Wolbachia strain *w*Mel induces cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*

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Wolbachia inherited bacteria are able to invade insect populations using cytoplasmic incompatibility and provide new strategies for controlling mosquito-borne tropical diseases, such as dengue. The overreplicating *w*MelPop strain was recently shown to strongly inhibit the replication of dengue virus when introduced into *Aedes aegypti* mosquitoes, as well as to stimulate chronic immune up-regulation. Here we show that stable introduction of the *w*Mel strain of *Drosophila melanogaster* into *Aedes albopictus*, a vector of dengue and other arboviruses, abolished the transmission capacity of dengue virus-challenged mosquitoes. Immune up-regulation was observed in the transinfected line, but at a much lower level than that previously found for transinfected *Ae. aegypti*. Transient infection experiments suggest that this difference is related to *Ae. albopictus* immunotolerance of *Wolbachia*, rather than to the *Wolbachia* strain used. This study provides an example of strong pathogen inhibition in a naturally *Wolbachia*-infected mosquito species, demonstrating that this inhibition is not limited to naturally naïve species, and suggests that the *Wolbachia* strain is more important than host background for viral inhibition. Complete bidirectional cytoplasmic incompatibility was observed with WT strains infected with the naturally occurring *Ae. albopictus Wolbachia*, and this provides a mechanism for introducing *w*Mel into natural populations of this species.

The Asian tiger mosquito *Aedes albopictus*, a native of southeast Asia that in recent decades has invaded Africa, the Americas, and southern Europe, is now an important rural/semiurban vector of dengue virus across the tropics (1). It likely is involved in maintaining sylvatic cycles of transmission and acting as a bridge vector from these to urban epidemic cycles; it also transmits other Flaviviruses, such as yellow fever and West Nile, and the Alphavirus chikungunya. Like the primary urban dengue vector *Aedes aegypti*, *Ae. albopictus* is a day-biting species and thus is not amenable to control/prevention using insecticide-treated bed nets. This factor, along with the absence of a vaccine for dengue and the expanding disease range, calls for new methods of control.

All known wild populations of *Ae. albopictus* are naturally infected with two strains of the maternally inherited bacterium *Wolbachia pipientis,* known as *w*AlbA and *w*AlbB (2, 3); *Ae. aegypti* is naturally uninfected with the bacterium. Recent work has shown that when an overreplicating strain of *Wolbachia* from *Drosophila melanogaster*, *w*MelPop, was transferred into *Ae. aegypti* (4), the dissemination of dengue virus was strongly inhibited, as was the dissemination of chikungunya virus (5). In addition, transfer of the *w*AlbB strain from *Ae. albopictus* into *Ae. aegypti* (6) led to reduced susceptibility to dengue (7). Both *Wolbachia* strains also induced cytoplasmic incompatibility (CI) in *Ae. aegypti*, whereby uninfected females mated with infected males produce embryos that die shortly after fertilization. This mechanism is used by *Wolbachia* to spread through insect populations because in contrast, infected females can mate successfully with either infected or uninfected males, giving them a frequency-dependent reproductive advantage (8–10). Thus, the combination of viral inhibition and a built-in self-spreading mechanism provides attractive prospects for the control of dengue transmission by *Ae. aegypti* (11).

In addition to life shortening, the *w*MelPop strain also causes chronic immune up-regulation in *Ae. aegypti* (5, 12). The Toll pathway, some components of which are up-regulated in *Ae. aegypti* in the presence of *w*MelPop (5, 12), has been shown to play a role in the control of dengue dissemination in *Ae. aegypti* (13, 14). A general role of immune up-regulation in pathogen inhibition is also supported by the knockdown of the major immune gene *TEP1*, which partially rescues the inhibitory effect of the presence of *w*MelPop on *Plasmodium berghei* development in transiently infected *Anopheles gambiae* (15). The fact that the *w*AlbB transinfection caused dengue inhibition in *Ae. aegypti* (7) even through the original host of this *Wolbachia* strain *Ae. albopictus* is a fairly efficient dengue vector suggests a significant contribution of host background to the dengue inhibition phenotype, which possibly could be mediated by the increased immune response to *Wolbachia* found in a novel insect host. Thus, it is unclear whether any *Wolbachia* strain can produce strong dengue inhibition in a naturally *Wolbachia*-infected mosquito such as *Ae. albopictus*, which would be expected to have acquired a degree of immune tolerance to *Wolbachia* over time.

