demonstrated the controlled spread of genetically modified symbionts in natural populations of triatominae vectors, thus reinforcing the feasibility of paratransgenesis as part of an integrated vector control program.^{10,11}

These achievements in insect vectors other than mosquito have strongly encouraged researchers to develop similar approaches to control MBD.

Symbiotic Control and Mosquitoes

Symbiotic control of insect pests and disease vectors has been developed through three main approaches:

1. the disruption of microbial symbionts required by insect pests/disease vector;
2. the manipulation of symbionts that can express anti-pathogen molecules within the host;
3. the introduction of endogenous microbes that affect life-span and vector capacity of the new hosts in insect populations.

In mosquito, the second and third approaches have been recently investigated in studies to assess their potential use in MBD control.

Manipulation of Symbionts to Express Anti-pathogen Molecules within the Mosquito-Host

As stated above, paratransgenesis aims to eliminate pathogens from host-vector populations through transgenesis of a symbiont of the vector. Thus, to develop an efficient paratransgenic approach to control MBD, suitable symbiotic microorganisms with specific and well defined features must be identified.

First of all, they should be cultured and stably genetically engineered. Furthermore, the fitness of the engineered symbionts should not be compromised and they should produce effective anti-parasite molecules.

Last but not least, an efficient means of distributing the symbionts must be developed.

To date, quite a few bacteria have already been engineered to express anti-pathogen effector molecules.

Riehle *et al.*¹² engineered *Escherichia coli* to display two anti-*Plasmodium* effector molecules on their outer membrane. With both molecules, a significant inhibition of *Plasmodium berghei* development was recorded when engineered bacteria were fed to mosquitoes 24 hours before an infective blood meal. Even though the prevalence and numbers of engineered bacteria increased dramatically following a blood meal, *E. coli* survived poorly in mosquitoes. Consequently, *Enterobacter agglomerans* was isolated from mosquitoes and selected for midgut survival by multiple passages through mosquitoes. After some passages, *E. agglomerans* survivorship showed an increase from 2 days to 2 weeks. These data coupled with the fact that *E. agglomerans* is non-pathogenic

and widespread, indicated *E. agglomerans* as an excellent candidate for a paratransgenic malaria control strategy. More recently, *Pantoea agglomerans*, a bacterial symbiont of *Anopheles* mosquitoes, has been engineered to express and secrete anti-*Plasmodium* effector proteins.¹³ The successful expression and secretion of anti-malaria molecules indicates *P. agglomerans* as a potentially useful tool for malaria paratransgenic control. This has been demonstrated by a study that used engineered *P. agglomerans* strains expressing some anti-*Plasmodium* effector molecules that inhibited development of the human malaria parasite *Plasmodium falciparum* and rodent malaria parasite *P. berghei* by up to 98% *in vivo*. This is a further demonstration that the use of an engineered symbiotic bacterium may be a powerful tool to combat malaria.¹⁴

Another potential candidate for use in mosquito paratransgenesis is the alpha-proteobacterium *Asaia*, which has attracted considerable interest due to its peculiar relationship with mosquitoes. This peculiarity relates to several elements: first of all, *Asaia* localizes in the gut, in the salivary glands and in the reproductive organs of mosquitoes of both sexes.^{15–17} Secondly, our group found *Asaia* in all the developmental stages of all tested malaria vectors and in *Aedes aegypti*, the main yellow fever vector, with very high prevalence within different populations (often reaching 100%) where it is the dominant bacterium.¹⁵ *Asaia* is easily cultivable outside the host in cell-free media, and we genetically transformed it to express fluorescent proteins. The introduction of modified symbionts unequivocally indicates that they reach the original organs in massive numbers, where they express the traceable markers in most of the members of the recipient populations.^{15,18} Moreover, *Asaia* uses several different routes of transmission within and between 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.^{15–17,19}

This fact of vertical transmission may make it possible to successfully introduce modified bacteria into mosquito populations in the field. Since a gravid mosquito will lay about 80–100 eggs,²⁰ about 50% of which develop into female adults, one could foresee a significant increase in the number of mosquitoes carrying *Asaia*, with rapid spread of the bacterium between members of a population.

Our group evaluated the fitness of two strains of *Asaia* modified to express different fluorescent proteins. Those strains of *Asaia* were delivered by allowing 3- to 4-day-old mosquitoes to feed overnight on cotton pads soaked with an *Asaia* suspension in 5% sucrose. Thereafter, mosquitoes were checked at frequent intervals for the presence of the modified

bacteria. We determined that *Asaia* remained in the mosquito gut as well as in the reproductive organs for at least 20 days, and in the salivary gland for at least 14 days when the assay was discontinued.¹⁷

At the present, attempts are underway to modify *Asaia* to produce strains able to express and secrete anti-*Plasmodium* effector molecules to be used in 'malaria transmission blocking' experiments.

