MINI REVIEW

Mosquito symbioses: from basic research to the paratransgenic control of mosquito-borne diseases

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Abstract

Mosquito-borne diseases pose significant concerns in public health. Microbial symbionts of mosquitoes are attracting quite a lot of interest in relation to the development of novel strategies aimed to reduce mosquito vectorial capacity with particular regard to paratransgenesis that relies on genetically modified mosquito symbionts to express molecules within the vector able to interfere with parasite development and transmission. Here, we review the present status of the knowledge of mosquito-symbionts relationships, focusing on perspective in the application of symbiotic control in developing an efficient management of mosquito-borne diseases.

Introduction

Over half of the world human population is exposed to the risk of contracting infections transmitted by mosquitoes. The lack of effective vaccines for many of these infections makes their control mainly dependent on the use of insecticides. Even though the combination of insecticide-based strategies with therapeutic and prophylactic treatment of human hosts has been very effective for some of these diseases in several regions, control based on chemical insecticides is not always feasible due to several factors among which the economic cost, the insecticide resistances and the ecological impact of the treatments.

Consequently, novel approaches to control mosquito-borne diseases are strongly needed. The idea of exploiting microorganisms as agents to combat mosquitoes was proposed over 20 years ago; in this context, particular attention has been posed on *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus*

(Lacey 2007). During sporulation, these bacteria produce some protoxin crystals that are highly toxic once ingested by susceptible mosquito larvae. As these toxins are generally specific for a given insect, they are considered environmentally safe; in addition, they are cost-effective and relatively easy to use, due to the ability of *B. thuringiensis* var. *israelensis* to grow on artificial media and the development of formulations that can be applied using conventional insecticide application technology (Federici 1995; Bhattacharya 1998). Several field trials have supported the potential of *B. thuringiensis* var. *israelensis* and *B. sphaericus* for controlling mosquitoes. On the other hand, the toxins of *B. sphaericus* and, in particular, those of *B. thuringiensis* var. *israelensis*, have a short persistence in nature which implies frequent applications, limiting their possible applications.

Furthermore, *B. thuringiensis* var. *israelensis* can be applied with success in particular environments, but its efficacy can be limited in other conditions.

Nevertheless, microbial insecticides for larval control have a great potential in the context of integrated vector management programmes and may synergistically improve other control methods targeted against adult insects.

In addition to the strategies based on the use *B. thuringiensis* and *B. sphaericus*, microorganisms associated with mosquitoes might offer novel ways to the control of these insects and of the diseases they transmit. For example, symbiotic microorganisms associated with mosquitoes might be exploited to interfere with the vector capacity of the insect. In this context, two approaches seem potentially very promising, the first being based on the possibility to eliminate those symbionts that contribute in some ways to the biology and vector capacity of the insect host, the second on the genetic manipulation of microbial symbionts, for the expression of anti-pathogen effector molecules aimed at rendering the vector incapable of transmitting pathogens (Riehle et al. 2007). The overall strategy of combating insect pests and vector-borne diseases through the use of symbiotic microorganisms is referred to as symbiotic control (SC), also known as paratransgenesis. In the context of SC, the strategy based on the manipulation of symbionts for the production of anti-pathogen effector molecule(s) is known as paratransgenesis (Douglas 2007; Coutinho-Abreu et al. 2010).

Until recently, investigations on the microbial community harboured by mosquitoes were underexplored. A pioneering study was published by Jadin et al. (1966) describing *Pseudomonas* sp. in the midgut of field-collected *Anopheles* mosquitoes from the Democratic Republic of Congo. More recently, several genera and species of midgut bacteria have been isolated from *Anopheles gambiae* sensu lato and *Anopheles funestus* mosquitoes caught in Kenya and Mali, with *Escherichia coli* and *Pantoea agglomerans* as the most frequently isolated (Straif et al. 1998). Gonzalez-Ceron et al. (2003) isolated different species of *Enterobacter* and *Serratia* from *Anopheles albimanus* mosquitoes from southern Mexico. In the last 5 years, further studies have been conducted on the microorganisms associated with mosquitoes, with the explicit aim of identifying bacteria that could effectively be used in the SC of mosquito-borne diseases (Lindh et al. 2005; Favia et al. 2007; Riehle et al. 2007; Damiani et al. 2008, 2010). This research area offers interesting perspectives both for the acquisition of a better knowledge of mosquito biology and its symbiotic relationships as well as for the development of novel control methods.