The *w*MelPop strain overreplicates and can approximately halve the lifespan of both its *D. melanogaster* (16) and *Ae. aegypti* (4) hosts. However, a *w*MelPop transinfection into *Ae. albopictus* also produced a greatly reduced egg hatch from intrastrain matings, and this appeared to preclude its application to disease control in *Ae. albopictus* (17). The *w*Mel strain, which is phylogenetically close to the *w*MelPop variant (18), does not produce the life-shortening phenotype of the latter in its native *D. melanogaster* host (16). However, *w*Mel can significantly delay the accumulation of RNA viruses, such as *Drosophila* C virus, in *D. melanogaster* (19–21). Thus, we selected *w*Mel for experimental transfer into *Ae. albopictus* to examine whether this strain is capable of producing dengue inhibition and CI in this new host background.

Results

Generation of the Uju.*w*Mel Line and Crossing Experiments. A stable infection of *w*Mel in a previously tetracycline-cured *Ae. albopictus* strain (UjuT) was generated. Cytoplasm from *D. melanogaster* was microinjected into UjuT, resulting in four G_0 females, one of which was positive and produced sufficient progeny to establish an isofemale line. This line was backcrossed with UjuT males each generation to minimize bottlenecking and was selected for

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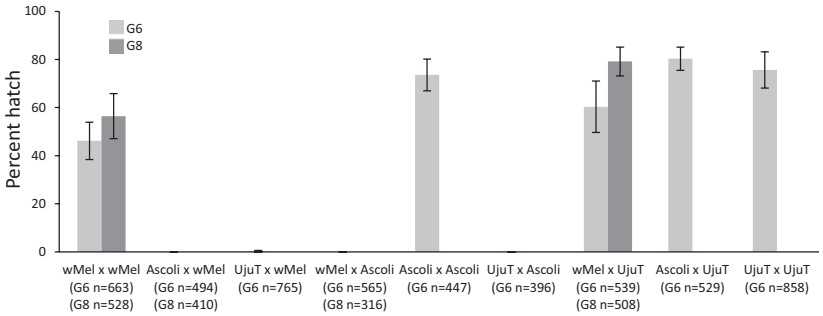

Fig. 1. Uju.*w*Mel crossing type. Experiments to characterize the crossing type of the Uju.*w*Mel line at G_6 were performed using UjuT and Ascoli strains. Error bars represent the SEM of hatch rates between females; 15 adult males and 15 females were used for each mass cross. A second round of crossing experiments was performed two generations later (G_8) using Uju.*w*Mel × Uju.*w*Mel, Ascoli × Uju.*w*Mel, Uju.*w*Mel × Ascoli, and Uju.*w*Mel × UjuT following to the same procedure as in the previous experiment. No statistically significant difference was found between G_6 and G_8 of the same cross using Wilcoxon tests. The number of eggs counted for each generation is shown under the *x*-axis.

maximal maternal inheritance; proportions confirmed as positive for *Wolbachia* by PCR were 70% ($n = 10$) for G_1, 81% ($n = 16$) for G_2, 89% ($n = 9$) for G_3, and 100% ($n = 10$) for G_4. The infection remained at 100% after G_4 ($n = 152$; tested up to G_9). The *Wolbachia* strain present was confirmed to be *w*Mel by sequencing of the *wsp* (*Wolbachia* surface protein) gene, which demonstrated 100% identity with published *w*Mel *wsp* sequence.

The transinfected strain initially showed reduced hatch rates compared with WT females, a 46.1 ± 7.8% hatch for Uju.*w*Mel in G_6 compared with 65.1 ± 10.7% for UjuT. After two successive generations of selection for high hatch rates, the Uju.*w*Mel × Uju.*w*Mel hatch rate had risen to 56.4 ± 9.4% by G_8 (Fig. 1). A similar effect was previously reported in newly transinfected *Drosophila* (22) and *Ae. aegypti* (6). The effects of *w*Mel on fecundity of *Ae. albopictus* remain to be characterized in detail; preliminary data suggest that *w*Mel has no major negative effect on fecundity, with an average number of eggs laid per female of 60.3 ± 7.0 ($n = 11$) for Uju.*w*Mel and 61.2 ± 5.0 ($n = 13$) for UjuT ($P = 0.9716$; Wilcoxon test).

