Wolbachia transinfection in Aedes aegypti: A potential gene driver of dengue vectors

Toon Ruang-areerate and Pattamaporn Kittayapong*

Center for Vectors and Vector-Borne Diseases and Department of Biology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand

The endosymbiotic bacteria in the genus *Wolbachia* are capable of inducing a wide range of reproductive abnormalities in their hosts, including cytoplasmic incompatibility (CI), which could lead to the replacement of uninfected host populations with infected ones. Because of this, *Wolbachia* have attracted considerable interest as a potential mechanism for spreading disease-blocking transgenes through vector populations. Here we report the establishment of double *Wolbachia* transinfection by direct adult microinjection of *Wolbachia* from naturally double-infected *Aedes albopictus* to *Aedes aegypti*, the most important mosquito vector of infectious viral diseases, and a mosquito in which natural *Wolbachia* infections are not known to occur. We further demonstrate that incomplete CI is induced in these double-transinfected mosquitoes. Comparisons of fitness traits between naturally uninfected and transinfected *Ae. aegypti* lines indicated one significant difference in favor of the latter, namely, an increased number of eggs laid. Levels of CI expression corresponded to the *Wolbachia* density. There were large differences in relative *Wolbachia* density between reproductive and nonreproductive tissues in both *Ae. albopictus* and transinfected *Ae. aegypti*, except Malpighian tubule, which implied the preferred establishment of *Wolbachia* within reproductive tissue. Results from a simulation model confirm that population replacement by transinfected *Ae. aegypti* is possible over time. The establishment of *Wolbachia* double infections in *Ae. aegypti* by direct adult microinjection and the demonstration of CI expression in this new host suggest that *Wolbachia* could be experimentally transferred into vector species and could also be used as a gene-driving system to genetically manipulate vector populations.

microinjection | transfer | replacement | tissue tropism | cytoplasmic incompatibility

Because of the inefficiency of vaccines for curing many vector-borne diseases, the genetic modification of arthropod vectors has been seriously considered as a means to control these diseases (1–4). Stable gene transformations of mosquitoes have been reported, indicating the possibility to genetically modify vectors to control diseases (2–5). Most such strategies, however, require a high frequency of transgenic vectors to spread in natural populations to achieve population replacement (6). To ensure the spread of disease-blocking transgenes and to reduce the number of transgenic vectors needed for release into natural populations, integration with gene drivers such as *Wolbachia* may be necessary and should be considered.

Wolbachia are maternally inherited endosymbiotic bacteria recognized to infect a broad range of arthropod species and some filarial nematodes (7). Various surveys have estimated that 16–76% of insect species are infected with *Wolbachia* (8, 9) and that they are capable of inducing a wide range of reproductive abnormalities in their hosts, including cytoplasmic incompatibility (CI) (10). CI results in a failure of karyogamy, perhaps by delaying nuclear envelope breakdown and mitosis (11), and consequently may promote *Wolbachia* invasion of uninfected populations because infected females are able to mate and produce offspring successfully with both infected and uninfected males, whereas uninfected females are unable to produce off-spring when they mate with infected males (10, 12). Theoretically, insects harboring CI-inducing *Wolbachia* would spread rapidly into uninfected populations, causing population replacement (12). The powerful spreading capability of *Wolbachia* via CI has attracted considerable attention as a potential gene-driving system (12, 13). The expression of transgenes could be straightforward if the population invasion ability of *Wolbachia* is used (12). However, within this theoretical framework, which still lacks empirical support, the capability of *Wolbachia* to invade and maintain themselves in host populations depends on three main parameters: (*i*) the strength of CI, (*ii*) maternal transmission efficiency, and (*iii*) fitness effects on the host (14).