Symbiosis in Insects

At least in terms of biomass and biodiversity, insects are among the most successful animals on Earth. The symbiotic relationships that insects have established with different types of microorganisms have probably played a key role in the evolutionary success of insects: symbiotic microorganisms can provide a variety of functions, integrating the functions under the control of the host genome and allow the insect a great ability to adapt to the environment (Feldhaar and Gross 2008). Symbiotic association in insects can take various forms and we refer to them in the broad sense, as defined by de Bary (1879) that intended symbiosis as the '*persistent living together of unlike organisms*'. There are relationships where the insect host and the symbiont cannot live independently (obligate symbiosis); these associations present high levels of specialization and coadaptation, for example that occurring between *Buchnera aphidicola* and aphids (Moran 2006). Other associations are obligatory only for the symbiont, as witnessed by the relationships between *Wolbachia* and several insect species (Saridaki and Bourtzis 2010). Finally, there are also microorganisms that appear to form association with insects while being also present in the environment (e.g. *Asaia* spp.; Chouaia et al. 2010); these could be indicated as facultative symbionts. Several insects harbour both obligate symbionts and facultative ones. Obligate symbiosis are generally ancient in origin and involve microorganisms that live in specialized host cells called bacteriocytes or mycetocytes (depending on whether the symbiont is a bacterium or a fungus); these bacteriocyte/mycetocyte symbionts generally provide the insect hosts with nutrients that are not present in their natural diet (Schiøtt et al. 2008). Facultative symbionts are more recent in origin and unlike obligate ones, can be cultured outside their host offering exciting possibility for genetic manipulation and reintroduction in the insect hosts to control the transmission of pathogens (Pontes and Dale 2006). Indeed, obligatory symbionts are generally intracellular, which makes their genetic manipulation quite difficult.

One of the symbionts of mosquitoes that has attracted a great deal of attention in the last 15 years for its potential for SC is *Wolbachia pipientis* (Wright et al. 1978). Several review articles have already been devoted to this symbiont and its possible application in the control of vector-borne diseases (e.g. Cook et al. 2008). Therefore, rather than concentrating on *Wolbachia*, we will review on facultative

symbionts associated with mosquitoes, that are opening interesting perspectives in the control of mosquito-borne diseases.

Symbiosis in Mosquitoes

As mentioned above, until recently, only a limited number of studies had been published regarding the structure of the bacterial community present in mosquitoes. These studies showed that different mosquito species of the genera *Anopheles*, *Aedes*, *Culex*, *Psorophora* host common bacterial genera as, for example, *Pseudomonas*, *Staphylococcus*, *Enterobacter*, *Escherichia*, *Serratia*, *Klebsiella* (Demaio et al. 1996; Touré et al. 2000; Pidiyar et al. 2002, 2004). These studies showed that the presence of genetically well-characterized bacteria in the midgut of mosquitoes could provide a mean of expressing specific products in mosquito vectors, opening the possibility of blocking the transmission of the pathogen by the insect. Furthermore, the presence of some bacterial species in the midgut of vector mosquitoes can *per se* interfere with the mosquito vectorial capacity. Several studies underline this aspect: Gonzalez-Ceron et al. (2003) investigated the impact of bacterial infections in the midguts of insectary and field-collected *An. albimanus* from southern Mexico on their vector capacity. *Serratia marcescens*, *Enterobacter cloacae* and *Enterobacter amnigenus*, *Enterobacter* sp. and *Serratia* sp. were isolated in field samples, while no bacteria were isolated from insectary specimens. *Anopheles albimanus* mosquitoes reared in aseptic conditions were infected with these bacteria, together with *Plasmodium vivax*. The number of infected mosquitoes, as well as the oocyst densities, was then assessed. *P. vivax* infections in mosquitoes co-infected with *En. amnigenus*, *En. cloacae* and *S. marcescens* were remarkably lower than in control mosquitoes; mortality was much higher in *S. marcescens*-infected mosquitoes compared with controls. This study clearly indicated that the midgut bacterial microflora can strongly influence the vector capacity and overall fitness of mosquitoes as also showed by some recent articles that report the influence of midgut bacteria on *Plasmodium* development (Dong et al. 2009; Meister et al. 2009; Kumar et al. 2010). Other studies have demonstrated that midgut bacteria can influence physiological traits of the mosquito: Fouda et al. (2001) clearly indicated that midgut bacteria strongly influence digestion and fecundity of mosquitoes and are essential for the completion of the embryonic development.

Recent Advances in Mosquito Symbiosis

In the last few years, investigations on mosquito symbiosis gained new life from studies on malarial vectors. The first article using 16S rRNA gene-based identification of bacteria associated with mosquitoes of the *An. gambiae* complex in Kenya (Lindh et al. 2005) light up the presence of several bacterial species belonging to different genera. Some of the culturable bacteria were further characterized; a novel species of *Janibacter* was named *Janibacter anophelis* and a bacterium of a novel genus was named *Thorsellia anophelis* (Kämpfer et al. 2006a,b). *Thorsellia anophelis* was also detected in aquatic environments where larvae develop (Briones et al. 2008) thus appearing as a promising system for the design of SC tools against *Plasmodium*.