Crossing experiments were performed to examine whether the *w*Mel strain was able to produce CI in this background, by crossing the Uju.*w*Mel line in both directions with the uninfected UjuT and WT Ascoli strain (infected with *Wolbachia* strains *w*AlbA and *w*AlbB) (Fig. 1). As expected, UjuT males were compatible with all females. Males of the Uju.*w*Mel line produced strong CI when mated to UjuT females (0.26% hatch). The Uju.*w*Mel and Ascoli lines showed complete bidirectional incompatibility, with 0% hatch when females of either strain were mated with males of the other strain.

Dengue Infection. At 14 d after challenge with dengue 2 virus provided in an artificial blood meal, the transinfected Uju.*w*Mel strain showed complete inhibition of dengue transmission capacity, with no infectious viral particles detected in the saliva of any tested mosquito. In contrast, the superinfected Uju.*w*AlbA/*w*AlbB strain (generated by the introgression of *Wolbachia* from Ascoli into UjuT to minimize any effect of host genetic background on dengue virus), and the uninfected UjuT strain were both able to transmit dengue 2 virus at day 14 postinfection (infection rate, 27.3% for the Uju.*w*AlbA/*w*AlbB strain and 8.3% for the UjuT strain) (Fig. 2). Mosquito saliva contained numbers of viral particles in the expected range for *Ae. albopictus* infected with dengue virus (23) (Fig. 2), with an average of 67 ± 139 viral particles for the Uju.*w*AlbA/*w*AlbB strain and 29 ± 29 viral particles for the UjuT strain.

Immune Gene Expression in Uju.*w*Mel. Because the *w*MelPop strain transinfection in *Ae. aegypti* has been shown to produce up-regulation of a number of immune genes, and given the possibility that this could be responsible for or contribute to the viral inhibition phenotypes, the effects of *w*Mel transinfection on transcription levels were investigated for four *Aedes albopictus* immune genes. These four genes were selected to represent a range of immune gene categories including important antimicrobial effectors [a cecropin, a peptoglycan recognition protein (PGRP), and a thioester-containing protein (TEP) (15, 24–27)], and also because their orthologs were previously shown to be up-regulated in the presence of *w*MelPop (12). The transcription of immune genes was measured by qRT-PCR using G_5 Uju.*w*Mel, UjuT, and Ascoli

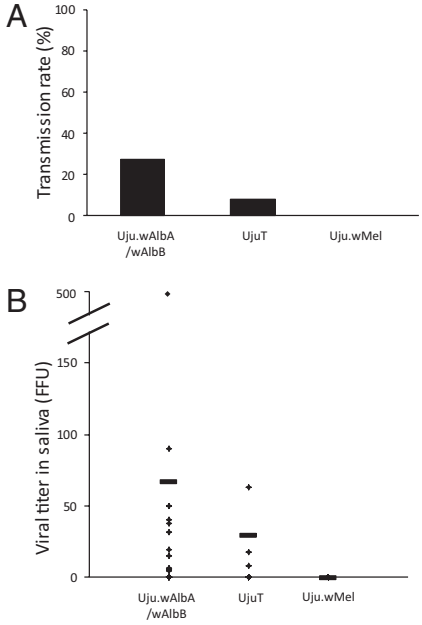

Fig. 2. Transmission capacity (*A*) and titer of dengue virus in mosquito saliva (*B*). Three *Ae. albopictus* strains (Uju.wAlbA/wAlbB, UjuT, and Uju.wMel) were orally infected with dengue 2 virus using glass feeders covered with a chicken skin membrane containing the infectious blood meal at a titer of 10^7 FFU/mL. After blood feeding, mosquitoes were transferred into cardboard containers and maintained in BSL3 insectaries at 28 °C. After 14 d, surviving mosquitoes were tested for the presence of viral particles in saliva collected using the forced salivation technique. The number of fluorescent foci in the saliva of each mosquito was estimated on C6/36 *Aedes albopictus* cell culture. The transmission capacity representing the percentage of mosquitoes with infectious saliva among tested mosquitoes was also calculated. The number of fed and assayed females was 44 for Uju.wAlbA/wAlbB, 26 for UjuT, and 44 for Uju.wMel.

females, as well as G_{10} Uju.*w*Mel, UjuT, and Uju.wAlbA/wAlbB females, at 11 d after eclosion (Fig. 3*A*). There was no significant difference in immune gene transcription between the cured UjuT strain and the *w*AlbA/*w*AlbB superinfected line. Significant immune up-regulation was observed in Uju.*w*Mel compared with the other two strains for three of the four genes assayed in the G_5 experiment, but no significant immune up-regulation was observed at G_{10}.