Aedes aegypti, one of the most important mosquito vectors, is commonly recognized to cause dengue and yellow fever epidemics (15, 16). *Wolbachia* infection has never been detected in this particular species (12, 17). Therefore, the establishment of *Wolbachia* infection and induction of CI expression in *Ae. aegypti* would be an essential prerequisite to the possible application of *Wolbachia* as a transgene drive mechanism to aid in genetic control of this important vector species (13, 18). Syncytial embryo microinjection has been used extensively for *Wolbachia* transfer in various insect groups (18–21). Recently this technique was successfully used to introduce a single strain of *Wolbachia* from *Aedes albopictus* into *Ae. aegypti* (22). In this article we demonstrate the successful *Wolbachia* transfer and the establishment of double strains of *Wolbachia* from *Ae. albopictus* in *Ae. aegypti* via direct adult microinjection.

Results and Discussion

Establishment of *Wolbachia*-Superinfected *Ae. aegypti*. The *Wolbachia* extract was directly microinjected into 76 newly emerged adult females of naturally uninfected *Ae. aegypti*. Forty-nine percent of injected mosquitoes survived, and 93% of surviving adults tested positive for *Wolbachia* DNA by PCR, indicating infection with *Wolbachia*. Isofemale lines were established by using surviving mosquitoes that were PCR-positive for *Wolbachia* and were designated AegW. Each generation of AegW was monitored for the transmission efficiency of *Wolbachia* by using a specific *wsp*-based PCR assay (23), and infected offspring were chosen to start a new generation as shown in Fig. 1. Transmission efficiency of *Wolbachia* was low in G_1 (39%, $n = 72$) but increased gradually (G_2, 44%, $n = 61$; G_3, 66%, $n = 38$; G_4, 76%, $n = 55$; G_5, 71%, $n = 66$) until it reached a maximum at G_6 (85%, $n = 27$). The transmission efficiency thereafter decreased to 45% in G_7 ($n = 60$) and was stably maintained at ≈40% until the last observation at G_{12} (G_8, 33%, $n = 90$; G_9, 34%, $n = 68$; G_{10}, 44%, $n = 18$; G_{11}, 39%, $n = 64$; G_{12}, 40%, $n = 15$). Since then, the AegW colony routinely has been maintained in the insectary at 25–27°C and 70–75% relative humidity by selection of the isofemale lines with PCR-positive results, and recently up
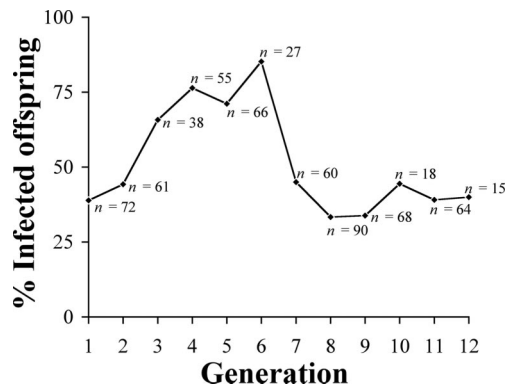
Fig. 1. Transmission efficiency in different generations of transinfected *Ae. aegypti* populations. Only infected mosquitoes were used to produce the next generation.

to 81.8% (G_{26}, $n = 11$) of infection rate has been observed in some of the selected isofemale lines.

To confirm the establishment of *Wolbachia* within AegW populations, *wsp* partial sequences were amplified and sequenced from multiple generations (G_4, G_6, G_8, and G_{10}). Both *w*AlbA and *w*AlbB strains were found in AegW. The sequences showed >99.2% identity to *w*AlbA and >98.4% identity to *w*AlbB, indicating that AegW were infected with *Wolbachia* derived from double-infected *Ae. albopictus*. The presence of *Wolbachia* in multiple generations of AegW shows that these bacteria are capable of being maternally transmitted in this novel host. The slight differences in DNA base changes within *wsp* sequences of AegW could be caused by PCR or sequencing (*Taq*) errors, stochastic cloning of relatively rare variants, or actual *Wolbachia* adaptation to the new host. However, the DNA base alteration occurred at the same sites and the same bases under different generations, whereas *Wolbachia* of transinfected *Ae. albopictus* that carried the same *Wolbachia* strains did not show DNA base alteration (100% identity; unpublished data). This finding implies that *Wolbachia* may have adapted to its new *Ae. aegypti* host, possibly through changes in *wsp* protein, which mediates between the host cell and the *Wolbachia* bacterium. However, we report here the successful establishment of double

Wolbachia strains (*w*AlbA and *w*AlbB) in *Ae. aegypti* host by direct microinjection into an adult stage.