Lindh et al. (2008), by 16S rRNA sequence analysis and temporal temperature gradient gel electrophoresis, identified several bacteria in *An. gambiae*, among which *Pantoea stewartii* and *Elizabethkingia meningoseptica*. These appear to be of particular interest, as they seem well adapted to mosquito midgut (*E. meningoseptica* was the most frequently isolated bacterium from adult mosquitoes and most of the newly emerged adults with bacteria in their abdomens had this species) and thus potentially useful for SC applications. Starting in 2007, our research group published a series of articles demonstrating the widespread diffusion of bacteria of the genus *Asaia* (Gram-negative, α -proteobacteria) (fig. 1) in different mosquito species (Favia et al. 2007, 2008; Damiani et al. 2008, 2010; Crotti et al. 2009). Based on this series of studies, we can summarize that bacteria of the genus *Asaia*: (i) are stably associated with mosquitoes belonging to different species (*An. gambiae*, *Anopheles stephensi*, *Anopheles maculipennis* and

Fig. 1 Transmission electron micrograph of *Asaia*, taken from the midgut content of a mosquito of the species *Anopheles stephensi*. The bacterium is characterized by the presence of an electron-dense cytoplasm and a filamentous nucleoid region.

Aedes aegypti); (ii) are localized in the gut and in the reproductive organs of both male and female adult mosquitoes; (iii) in tested species, are present in all developmental stages, indicating a vertical transmission; (iv) present high prevalence in mosquitoes, which reaches 100% in laboratory colonies.

Asaia is easily cultivable in cell-free media. This has quickly allowed the modification of these bacteria, for the production of recombinant proteins. Engineered bacteria, transformed to produce fluorescent proteins, have been added to the food (sugar solution or blood) of mosquitoes, and showed a remarkable capacity to colonize the midgut, reproductive organs and salivary glands of recipient insects (Favia et al. 2007; Damiani et al. 2008, 2010; Crotti et al. 2009). These studies represent a proof of principle of the possibility of using this bacterium to express anti-parasite molecules within the mosquito body, to interfere with pathogen development/transmission. One additional important feature of the

Asaia–mosquito relationship, both for investigations on the biology of the insect and for applications, is represented by the multiple routes by which *Asaia* can be transmitted, vertically to the progeny and horizontally between mosquito individuals, even belonging to different species. This bacterium can indeed be transferred horizontally by mating and co-feeding and vertically by maternal, paternal and trans-stadial routes (Favia et al. 2007, 2008; Damiani et al. 2008, 2010). Vertical transmission is important because it offers the chance of introducing engineered bacteria into mosquito populations in the field. In *An. gambiae*, we have recently provided evidence that vertical transmission is likely ensured by an egg-smearing mechanism, where the extracellular *Asaia* symbionts are smeared onto the egg surface (fig. 2); we hypothesize that larvae, after emergence, are infected by the bacteria possibly by feeding on them and on egg remnants (Damiani et al. 2010). If, as our data strongly suggest, in the breeding water

Fig. 2 Detection of naturally occurring *Asaia* in *Anopheles gambiae* eggs isolated from ovaries of gravid mosquitoes by FISH. Phase contrast (a, c) and fluorescence (b, d) images showing different stages of the eggs maturation, bacteria completely surround the eggs surface. *Asaia* specific nucleotide probes were stained with fluorochrome Cy3. Length bars are: 100 μm in (a) and (b); 125 μm in (c) and (d).

larvae are infected by bacteria, this would offer an easy way to spread recombinant bacteria in the field. In addition, the vertical transmission of *Asaia* symbiont would allow the perpetuation of the infection through the generations.

Paternal transmission is a very peculiar aspect of the symbiosis between *Asaia* and mosquitoes. In insects, this route of transmission has been reported previously only for a type of symbiont of aphids (Moran and Dunbar 2006). This mode of transmission offers an alternative way to release engineered bacteria. Indeed, in addition to the possible direct release in the larval breeding site to contaminate larvae and consequently adults by trans-stadial routes, the paternal transmission suggests the possibility of releasing male mosquitoes (that not take the blood meals), previously colonized with engineered bacteria (Damiani et al. 2010). Thus far, *Asaia* appears as one of the best candidates for a paratransgenic approach aimed at the control of malaria infection. Its localization in the midgut and salivary glands overlap that of malaria parasites, while localization in reproductive organs guarantees its passage to successive generations. These characteristics, coupled with the fact that bacteria have already been successfully tested for the ability to express anti-*Plasmodium* molecules in mosquito midgut (Yoshida et al. 2001; Riehle et al. 2007), strongly support the feasibility of a paratransgenic approach. In fact, the recent demonstration that effector genes [as for example the one encoding for the small peptide called SM1 (Ito et al. 2002) or that expressing the bee venom phospholipase A2 (PLA2) (Moreira et al. 2002)] can be introduced into the mosquito germ-line impairing its ability to transmit the malarial parasite raised more hope towards the fight of malaria.