To compare the contribution of *Wolbachia* strain type with host species background, transient somatic infections of *w*MelPop and *w*AlbB were also created in *Ae. aegypti* and *Ae. albopictus* using intrathoracic inoculation as described previously (15, 28). Adult females were injected with suspensions of *Wolbachia* purified from *Ae. albopictus* cell lines (Aa23) approximately 3 d after eclosion, and the transcription of immune genes was measured by qRT-PCR at 5 d after injection (Fig. 3*B*) (15, 29). Strong immune up-regulation was observed in *Ae. aegypti* with both *w*MelPop and *w*AlbB strains compared with noninjected, buffer-injected, and heat-killed *E. coli*-injected controls. However, no significant immune up-regulation was observed in *Ae. albopictus* injected with either *Wolbachia* strain, with the exception of *CECD* at day 9. Live *Wolbachia* was confirmed in *w*MelPop- and *w*AlbB-injected mosquitoes at 5 d and 9 d after injection by RT-PCR on the cDNA generated for this experiment.

Concentration of *Wolbachia* in Uju.*w*Mel. The concentration of *w*Mel in Uju.*w*Mel was compared with the combined concentration of both *w*AlbA and *w*AlbB in the Ascoli strain in adults at 11 d after eclosion. The ratio of *Wolbachia wsp* DNA to host *S17* DNA was used to estimate the *Wolbachia* concentration The concentration of *w*Mel was found to be approximately 7 times greater than the total concentration of *Wolbachia* in the superinfected Ascoli strain (Fig. 4).

Discussion

Our results show that *w*Mel infection can block dengue virus transmission in the increasingly widespread vector species *Ae. albopictus*. RNA viral inhibition by *w*Mel has been previously demonstrated in *Drosophila* (20), and we have shown that this viral interference is also produced when it is transferred into *Ae. albopictus*. The inhibition appears to be limited to specific strains of *Wolbachia*, given that *Ae. albopictus* is naturally infected with two strains of *Wolbachia*, *w*AlbA and *w*AlbB, which seem to have no inhibitory effect on the virus. This is the first