Syncytial embryonic microinjection is commonly used to establish *Wolbachia* infections in desirable insects because it allows *Wolbachia* placement within developing pole cells (and thus germ-line tissues) and yields high rates of establishment of successful transinfected lines. However, until recently high mortality rates in mosquitoes resulted in low transinfection frequencies relative to the starting number of embryos (22). A different microinjection approach, direct adult microinjection, has been taken in this work for establishing *Wolbachia* infection in *Ae. aegypti*. Unlike the earlier approach, it is much simpler and has several benefits, including no need for the expertise required for embryonic microinjection. A disadvantage of this approach is the low *Wolbachia* number caused by indirect establishment in germ cells in G_0, which results in a high proportion of uninfected cytoblasts (11). This finding contrasts with embryonic microinjection in which syncytial eggs are directly infected with *Wolbachia* in the pole cell region, resulting in codevelopment of *Wolbachia* and the host's embryo, and especially germ-line tissues, potentially leading to a higher number of *Wolbachia* within the reproductive tissues.

CI Expression in Transinfected *Ae. aegypti*. To determine the capability for CI expression and the effect of multiple male and female matings on CI expression, test crosses were established between transinfected *Ae. aegypti* (AegW; G_5 and G_6) and naturally uninfected *Ae. aegypti* (Aeg) as shown in Table 1. Two crossing experiments were conducted. In the first experiment (Table 1, Exp. 1), comprising single-pair copulations with one male and one female, we found that crosses between AegW males and Aeg females (c) produced 50.68% egg hatch, which was significantly lower than that for naturally uninfected crosses [d, 87.53%; Mann–Whitney U test (U), $P = 0.002$] and is indicative of incomplete CI expression. In contrast, the other reciprocal cross (Aeg males and AegW females) did not give any significant reduction in the average hatch rate (a vs. b; U, $P = 0.262$). The occurrence of incomplete CI probably reflects the low *Wolbachia* numbers in G_5 and G_6 of AegW used in the experiments (19). To clarify any effect of multiple mating on the net egg hatchability, test crosses were conducted with swarming females and males to allow freedom of mate choice and number

Table 1. CI expression, as shown by the hatch rate of eggs produced, in crosses between *Ae. aegypti* transinfected with *Wolbachia* from double-infected *Ae. albopictus* and naturally uninfected *Ae. aegypti*

Cross (female × male)	Mean % egg hatch ± SE	Total no. of eggs counted	No. of crosses	Comparison	P value (U)
Exp. 1					
AegW × AegW, a	78.79 ± 6.77	785	12		
AegW × Aeg, b	89.19 ± 2.47	1,007	15	b, a	0.262
Aeg × AegW, c	50.68 ± 8.65	1,092	22	c, d	0.002
Aeg × Aeg, d	87.53 ± 5.15	1,076	15		
Exp. 2					
AegW × AegW, e	87.88 ± 1.71	1,962	36		
AegW × Aeg, f	85.36 ± 3.18	1,934	37	f, e	0.623
Aeg × AegW, g	50.94 ± 5.36	1,720	37	g, h	<0.001
Aeg × Aeg, h	89.04 ± 1.17	1,936	38		

Two crossing experiments (Exp.) were conducted: Exp. 1 used single-pair copulations; in Exp. 2, males and females were allowed to swarm and thus were free to choose mates and number of copulations. Crosses were conducted under controlled conditions (27°C, 72% relative humidity, and 10% sucrose) for 3 days before females were allowed to blood-feed and were individually isolated into vials filled with egg-collecting paper. The paper was removed and dried after 3 days. The eggs were counted and incubated for 24 h in deoxygenated water, after which hatched offspring were counted. All females were checked for insemination by examination of spermathecae for sperm. Unmated females were excluded from analysis. In the single-pair cross, only crosses in which AegW males were shown to be PCR-positive were included.