In recent years, several other endosymbionts have been described as stably associated with different mosquito species. Some are newly identified bacterial species, and extensive studies are rapidly increasing the number of potential candidates to be used in paratransgenic approaches to control MBD.^{21–24}

To develop such approaches, it is also possible to refer to other microorganisms rather than bacterial endosymbionts; viruses, fungi, and yeasts have recently been proposed. Densoviruses, capable of infecting and disseminating in *Anopheles gambiae*, have been recently proposed as a paratransgenic tool for malaria control strategies.²⁵ The *Wickerhamomyces anomalus* yeast, which has been indicated as a symbiont of some mosquito vector species, has been found in the midgut and reproductive organs of the host.^{26,27} This mosquito symbiont can be cultured in cell free media and thus may be a good candidate for the expression of effector molecules in the midgut of mosquito vectors. A recent study describes the use of the transgenic *Metarhizium anisopliae* fungus to inhibit malaria transmission, abolishing parasite development within the mosquito.²⁸

All these studies indicate that paratransgenesis offers a potentially feasible technological approach to manipulate mosquito functions and control MBD by blocking pathogen transmission in the mosquito vector.

The Introduction of Endogenous Microbes that Affect Life-Span and Vector Capacity of the New Hosts in Insect Populations

It is well known that natural endosymbionts may inhibit pathogen development in mosquito. There is quite a vast literature in that sense concerning several bacterial genera.²⁹ For example, *Plasmodium vivax* infections in mosquitoes co-infected with *Enterobacter amnigenus*, *Enterobacter cloacae*, and *Serratia marcescens* were significantly lower than in control mosquitoes, while mortality was much higher in *S. marcescens*-infected mosquitoes than in controls.³⁰

Dong and collaborators³¹ have recently demonstrated that the microbial flora associated with some malaria vectors can modulate the mosquito's vectorial capacity by inhibiting the development of *Plasmodium* and other human pathogens through the modulation of some immune genes with anti-*Plasmodium* effects.

Thus, the idea to use bacterial infection of mosquito to prevent parasite transmission has been pursued in recent years. In this context, the most promising results have been achieved with the maternally transmitted endosymbiont *Wolbachia*, which has been detected in arthropods and filariae.^{32,33} Recent estimates indicate that 40% of terrestrial arthropod species are infected, confirming that *Wolbachia* is the most abundant endosymbiont among arthropod species.³⁴ *Wolbachia* is able to manipulate the reproduction of its arthropod hosts, and the role of so-called 'cytoplasmic incompatibility' (CI), which the bacteria use to increase the number of infected individuals in the host population, is particularly significant in this context.

CI causes reduced fertility when an uninfected female mates with a *Wolbachia*-infected male.³⁵ On the other hand, *Wolbachia*-infected females can produce viable progeny when mating with either infected or uninfected males. Thus, infected females have a selective reproductive advantage in respect to uninfected females.³⁶

This ability of *Wolbachia* to spread rapidly through host populations opens the door to the development of several *Wolbachia*-based potential applications to control MBD.

Though, as mentioned above, a huge number of arthropod species is naturally infected with *Wolbachia*, it has never been found in the *Anopheles* genus (the only known vectors of human malaria belong to this genus) and in *Ae. aegypti* (one of the main vector of dengue virus and other disease agents).^{37–40}

The infection of *Wolbachia*-negative mosquitoes (like *Anopheles* spp. and *Ae. aegypti*) with specific *Wolbachia* strains has been regarded as a very promising strategy for the development of novel methods for pathogen transmission, and the results obtained to date are indeed encouraging.

Infecting *Ae. aegypti* with *Wolbachia* may cause different effects in the host. In particular, two effects open interesting perspectives in diseases control:

1. shortened mosquito life-span;⁴¹
2. refractoriness to dengue infection and inhibition of filarial infective stage development, due to up-regulation of the mosquito immune response.⁴²

The life-shortening effect was first discovered in *Drosophila melanogaster* and the strain of *Wolbachia* responsible for this phenomenon was named *wMelPop*.⁴³ This life-shortening *Wolbachia* strain was artificially introduced into *Ae. aegypti* recipient mosquitoes, with a consequent halving of the life-span of these insects. The '*wMelPop-Ae. aegypti*' association proved to be stable and the *Wolbachia* strain was shown to be maternally inherited at high frequency and able to induce CI, thus demonstrating its

potential to spread into *Ae. aegypti* populations and to serve in dengue control strategies.