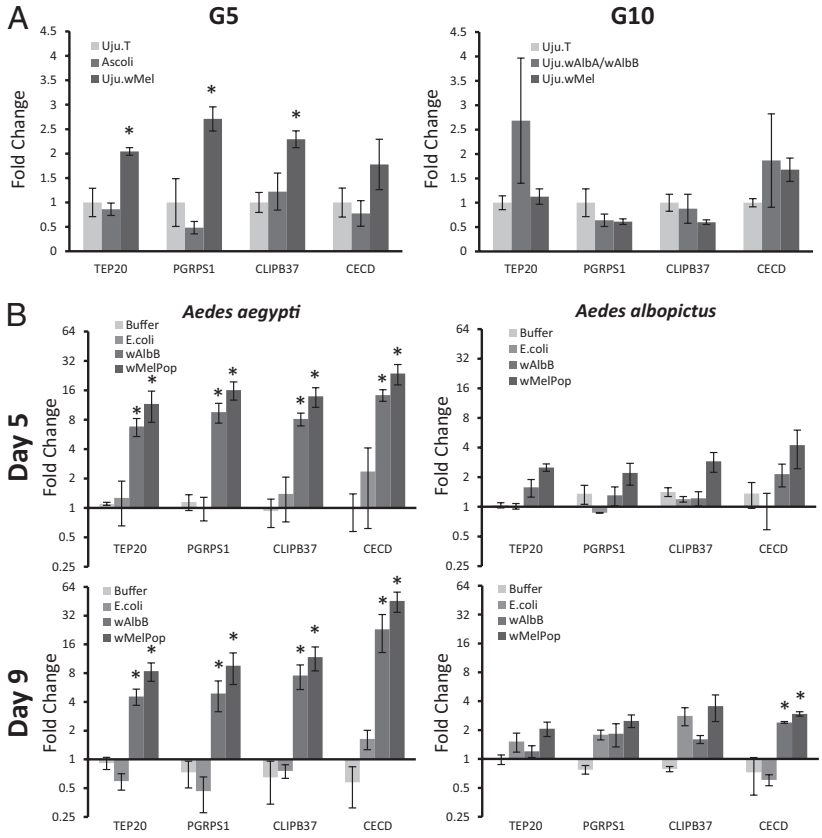

Fig. 3. Immune gene expression in *Wolbachia*-infected and uninfected mosquitoes. (*A*) RNA was extracted from adult females of G_5 Uju.*w*Mel, UjuT, and Ascoli, and separately from G_{10} Uju.wMel, UjuT, and Uju.*w*AlbA/*w*AlbB, at 11 d posteclosion. The expression of four *Ae. albopictus* orthologs for *Ae. aegypti* immune genes was analyzed by qRT-PCR: a peptidoglycan recognition protein (*PGRPS1*), cecropin D (*CECD*), CLIP-domain serine protease (*CLIPB37*), and a thioester-containing protein (*TEP20*). Expression was normalized to that of the UjuT adult females. Error bars show the SEM of three biological replicates, each containing four adult females for the G_5 experiment and six adults for the G_{10} experiment (a total of 12 and 18 mosquitoes per condition). $*P < 0.05$ compared with UjuT, Wilcoxon test. (*B*) Adult females were transiently infected with *Wolbachia* using intrathoracic injections with either *w*MelPop or *w*AlbB, controls of either heat-killed *E. coli*, or the buffer alone, approximately 3 d after eclosion. RNA was extracted from half of the females at 5 d postinjection and from the other half at 9 d postinjection. The expression of four *Ae. aegypti* immune genes was analyzed by qRT-PCR: a peptidoglycan recognition protein (*PGRPS1*) cecropin D (*CECD*), CLIP-domain serine protease (*CLIPB37*), and a thioester-containing protein (*TEP20*). Orthologs for these genes in *Ae. albopictus* were also analyzed by qRT-PCR. Expression was normalized to noninjected adult females of the same age from the same colony. Error bars show the SEM of three biological replicates, each containing five adult females (a total of 30 mosquitoes per condition). $*P < 0.05$ compared with noninjected control, Wilcoxon test.

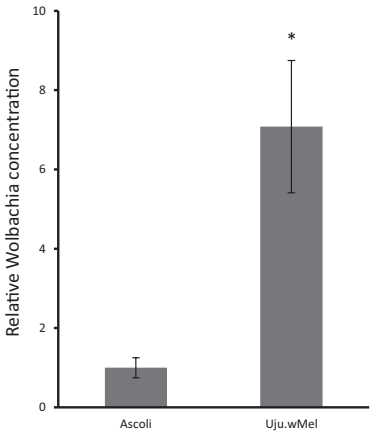

Fig. 4. Concentration of *Wolbachia* in Ascoli and Uju.*w*Mel lines. DNA was extracted from adult females of Uju.*w*Mel and Ascoli at 11 d after eclosion. The ratio between *Wolbachia wsp* DNA and host *S17* DNA was used to estimate the concentration of *Wolbachia*. The concentration of *w*AlbA/*w*AlbB in the Ascoli strain was arbitrarily designated "1," and the concentration of *w*Mel in Uju.*w*Mel was plotted relative to this. Error bars show the SEM of three biological replicates, each containing four adult females (a total of 12 mosquitoes per condition). *$P < 0.05$, Wilcoxon test.

time that *Wolbachia*-mediated dengue virus inhibition has been demonstrated in *Ae. albopictus,* and this result has significant implications for dengue control.