MICROBIOLOGY

of matings (Table 1, Exp. 2). It was found that expected incompatible crosses (g) produced 50.94% egg hatch, which was significantly lower than that of uninfected crosses (h, 89.94%; U, $P < 0.001$), and the egg hatch rate in reciprocal crosses (e vs. f; U, $P = 0.623$) was not significantly lower. Nor was there any significant difference in offspring production between these two crosses, thus indicating that multiple matings, if they occurred, and free mate choice did not cause a negative effect on CI.

Fitness of Transinfected *Ae. aegypti*. The fitness of AegW (G_6, $n = 28$) as measured by percentage of egg hatch, percentages of survival of larvae, pupae, and adults, and number of eggs laid was compared with that of Aeg ($n = 28$). Differences were not significant for percentage of egg hatch (AegW = 82.1 ± 2.3 and Aeg = 86.8 ± 2.0; U, $P = 0.11$), percentage of larval survival (91.2 ± 2.0 and 92.6 ± 1.4; U, $P = 0.74$), percentage of pupal survival (97.8 ± 0.9 and 99.1 ± 0.5; U, $P = 0.09$), and percentage of adult survival (88.7 ± 0.2 and 91.7 ± 0.1; U, $P = 0.902$), whereas a significant difference was found for the number of eggs laid (78.0 ± 6.2 and 48.8 ± 2.8; $t = 4.276$, df = 37.65, $P < 0.001$) (Fig. 2*A*). Transgenic mosquitoes produced fewer eggs in the study by Irvin *et al.* (24). However, in this study we found a high fecundity of transinfected AegW females. In addition, the ratio of male-to-female offspring did not significantly deviate from 1:1, and no significant differences were found between offspring sex ratios [AegW ($n = 28$) = 1.2 ± 0.1 and Aeg ($n = 28$) = 1.2 ± 0.2; U, $P = 0.555$]. The equality of sex ratio and the higher fecundity encourage CI expression by two beneficial outcomes: infected females increase infected offspring, whereas infected males decrease uninfected offspring in the next generations. These phenomena distort the ratio of infected to uninfected individuals in successive generations. Because our fitness determination was for the first gonotrophic cycle only, additional observations of fitness in multiple generations and gonotrophic cycles are important to be sure of beneficial effects of *Wolbachia* on AegW fitness.

