Wolbachia prevalence, diversity, and ability to induce cytoplasmic incompatibility in mosquitoes
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Topropect humans and domestic animals from mosquito borne diseases, alternative methods to chemical insecticides have to be found. Pilot studies using the vertically transmitted bacterial endosymbiont Wolbachia were already launched in different parts of the world. Wolbachia can be used either in Incompatible Insect Technique (IIT), to decrease mosquito population, or to decrease the ability of mosquitoes to transmit pathogens. Not all mosquito species are naturally infected with Wolbachia: while in Culex pipiens and Aedes albopictus almost all individuals harbor Wolbachia, putative infections have to be further investigated in Anopheles species and in Aedes aegypti. All Wolbachia-based control methods rely on the ability of Wolbachia to induce cytoplasmic incompatibility (CI) resulting in embryonic death in incompatible crossings. Knowledge on CI diversity in mosquito is required to find the better Wolbachia-mosquito associations to optimize the success of both ‘sterile insect’ and ‘pathogen blocking’ Wolbachia-based methods.

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Introduction
Mosquitoes are vectors for major pathogens such as arboviruses, nematodes and protozoans. To protect humans and domestic animals from these pathogens, strategies targeting the vectors aim at decreasing vector population density and/or at diminishing their ability to transmit pathogens [1**]. Presently, the most common vector control actions are intended to decrease the longevity and the density in vector populations, mainly by using chemical insecticides, which have reached their limits because of genetic resistance and negative consequence on non-targeted invertebrate species [2]. In this context, new vector control strategies, hopefully more environmental friendly, have to be proposed. Bio-control strategies of vectors based on the knowledge of their microbiota are promising [3], and particularly those based on $\alpha$-proteobacteria of the genus Wolbachia which manipulate many aspects of their mosquito host biology [4–6]. Because these symbionts can influence both mosquito reproduction and their pathogen loads, Wolbachia-based control methods can be deployed to reduce vector populations and/or to diminish their capacity to transmit pathogens.

The Wolbachia, which are maternally transmitted endosymbionts, have long been studied because of their ability to manipulate their host reproduction to increase their prevalence within host populations [4]. Cytoplasmic incompatibility (CI) is the most frequent manipulation used by Wolbachia to spread within insect populations [4]. During the invasion process, CI occurs when males infected with a given Wolbachia breed with uninfected females which, then, produce non-viable embryos (Figure 1). As crossings between individuals infected with compatible Wolbachia give normal viable embryos, the consequence of CI is that the prevalence of Wolbachia increases within the host population so that prevalence can reach 100%.

The ability of Wolbachia to induce CI is the cornerstone of the two major Wolbachia-based control methods developed to date (Box 1). The first method, called ‘Incompatible Insect Techniques’ (IIT) [1**], and aims at decreasing mosquito population size by releasing Wolbachia infected males that are incompatible with local females. In this strategy, the local females produce non-viable embryos resulting locally and temporally in the vector population crash-down [7,8,9**,10]. The second method uses CI induction by Wolbachia, not to reduce the density of a focal vector population, but to sustainably replace its uninfected individuals by Wolbachia infected ones. Indeed it has been shown that Wolbachia can interfere negatively with the transmission of disease pathogens including the major arboviruses Chikungunya, Dengue, Rift Valley, West-Nile, Zika, and so on [11*,12**,13**,14–18,19*]. In this strategy, CI allows the progressive invasion of a local vector population with individuals harboring Wolbachia which mediate blocking of arboviruses transmission [20–22].

Wolbachia being a promising weapon against mosquitoes, we synthetize in this review the current knowledge...
Figure 1

Unidirectional CI between Wolbachia infected males and uninfected females

(a) The simplest type of CI occurring between Wolbachia infected males and uninfected females allows Wolbachia to invade uninfected populations. In Culex pipiens and Aedes albopictus, Wolbachia has reached fixation in nature; this type of CI is, thus, only observed in laboratory conditions when females are artificially cured of their Wolbachia with an antibiotic treatment. This type of CI was also observed when Ae. aegypti and An. stephensimales were transfected with a Wolbachia strain from other species and crossed with naturally uninfected females. (b–c) Other types of CI can occur between males and females both infected with different Wolbachia strains. In such crosses CI can be unidirectional (i.e. only one of the reciprocal crosses is compatible, the other is incompatible) (b) or bidirectional (both reciprocal crosses are incompatible) (c). Unidirectional CI can be observed in Ae. albopictus when bi-infected males (infected with both wAlbA and wAlbB) are crossed with laboratory females infected only with wAlbA. In Culex pipiens, really complex CI crossing types are observed, including unidirectional and bidirectional CI depending on the wPip strains present in crossed individuals.