Uju.*w*Mel produces complete bidirectional CI with the wild-type Ascoli line (containing a *w*AlbA and *w*AlbB superinfection, like all known wild populations of this species). The *w*Mel crossing type differs from the crossing type of a *w*MelPop transinfection into *Ae. albopictus* (17), unexpectedly given the phylogenetic similarity of *w*Mel and *w*MelPop (18). Bidirectional CI provides a method for stably introducing *Wolbachia* into populations, because bidirectionally incompatible crossing types cannot stably coexist (30); whichever strain is at a local majority has a reproductive advantage, because its females will more frequently encounter and mate with males with which they are compatible. Assuming that complete bidirectional CI also will be produced under field conditions, once the *w*Mel infection reaches a population majority, it would then be expected to go to local fixation and be stable to further immigration of *w*AlbA/*w*AlbB-infected (WT) individuals with which they are incompatible. Large-scale female releases would not be essential for this strategy to be successful; because *Aedes* pupae are readily separated by sex, heavily male-biased releases could be done, which would suppress the female population (31). Given that most populations are seasonal, appropriately timed releases at the start of the rainy season could achieve local replacement with *w*Mel.

Future experiments are needed to examine whether the creation of a stable *w*Mel/*w*AlbA/*w*AlbB triple infection is possible. Based on previous work (32), we expect the crossing type of this triple infection to produce unidirectional incompatibility with the parental lines and thereby have the potential to spread *w*Mel more efficiently through field populations. In any event, at the initial field trial stage, using the bidirectionally incompatible line would be preferable, to maintain better control over the geographical extent to which population replacement occurs.

There was 7 times more *Wolbachia* in the Uju.*w*Mel strain compared with the superinfected (*w*AlbA and *w*AlbB) Ascoli strain. This result is quite surprising given the relatively high concentrations found in the natural superinfection. High levels of *Wolbachia* are expected in recently transinfected species (33) and may decrease over time as coadaptation of strain and host occur. It is possible that the increased density of *Wolbachia* had a negative effect on egg hatch rates from intrastrain matings in the early generations.

The immune gene up-regulation caused by *w*Mel in the stably infected Uju.*w*Mel line (Fig. 3*A*) was statistically significant for three of the four genes in one experiment, but on a much lower scale than that observed in a stably *w*MelPop-infected line of *Ae. aegypti* (12) or transiently *w*MelPop-transinfected *Anopheles gambiae* (15). By G_{10}, no significant up-regulation was observed, possibly due to coadaption between the host and the transinfecting strain. Immune up-regulation was observed when *Ae. aegypti* was transiently infected with *w*AlbB (Fig. 3*B*), and there was no significant difference between the immune up-regulation caused by *w*MelPop and *w*AlbB in *Ae. aegypti,* demonstrating that this effect is not limited to the overreplicating *w*MelPop strain. The lack of significant immune up-regulation observed in *Ae. albopictus* when transiently infected with *w*AlbB, one of its natural *Wolbachia*, or with *w*MelPop for three of the four genes assayed, together with our previous findings (15), suggests that whether or not the host species is naturally infected with *Wolbachia* is a more important factor in the level of immune response than the *Wolbachia* strain used.

The fact that *Ae. aegypti* stably transinfected with *w*AlbB showed reduced transmission of dengue (7) suggests a host component, possibly immune-related, to the viral inhibition in that transinfection. However, the relatively modest immune up-regulation observed in the transinfected *Ae. albopictus* Uju.*w*Mel line, together with the fact that no dengue virus was detected in saliva after the challenge, suggests that priming of the host immune system might not be the most important factor in this case of viral inhibition. The *Wolbachia* strain used seems to be the critical consideration here. Possible mechanisms for direct viral inhibition by *Wolbachia* include the production of reactive oxygen species by the bacterium (34) and resource competition, such as for cholesterol (5).

Future research using later generations of the line will more clearly identify whether *w*Mel has any effects on the fitness or fecundity of *Ae. albopictus* similar to those demonstrated by the *w*AlbA/*w*AlbB superinfection (35, 36). Our results so far show no major effect on fecundity, unlike the significant fecundity reduction previously observed with *w*Pip infection of *Ae. albopictus* (37). Furthermore, our *w*Mel-transinfected line had a much higher hatch rate than that previously observed for a *w*MelPop strain transinfection in *Ae. albopictus*, which averaged only in the 10–20% range (17). This study has yielded an *Ae. albopictus* line that may provide the basis for a viable new option for dengue control in this species. A pair of studies published while this article was under review reported the generation of a *w*Mel-