Effect of *Wolbachia* Density on CI Expression of Transinfected *Ae. aegypti*. Lower CI expression in transinfected lines has been observed in many studies (19, 21) and suggests that CI expression depends on host factors, *Wolbachia* adaptation, or a threshold level for *Wolbachia* densities. However, the successful transfer of the same *Wolbachia* strains to tetracycline-treated *Drosophila simulans*, the ability of the *Wolbachia* strains to induce CI, and their cytoskeleton associations implied *Wolbachia* adaptation to the new host (18). To investigate the correlation of *Wolbachia* density with CI expression, the densities of *Wolbachia* were measured by using quantitative real-time PCR. Comparison of internal host gene control between AegW and KLPP was conducted, and it was found that host copy numbers of AegW were not significantly different from KLPP [AegW = (3.2 ± 0.3) × 10^9 and KLPP = (2.5 ± 0.3) × 10^9; $t = 1.832$, df = 38, $P = 0.075$] (Table 2); therefore, AegW and KLPP have approximately the same cell numbers under the control condition/environment. The correlation between relative *Wolbachia* density in adults and the degree of CI expression in AegW and KLPP mosquitoes is shown in Table 2. Transinfected AegW ($n = 33$) were found to have *Wolbachia* density 165 times lower than that of the naturally double-infected *Ae. albopictus* (KLPP, $n = 25$) ($t = 6.304$, df = 24, $P < 0.001$). This finding corresponded to the level of CI expression in the two mosquitoes, in which the percentage of unhatched eggs in the nonpermissible crosses in *Ae. aegypti* (Aeg female × AegW male; $n = 59$; 49.16 ± 4.61) was significantly lower than that of the corresponding crosses in *Ae. albopictus* [uninfected female (KLPPT) × KLPP male, $n = 58$; 100 ± 0.0] (U, $P < 0.001$). Low *Wolbachia* density could result in a proportion of uninfected cytoblasts as a result of stochastic loss of *Wolbachia* during mitosis and the production of sperma-
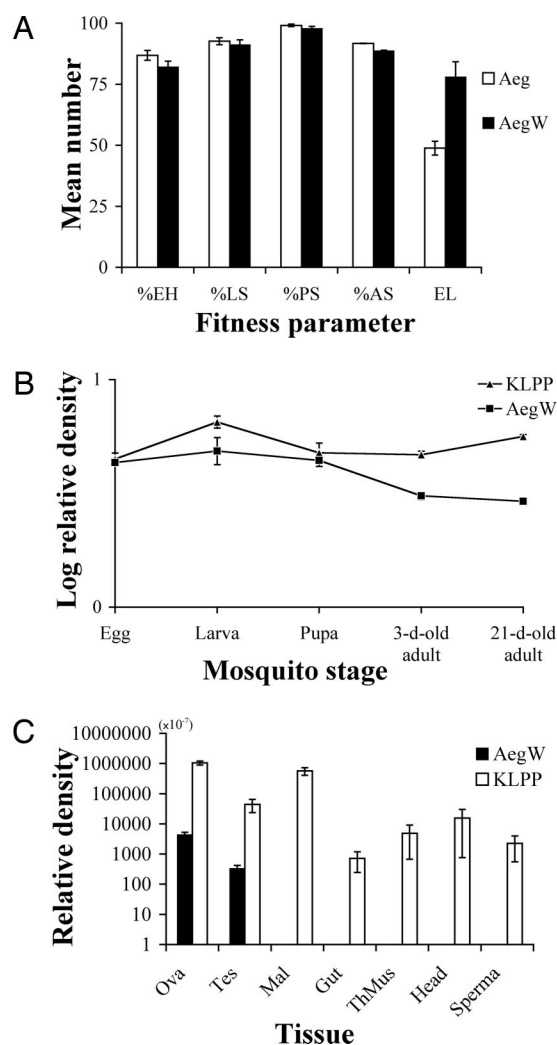
Fig. 2. Fitness and levels of relative *Wolbachia* density. (*A*) Fitness of AegW (G_6) and Aeg, i.e., mean percentage of egg hatch (EH), percentage of larval survival (LS), percentage of pupal survival (PS), percentage of adult survival (AS), and number of eggs laid (EL). (*B*) Log relative *Wolbachia* density at different developmental stages in AegW and KLPP. (*C*) Relative *Wolbachia* density in different tissues in AegW and KLPP. The error bars represent standard errors.

tids containing amounts of *Wolbachia* insufficient to elicit CI (19), which may explain the incomplete CI of AegW (G_5 and G_6). To determine the influence of host factors on AegW, relative *Wolbachia* density was observed in different stages of AegW and KLPP. The relative *Wolbachia* density of KLPP constantly remained at the same level in all developmental stages (Fig. 2*B*), whereas AegW did not change at the beginning stages (egg, larva, and pupa stages) but started to decrease when it entered the 3-day-old adult stage. No significant differences in *Wolbachia* densities were detected at the egg stage (t test, $t = 0.5$, df = 7, $P = 0.632$), the larval stage ($t = 1.959$, df = 15.331, $P = 0.069$), and the pupal stage ($t = 0.548$, df = 6, $P = 0.603$). The lack of differences in *Wolbachia* density in the eggs and the immature stages between AegW and KLPP indicates that host factors may not be important during these stages. However, relative *Wolbachia* density of AegW was significantly lower in the 3-day-old adults ($t = 8.064$, df = 19, $P < 0.001$) and also in the 21-day-old adults ($t = 20.023$, df = 22, $P < 0.001$) than in those of KLPP. The possibility that host factors may suppress an increase of *Wolbachia* in AegW is not conclusive and requires further

Ruang-areerate and Kittayapong

Table 2. Levels of relative *Wolbachia* density of the host cell numbers and levels of CI expression measured by the mean percentage of unhatched eggs in expected CI crosses in transinfected *Ae. aegypti* (AegW, recipient) and double-infected *Ae. albopictus* (KLPP, donor)