In the second case, genetically identical *Ae. aegypti* lines infected and uninfected with *wMelPop* were compared to determine whether differences in gene expression between the two lines were related with the life-shortening phenotype. This study indicated that *wMelPop* induces an up-regulation of the mosquito's innate immune system and that its presence inhibits the development of filarial nematodes in the mosquito. Once again, these data suggest that *wMelPop* could be used in control programs to eradicate lymphatic filariasis and other MBDs.

These data have been further corroborated by a more recent study on *An. gambiae*, the main African malaria vector.⁴⁴ Infection of an *An. gambiae* cell line or transient somatic infection of *An. gambiae* mosquitoes with *wMelPop* stimulated expression of several immune genes. Furthermore, transient somatic infection significantly reduced the intensity of *Plasmodium* infection through a TEP1-dependent mechanism.

In addition to these studies at the 'bench' level that reflect the potential of using *Wolbachia* strains to control MBD, very recent evidence from the field also supports the use of *Wolbachia* to defeat dengue diseases.

Walker *et al.*⁴⁵ have demonstrated that the *wMel* *Wolbachia* strain from *Drosophila melanogaster* can block dengue transmission in *Ae. aegypti*. Furthermore, Hoffmann *et al.*⁴⁶ showed that *wMel* *Wolbachia* can successfully invade the natural Australian population of the dengue vector *Ae. aegypti*.

These small trials look particularly promising. In fact, within 14 weeks of the release of around 250 000 *Wolbachia*-infected *Ae. aegypti*, around 90% of the populations in the test areas were positive for *wMel* *Wolbachia*. Furthermore, this study pointed out that *Wolbachia*-infected mosquitoes suffered relatively small fitness costs. It is worth noting that the *wMel* strain differs from *wMelPop* in that it is not a life-shortening *Wolbachia* strain, and thus has fewer fitness cost implications for mosquito hosts.

On the basis of these developments, new field trials have been planned in Brazil, Thailand, Indonesia, and Vietnam; this approach to 'vaccinate' mosquitoes rather than people is attracting considerable interest.

One more *Wolbachia*-based approach that has found several applications in the field of insect control is the so-called Insect Incompatible Technique (IIT). IIT resembles the Sterile Insect Technique because it is based on the release of large numbers of sterile insects to compete for mates in the wild, leading to population suppression.⁴⁷ IIT differs from Sterile Insect Technique because rather than using irradiation to induce sterility, it relies on the mechanism of *Wolbachia*-induced cytoplasmic

incompatibility. IIT has been successfully developed for some major agricultural pests^{48,49} and recently has also been explored for its effectiveness in the control of mosquito populations in the field. Recently, Atyame and collaborators⁵⁰ showed that the *wPip(Is)* *Wolbachia* strain, naturally infecting *Culex pipiens pipiens* from Turkey, can be used in the context of IIT to sterilize *Cx. p. quinquefasciatus* females from several islands of the southwestern Indian Ocean, confirming the feasibility of IIT in mosquito control.

Conclusions

The field of mosquito symbiosis is still largely unexplored, and it is likely that in the near future, new associations will provide opportunities to establish effective methods to control MBD.

This paper described the state of the art of SC-based approaches for fighting MBD.

Concerning approaches that modify gut symbiont for 'in-situ' expression of anti-pathogens effector molecules, large-cage studies before any open field releases are necessary, in order to assess the ecological parameters of paratransgenic mosquito in natural habitats, and to evaluate any potential risks, such as those associated with horizontal gene transfer from the modified bacteria to environmental microbes.

Furthermore, all the approaches based on symbiotic control need to address regulatory and ethical concerns raised by specialists and local populations in the last few years.

Despite these concerns, mosquito symbiotic control offers several favourable features compared to other control methods. First, genetic manipulation of symbionts is much simpler and faster than genetic manipulation of mosquitoes, and transformed symbionts are much easier to introduce into mosquito populations than transgenes. Second, use of transformed symbionts bypasses the genetic barriers of reproductively isolated mosquito populations that often occur in areas of high malaria transmission, and thus which present a great obstacle to the spread of mosquito transgenes. Third, microorganisms can be largely produced at low cost, even in some developing countries, and possible inactivation of bacterial transgenes in the field is not a major concern because of the easier logistics of introducing newly transformed bacteria.

Finally, concerning regulatory and ethical requirements, already-existing regulations on the release of modified bacteria into the environment are a sufficient starting point for formulating more specific guidelines for field applications of mosquito symbionts.

Acknowledgements

This work was supported by Fibr-Ideas (grant RBID 082MLZ) and Prin 2009 (grant 2009L27YC8_003),

both from the Italian Ministry of Education, University and Research (MIUR) and by the EU-FP7 Capacities-Infrastructure 2008 (grant 228421) to GF. We would like to thank Sheila Beatty for editing the English usage in the manuscript.

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