Table 1. Oligonucleotides designed for amplification from *Ae. albopictus*

Gene	Oligonucleotide: forward; reverse
PGRPS1	GCAACTTACTGGCCGCTCGC; CGTTGGAGCGCATACCCGTG
CECD	TTCACGAAGTTGTTCGCAAT; GGCATTGAAGACTCGTTTGC
CLIPB37	ACCCGAACCAGGTTGTGTAGCG; GGATGCAACCAGTACGCCGTCC
TEP20	TGCCCAGCGGATTTGTAGCAGAAG; AAACAGTCTGATTCGGGTCCCATGT
S17	AAGCCCCTGCGTAACAAGAT; GTTATCTCTGCGCTCACGTTC

transinfected *Ae. aegypti* line, dengue inhibition in this line (38), and successful field trials in Queensland, Australia (39), demonstrating the feasibility of field implementation of a *Wolbachia* population replacement strategy. Our study demonstrates that both of the two main vectors of dengue globally are amenable to such a strategy.

Materials and Methods

Mosquito Strains. The *Wolbachia*-uninfected *Ae. albopictus* strain UjuT was generated by tetracycline treatment (40). The Ascoli strain of *Ae. albopictus* was colonized from San Benedetto del Tronto, Italy in 2006 by G. Favia and colleagues, and the *Ae. aegypti* Rockefeller strain originated in the Caribbean in the 1930s. All colonies and lines were maintained at 27 °C and 70% relative humidity on a 12-h light/dark cycle.

The *w*AlbA and *w*AlbB *Wolbachia* strains were introgressed into the UjuT background for four generations by removing all male pupae from one colony of the Ascoli strain and providing an approximately equal number of UjuT males. The resulting line was ∼94% UjuT nuclear background and contained both *w*AlbA and *w*AlbB. This line was generated to partially control for any effects of host background.

Embryo Microinjection and Line Establishment. The *w*Mel strain of *Wolbachia* was transferred from *D. melanogaster* yw^{67c23} embryos into *Ae. albopictus* (UjuT) by the transfer of cytoplasm. Adult *Drosophila* were encouraged to oviposit using apple juice agar plates and yeast paste. Eggs were collected at ∼30 min after oviposition. *Ae. albopictus* were encouraged to lay eggs by placing ∼15 females, blood-fed 7 d earlier, into a small (3 cm diameter, 10 cm tall) plastic vial with moist filter paper on the bottom. Eggs were collected at ∼30 min after oviposition. *Ae. albopictus* eggs were allowed to desiccate for 15–30 min. Both donor and recipient eggs were aligned on a nitrocellulose membrane and transferred to a glass slide using double-sided tape. The eggs were then covered with Voltalef oil ready for injection. Cytoplasm was aspirated from the posterior of the donor eggs using a FemtoJet microinjector (Eppendorf) and injected into the posterior of recipient eggs. After a short incubation time, eggs were transferred onto wet filter paper, stored at 100% humidity at 27 °C for 5 d, and then hatched in deoxygenated water. G_0 larvae were reared under standard conditions. Females were separated 1–2 d after blood feeding into small plastic vials with moist filter paper on the bottom. Once females laid eggs, they underwent PCR analysis for the presence of *Wolbachia* using universal *wsp* primers 81F and 691R (3). These primers also were used for sequencing the *wsp* gene to confirm that the *Wolbachia* was the *w*Mel strain. After initial optimization trials, the experiment from which the line was established involved microinjection of ∼100 embryos.

While the transinfected line was being established, only eggs from PCR-positive females were hatched. After G_6, batches also were selected for high egg hatch. Eggs from individual *Wolbachia*-positive females were counted and hatched (with deoxygenated water in the small plastic vials), and then second instar larvae were counted. Approximately three-quarters of the broods with the highest hatch rates were pooled to form the next generation.

qRT-PCR and qPCR. Gene transcription levels were tested by quantitative RT-PCR (qRT-PCR). RNA was extracted from adult mosquitoes using TRIzol reagent. cDNA was generated from 1 μg of this RNA using SuperScript Vilo (Invitrogen). cDNA was diluted to 1:20. The dsDNA dye SYBR Green (Invitrogen) was used for amplicon detection in a DNA Engine thermocycler (MJ Research) with a Chromo4 real-time PCR detection system (Bio-Rad). The following cycling conditions were used: 95 °C for 15 min, followed by 45 cycles of 95 °C for 10 s, 59 °C for 10 s, and 72 °C for 20 s, with fluorescence acquisition at the end of each cycle and a melting curve analysis.