As mentioned, *Asaia* has not only been detected in malarial vectors, but also in *Ae. aegypti* (Crotti et al. 2009; Gusmão et al. 2010). We were able to demonstrate that *Asaia* is capable of cross-colonizing other sugar-feeding insects of phylogenetically distant genera and orders. PCR, real-time PCR and *in situ* hybridization experiments showed *Asaia* not only in the body of the mosquito *Ae. aegypti*, but also in the leafhopper *Scaphoideus titanus* (Crotti et al. 2009). Cross-colonization patterns of the body of *Ae. aegypti*, *An. stephensi* and *S. titanus* have been investigated using *Asaia* strains isolated from *An. stephensi* or *Ae. aegypti*. By a multifaced approach we showed that *Asaia* from an insect of a given genus, administered with the meal, efficiently colonizes guts, male and female reproductive systems and the salivary glands

of insects of the other genera. The ability of cross-colonizing insects of different genera, and even of different orders, is an important feature for the development of symbiont-based control of different mosquito-borne diseases. Even though, *Asaia* is attracting a great deal of attention for its potential in mosquito-borne diseases control, its function in mosquito physiology has not yet been defined. Preliminary results seem to indicate a beneficial role in larval development and in mosquito fecundity (G. Favia, unpublished observation). A clear definition of its function(s) is obviously need, both for a better knowledge of mosquito biology, as well as for the development of effective symbiont-based control methods.

Conclusions and Perspectives

The field of mosquito symbiosis is expanding and is providing an important contribution to the knowledge of the biology of mosquitoes. Curiosity-driven studies have lead to new insight into the biology of facultative symbionts that may affect important traits of mosquito vectors. Genetic transformation of the symbionts with genes encoding for anti-pathogen effector molecules is an interesting field of application, that could contribute to the set-up of integrated control strategies, as well witnessed by the experience with genetically modified *Rhodnius prolixus*, symbionts of the triatomine bug *Rhodococcus rhodnii*, that have already been tested with success for their capacity to block the transmission of Chagas' disease (Beard et al. 2001).

Some bacteria have been already tested for their ability to express anti-*Plasmodium* molecules in mosquito midgut (Riehle et al. 2007). *Escherichia coli* expressing the above-mentioned SM1 and PLA2 effector molecules were able to inhibit *Plasmodium berghei* development in *An. stephensi* midguts, even though the survival of the modified bacteria after the blood meal was limited to no more than 3 days. The peculiar features of some mosquito symbionts, like that described for *Asaia*, open new perspectives in the control of vector-borne diseases. If compared with the use of genetically modified mosquitoes to control mosquito-borne diseases, the paratransgenic approach can overcome some problems associated with transgene introgression and horizontal transgene transfer.

In particular, it is worth to remark that bacteria can be much easily introduced into mosquito populations than transgenes and by paratransgenesis is possible to bypass the genetic barriers of reproduc-

tively isolated mosquito populations that occur very frequently in endemic areas, and may strongly affect the spread of mosquito transgenes. It is also important to underline that modified bacteria can be cheaply produced in large amount even in developing countries.

As new technologies must be added to current applications for successful integrated control approach in reducing the burden of mosquito-borne diseases, paratransgenesis can be considered as part of new integrated approach. In fact, even though further studies have to be performed to verify if transformed symbionts can replace wild-type bacteria in the field, in our view, paratransgenic-based SC have a great potential as symbionts seem not to have fitness load on mosquitoes and they are able to spread within and through mosquito populations by vertical and horizontal transmission routes. Furthermore, unlike transgenic mosquitoes, the possible inactivation of bacterial transgenes after some generations in the field is a surmountable problem since the easier logistics of introducing 'newly transformed' bacteria. One more element that supports the paratransgenic control of vector-borne diseases, is that regulation about the evaluation of bacteria release into the environment have been already developed, unlike the case for transgenic insect release, and regulatory issues developed for the release of paratransgenic bacteria for the control of trypanosomiasis may be easily adapted to the applications with mosquitoes.

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