The different types of CI in mosquitoes.

The ability to induce CI in the major vector mosquito species of Aedes, Anopheles, and Culex genera.

Prevalence and diversity of Wolbachia in mosquitoes

The diversity hidden behind the term ‘Wolbachia pipiens’ in arthropod and nematode hosts is presently organized in 17 phylogenetic clades called supergroups (A to Q) [23–27]. Within each supergroup, the unit of diversity is called a ‘strain’. Most of the Wolbachia strains were named according to their host species (e.g. wPip in Culex pipiens and wAlb in Aedes albopictus). If genetic differences are identified within an already defined ‘strain’, new information including sampling location or phylogenetic position can be added to name the new strains. The increase in genomic analyses to investigate more and more accurately Wolbachia diversity, between and within host species, may lead to important changes in the definition of new ‘strains’ in the near future [28].

Anopheles mosquitoes, the major vectors of Plasmodium, were considered to be exempt of Wolbachia because classic PCR diagnostic tests were always found negative [29–31]. However, very deep sequencing of Wolbachia-specific 16S rRNA recently suggested putative natural infections of Anopheles coluzzii and Anopheles gambiae in Burkina Faso [32,33]. The Wolbachia 16S sequences obtained were attributed to a new strain named ‘wAnga’. Positive mothers did not produce only positive offspring ruling out both an insertion in host genome and a perfect vertical transmission of Wolbachia [32]. Such a genetic detection of Wolbachia has now been extended to
Box 1 Wolbachia anti-vectorial methods: either decrease the density or modify the physiology of mosquitoes

a In the Incompatible Insect Technique (ITT) large numbers of Wolbachia-infected males are released. The Wolbachia harboring by these males has to be carefully chosen to ensure that these males will be able to kill, due to CI, embryos of females from the focal population. This requires that females either do not harbor the same Wolbachia strain (for instance in Aedes albopictus and C. pipiens cases) or that are putatively not infected with Wolbachia (for instance in Ae. aegypti and An. gambiae). After repeated releases of incompatible males, the vector population will decrease. To be successful, the Wolbachia strain in the released males should be involved in bidirectional CI with the Wolbachia strain from the targeted population (Figures 1 and 2). This way, the released Wolbachia has almost no chance to settle in the introduced environment because even if females are concomitantly released with infected males, they would not be able to produce offspring with the local males. The release of the same line of Wolbachia infected males can stay efficient through years. For even more efficiency, ITT may be combined with sterile insect technique (SIT) by irradiating Wolbachia-infected mosquitoes.

b Wolbachia can also be used to modify the physiology of mosquito. In this method, both Wolbachia infected male and female mosquitoes are released. CI allows the progressive invasion of local vector population with individuals harboring Wolbachia which mediates blocking of arboviruses transmission. To be successful, the Wolbachia of the released individuals have (i) to block viruses and (ii) to exhibit a unidirectional CI relationship with the targeted populations that allows the spread and sustainability of protective Wolbachia (Figure 2).

Anopheles funestus from Senegal [34], Anopheles arabiensis in Tanzania [35], 16 Anopheles species among a total of 25 in Gabon [36], and in 5 species among 17 from Ghana, Democratic Republic of the Congo (DRC), Guinea, Uganda and Madagascar [37]. Wolbachia 16S sequences exhibited much larger diversity than usually expected within one strain suggesting multiple infections. Indeed, these sequences can be clustered with those from Wolbachia strains belonging to the superfamilies A and even, more surprisingly, to supergroup C [36,38**]. The reported proportions of positive individuals vary among species and localities but remain low for most of the Anopheles species. Besides the interesting case of Anopheles moucheti in Gabon and DRC, for which the prevalence of Wolbachia seems very high, calls for further studies [36,37]. The low detection of Wolbachia in most Anopheles species could be due to a low prevalence of the symbiont that required more individuals and screened populations to be detected. However, if Wolbachia are really present in these Anopheles species their density must be very low as several screening techniques revealed discordant results, even requiring nested PCR or quantitative PCR with a very high number of amplification cycles for detection [34]. The main problem is that the putative presence of Wolbachia in all these Anopheles species is mostly based on its genetic detection which is not an actual proof of real infection and could result from contaminations, at least for certain species [38**]. To our knowledge, no electronic microscopy observations that would provide a direct proof of infections, have been yet conducted. Only one study reported fluorescent in situ hybridization (FISH) labelling to monitor the presence of Wolbachia, found at low density in the ovaries of some An. coluzzii [33].