Quantitative PCR (qPCR) was used to determine *Wolbachia* copy number. DNA was extracted from adult mosquitoes using the Livak method (41). DNA was diluted to 100 ng/μL using a NanoDrop spectrophotometer. Thermocycler conditions and reaction chemistry followed the same protocol as for qRT-PCR. Primer pairs used for *Ae. albopictus* qRT-PCR and qPCR are listed in Table 1. *Ae. albopictus* primers were designed using sequence data generated using degenerate primers based on *Ae. aegypti* Vectorbase sequences or from *Ae. albopictus* EST data from the National Center for Biotechnology Information. Primers for *Ae. aegypti PGRPS1* (AAEL009474), *CECD* (AAEL000598), *CLIPB37* (AAEL005093), and *TEP 20* (AAEL001794) were as listed by Kambris et al. (12), and those for Actin 5c (AAEL011197) were as described by Kambris (15). Concentrations of all *Wolbachia* strains were measured using *wsp* primers (42).

CI Crosses. Crossing experiments designed to characterize the crossing type of *w*Mel were performed using UjuT, Ascoli, and Uju.*w*Mel lines. All individuals were sexed as pupae. Adults were blood-fed at age 6 d, and the females were separated out into plastic vials for individual laying. Eggs were dried and allowed to mature at 27 °C and ∼70% relative humidity for 5 d, counted, and hatched in deoxygenated water containing algae and yeast. Larvae were fed with dried liver powder. Second instar larvae from each female were counted to give hatch rates. Females with no egg hatch were dissected to check for successful mating; egg hatch rates from unmated females were disregarded. Adults were given a constant supply of water and sucrose.

Dengue Infection. One-wk-old Uju.*w*Mel, UjuT and Uju.*w*AlbA/*w*AlbB females were deprived of sucrose solution for 24 h before exposure to the infectious blood meal containing 10^7 FFU (foci fluorescent units)/mL of virus. The dengue serotype 2 virus strain (provided by Leon Rosen) was isolated in 1974 from a human sera from Bangkok, Thailand. The artificial blood meal provided in glass feeders covered with a chicken skin membrane and maintained at 37 °C, consisted of a virus suspension (1/3 vol/vol), washed rabbit erythrocytes (2/3 vol/vol), and 5 mM ATP as a phagostimulant. Engorged mosquitoes were transferred into cardboard containers, provided with sucrose solution, and maintained in BSL-3 insectaries at 28 °C for 14 d. Saliva was collected using the forced salivation technique, which consists of inserting a capillary tube containing FCS into the proboscis of females whose legs and wings had been removed. After 45 min, saliva was collected and titrated by focus fluorescent assay on C6/36 *Ae. albopictus* cell culture. The transmission capacity was estimated as the percentage of mosquitoes with infectious saliva among tested mosquitoes.

***Wolbachia* Purification and Intrathoracic Inoculation.** *Wolbachia* were maintained in the *Ae. albopictus* cell line Aa23 (43). Cells were grown in 75-cm^2 culture flasks to ∼50% confluence in Schneider's media containing 10% FBS, 140 U/mL of penicillin, and 140 μg/mL of streptomycin. Cells were passaged every 3–5 d. *Wolbachia* was extracted from cells and purified as described previously (44) at 3 d after the previous passage. The *Wolbachia* pellet was resuspended in Schneider's media with 10% FBS (without antibiotics) to an optical density of OD = 0.06 at a wavelength of 400 nm. For *Escherichia coli* controls, an OD of 0.01 at 400 nm was used. Then 69 nL of *Wolbachia* suspension (or 69 nL of Schneider's/*E. coli* suspension for the controls) was microinjected into the thorax of ∼3-d-old *Ae. aegypti* Rockefeller/*Ae. albopictus* UjuT strains using a Nanoject microinjector (Drummond). The mosquitoes were supplied with water and sucrose and left for 5 d before the qRT-PCR experiments.

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