Aedes aegypti and Ae. albopictus, the major arboviruses vectors, although belonging to the same genus exhibit strongly different patterns in terms of Wolbachia infection. As for Anopheles, classic PCR tests were always negative on Ae. aegypti placing this species among uninfected ones. However, deep sequencing of Wolbachia-specific 16S rRNA from both larvae and adults in USA and Thailand were recently found positive, indicating the putative presence of Wolbachia in some individuals [39,40]. Nevertheless, as it is the case with the Anopheles, if Wolbachia cells are present in this vector it must be at low prevalence and at a ‘cryptic’ load [41]. Further investigations including symbiont visualization must be conducted in the future to confirm the presence of Wolbachia at low prevalence and titer in Ae. aegypti.

In contrast, Ae. albopictus is found infected with Wolbachia everywhere in the world [42,43]. All individuals are usually infected with two Wolbachia strains namely wAlbA and wAlbB belonging to the superfamilies A and B, respectively [44]. However, a polymorphism of the infection status exists: (i) wAlbB mono-infected males (but not females) have been reported in La Réunion Island and Madagascar field populations [45], and (ii) wAlbA mono-infected laboratory lines were obtained from individuals initially sampled in Thailand and Mauritius [46,47]. The genetic variation within both wAlbA and wAlbB strains is yet considered to be low as no variation was detected within each strain based on 16S rRNA, wsp and ftsZ gene sequences [42,46–48] suggesting that Wolbachia could have recently, invaded and spread throughout populations of this mosquito species to finally reach fixation [42].

In C. pipiens (s.l.), all individuals are infected with wPip Wolbachia that were also found non-polymorphic using MLST genes [23,24]. However, MLSTs including a larger number of highly polymorphic genes (MutL, ank2, pk1, pk2, GP12, GP15, and RepA) allowed to uncover a previously hidden diversity [49]. All wPip strains are monophyletic and closely related, and they form five groups from wPip-I to wPip-V. As the thousands C. pipiens individuals tested around the world [50–53] harbored a wPip strain belonging to one of the five groups, the infection is considered to have reached fixation in this species. However, few individuals in South Africa, France, Scotland and Tunisia were found negative to Wolbachia genetic tests [54,55]. A phylogenetic analysis based on mitochondrial markers demonstrated that all these uninfected mosquitoes form a new species named’
*Culex juppi* nov. sp. is independent from all the infected *C.
pienis* [55].

CI induction in natural *Wolbachia*-mosquito associations

In *Anopheles* no cytoplasmic incompatibility has been shown in laboratory crosses between males putatively infected with *Wolbachia* and uninfected females [33]. Such laboratory observations are in accordance with the low detection of the symbionts in *Anopheles* natural populations. However, an acceleration of egg laying in *Wolbachia* positive females has been reported [33]. In *Ae. albopictus*, both *wAlbA* and *wAlbB* were reported to increase host fecundity [8]. CI does not occur between individuals from lines originating from distant parts of the world since most individuals are bi-infected with *wAlbA* and *wAlbB* showing no or low polymorphism [43] (Figure 1). Consequently, there is only one dominant crossing type in *Ae. albopictus* natural populations all over the world, resulting in compatibility between all lines. Nevertheless, females mono-infected with only *wAlbA* strain produce unviable embryos when crossed with normally bi-infected males resulting in unidirectional CI [46,47] (Figure 1). This clearly demonstrated that *wAlbB* strain is able to induce CI but that this CI phenotype rarely occurs in nature because of the high frequency of bi-infections with *wAlbA* and *wAlbB*.

In contrast to the absence of CI recorded in *Anopheles* and the poor crossing type diversity observed in *Ae. albopictus*, the hundreds of crosses performed between *C. pipiens* lines sampled worldwide have revealed an unrivaled diversity of crossing types [56,57,58] (Figure 1). Genetic diversity within the *wPip* clade is responsible for this unique CI polymorphism since (i) no other manipulative endosymbiont was detected in this host species, (ii) the host genetic background did not influence the crossing types, and more importantly (iii) *C. pipiens* lines harboring *wPip* belonging to the same phylogenetic group (*wPip*-I–V) are generally compatible, whereas ‘inter-group crosses’ are more likely to be incompatible [57]. Infected males harboring a *wPip* strain (from any *wPip* group) induce total CI (i.e. no embryo will develop) when crossed with uninfected females while the reciprocal crossing is fertile [59–61,62]. Such unidirectional CI pattern between uninfected and infected individuals has certainly prevailed during the spread of the *wPip* infection in *C. pipiens* populations, but is no longer observed in the wild since infection reached fixation. To date, crosses can only occur between (i) individuals infected with the same *wPip* group (usually resulting in normal reproduction) or (ii) individuals harboring *wPip* from two different groups. Such ‘inter-group crossings’ can have three outcomes (Figure 1): (i) production of living offspring; (ii) unidirectional CI (one cross direction is compatible while the reciprocal one is incompatible) or (iii) bidirectional CI (both cross directions are incompatible).

CI induction in artificial *Wolbachia*-mosquito associations

When *Wolbachia* have been experimentally introduced by transinfection in two ‘non-infected mosquito species’ namely *Ae. aegypti* and *Anopheles stephensi*, CI has been observed showing that *Wolbachia* molecular targets responsible for CI are present in these species. Indeed, *Ae. aegypti* has been successfully transinfected independently with eight *Wolbachia* strains (*wMel*, *wMelPop-CLA*, *wMelCS*, *wRi*, *wAu*, *wAlbA*, *wAlbB*, and *wPip* [14,19,63–65]) (Figure 2); and all induced unidirectional CI with natural uninfected *Ae. aegypti* except *wAu* which is a *Wolbachia* strain from *D. simulans* that also does not induce CI in its natural host [19]. *An. stephensi* has also been successfully transinfected with *wPip* from *Ae. albopictus* which induced CI enabling *Wolbachia* to invade uninfected laboratory populations [66]. Transinfections have also been conducted in *Ae. albopictus*, which is naturally infected, in order to create new crossing types. Both *wPip* and *wMel* strains have been introduced in *Wolbachia*-cured lines resulting in bidirectional incompatibility between transinfected lines and naturally infected ones [67–69]. Moreover, a triple-infected (*wAlbA*, *wAlbB*, and *wPip*) *Ae. albopictus* line has been established: it expresses unidirectional CI when crossed with naturally double-infected mosquitoes.

*C. pipiens* has not yet been transinfected with other *Wolbachia* since the natural crossing type diversity demonstrated in this species can provide with unidirectional and bidirectional crossing types required in *Wolbachia*-based control methods (e.g. [53]).

Cellular mechanism of CI in mosquitoes

The cellular mechanism of CI has only been yet studied in details in *C. pipiens* [62]. To do so, the early embryogenesis was monitored using fluorescence confocal microscopy in (i) fertile intra-group crosses, (ii) incompatible crosses between infected males and infected females (i.e. inter-group crosses), and (iii) incompatible crosses between infected males and uninfected females. Despite the diversity of the crosses involving various *wPip* strains, common embryonic defects resulting in the death of the embryos were detected. These defects consisted in paternal chromatin condensation and segregation impairments during the first embryonic division as for *Drosophila* and *Nasonia* [62*,70–74] (Figure 3).

*Wolbachia* genes involved in CI in mosquitoes

Cytological observations in *C. pipiens* suggest that a toxin, deposited in maturing sperm, would prevent the development of embryos by impairing paternal...
chromatin normal segregation unless they are rescued by an antidote [75]. A combination of approaches on different insects demonstrated that the Wolbachia cidA and cidB genes, first identified by the presence of CidA protein in C. pipiens sperm [76], were the determinant in the induction and rescue of CI in insects [77**,78**]. Biochemical analyses revealed that CidB protein could act as toxin since it encodes a putatively toxic deubiquitylase (DUB). Convincingly, when cidA and cidB were transgenically expressed in uninfected Drosophila males, these males were incompatible with uninfected females: embryos were unviable, and the first embryonic mitosis displayed the same characteristics as in CI [77**,78**]. CidA is most probably the antidote against the toxic activity of CidB since its expression during early oogenesis restored the viability of uninfected eggs fertilized by Drosophila infected males [79]. Both cidA and cidB genes are monomorphic in wAlbB. No genomic data are yet available on wAlbA and on the putative strain wAnga from Anopheles. However, in C. pipiens, these genes are amplified and diversified within each wPip genome constituting the fuel for the diversity of crossing types described in this species [80]. This cidA/cidB gene amplifications and diversifications in wPip may also account for the impressive CI penetrance observed in C. pipiens [62*].
Conclusion
Prevalence and diversity of Wolbachia are quite contrasted between mosquito species, Ae. albopictus and C. pipiens individuals all harbor diverse Wolbachia that can induce CI and influence their life history traits at each generation. In contrast, the major arboviruses vector Ae. aegypti and the major malaria vectors, Anopheles spp., are only suspected to be infected. Further studies are required to investigate infection status of these last species. Recent studies on C. pipiens along with those conducted on Drosophila brought new elements on CI mechanisms, both at cellular and molecular levels that constitute the cornerstone for an efficient use of Wolbachia genetic resources in vector control.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:
• of special interest
•• of outstanding interest

This review focused on new vector control methods based on the release of genetically modified or Wolbachia-infected mosquitoes.


The results presented in this study constituted the cornerstone to encourage methods based on the introduction of Wolbachia into pest and vector species of economic and health relevance to suppress or modify natural populations.


In this study, the ‘protective effect’ of Wolbachia against the Drosophila C virus in Drosophila melanogaster was revealed. The infected flies showed a delay in mortality compared to flies cured of Wolbachia infection. These results indicated that Wolbachia could exhibit an antiviral effect.

In this study, the ‘protective effects’ of Wolbachia against RNA viruses (Drosophila C virus, Nora virus and Flock House virus) and DNA virus...
(Insect iridescent virus 6) in Drosophila melanogaster were analyzed. 'Protective effects' were found in RNA viruses but not with one DNA virus.


This study showed that Wolbachia infection directly inhibits the ability of a range of pathogens to infect Aedes aegypti. The effect is Wolbachia strain-specific and relates to Wolbachia priming of the mosquito innate immune system and potentially competing for limited cellular resources required for pathogen replication.


In this study, the ‘protective effects’ of several Wolbachia strains transmitted in the Aedes aegypti mosquito against arboviruses (Semliki Forest, Dengue and Zika) were analyzed. Among these Wolbachia strains, the wAu strain from Drosophila simulans provided highly efficient transmission blocking of arboviruses. However, wAu did not induce CI and must be associated with a CI-inducing strain such as wAlbD from Aedes albopictus to be used in vector-control strategies.


This study suggested for the first time putative Wolbachia infections in Anopheles gambiae long time thought as not infected with Wolbachia.


The Wolbachia infection status of Anopheles gambiae was questioned in this study as the evidences of Wolbachia infection in this species have unusual properties: high Wolbachia 16S sequence diversity, low abundance and lack of congruence between hosts. The authors believe that the unusual characteristics of Wolbachia sequences render them insufficient to diagnose Wolbachia infection and that sequence-independent evidence such as microscopic observation are required to confirm the infection.


Wolbachia in mosquitoes
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Deubiquitylating enzyme induces cytoplasmic incompatibility. *Nat Microbiol* 2017, 2, 17007. In this paper, the capacity of the genes *cidA* and *cidB* from the *Wolbachia* wPip to induce CI when transgenetically expressed in *Drosophila melanogaster* was studied. *D. melanogaster* males expressing both *cidA* and *cidB* genes were able to induce CI when crossed with uninfected females, demonstrating the implication of these two genes in CI induction.

This study demonstrated the presence of several different *cidA* and *cidB* variants in each wPip strain genomes studied and the association of specific *cidA* and *cidB* variants with a given CI phenotype strongly supporting the implication of these genes in the unrivaled CI diversity described in *Culex pipiens*.