	% of unhatched eggs	*Wolbachia* density, $\times 10^{-3}$	Host cell no., $\times 10^9$
KLPP donor	100 ± 0.0 (58)	3.3 ± 0.6 (25)	2.5 ± 0.3 (20)
AegW recipient	49.16 ± 4.61 (59)	0.02 ± 0.003 (33)	3.2 ± 0.3 (20)

The numbers of KLPP donors and AegW recipients are listed in parentheses.

investigation. *w*MelPop in *D. simulans* was found to have a *Wolbachia* density similar to *w*MelPop in *Drosophila melanogaster* after *w*MelPop was introduced into *D. simulans* (18). *D. simulans*, therefore, appears to have no repressing factors, and *Wolbachia* may easily establish without host interference (18, 25). One possible way to prove that differences in *Wolbachia* densities are likely due to host factors could be to reintroduce *Wolbachia* back into uninfected *Ae. albopictus* and determine *Wolbachia* density.

Relative *Wolbachia* density was determined in different tissues, including ovaries, testes, Malpighian tubule, midgut, thoracic muscles, head, and spermatheca to demonstrate tissue tropism of AegW and KLPP. *Wolbachia* was detected in all tissues of KLPP, whereas infection was found only in the ovaries and testes of AegW. There were differences in *Wolbachia* density between AegW and KLPP: ovaries, AegW = $(4.4 \pm 0.9) \times 10^{-4}$ ($n = 20$), and KLPP = $(1.0 \pm 0.2) \times 10^{-1}$ ($n = 12$); testes, $(3.3 \pm 0.9) \times 10^{-5}$ ($n = 10$) and $(4.4 \pm 2.1) \times 10^{-3}$ ($n = 6$); Malpighian tubule, 0.0 ± 0.0 ($n = 12$) and $(5.7 \pm 1.6) \times 10^{-2}$ ($n = 9$); midgut, 0.0 ± 0.0 ($n = 12$) and $(7.1 \pm 4.7) \times 10^{-5}$ ($n = 12$); thoracic muscles, 0.0 ± 0.0 ($n = 12$) and $(4.9 \pm 4.2) \times 10^{-4}$ ($n = 12$); head, 0.0 ± 0.0 ($n = 12$) and $(1.6 \pm 1.5) \times 10^{-3}$ ($n = 12$); spermatheca, 0.0 ± 0.0 ($n = 12$) and $(2.3 \pm 1.7) \times 10^{-4}$ ($n = 9$) (Fig. 2C). The nondetection of *Wolbachia* in nonreproductive tissues of AegW does not definitely indicate no infection. AegW could display different tissue tropism in different host species, or it is possible that the nonreproductive tissues of AegW harbor *Wolbachia* densities under the minimum detection level of quantitative PCR. Differences in relative *Wolbachia* density were observed among different tissues of KLPP, with the average *Wolbachia* density of nonreproductive tissues being comparatively lower than that for reproductive tissues except in Mulpighian tubules. However, the highest average density of *Wolbachia* in AegW and KLPP was found in the reproductive tissues. The large differences in average *Wolbachia* density between reproductive and nonreproductive tissues in both AegW and KLPP could imply that *Wolbachia* prefers reproductive tissues over nonreproductive tissues.

Transinfected *Ae. aegypti* as a Potential Tool for Vector Population Replacement. To predict population replacement by AegW, the Dobson model was chosen and applied with our parameters as shown in Fig. 3. The Dobson model is specifically modified to study population replacement of uninfected *Ae. albopictus* by double-infected *Ae. albopictus* and could describe infections affording a host fecundity advantage (26). By using 10% initial AegW and the highest transmission efficiency observed in G_6 (85%), infection frequency exceeds 82.6% by generation 23 and remains fixed at 82.6% (Fig. 3, line 3). One hundred percent infection frequency cannot be reached because the chosen 85% transmission efficiency of AegW results in 15% uninfected progeny in the next generation. If the initial AegW is increased to 30%, infection frequency can exceed 82.6% by G_{16} (Fig. 3, line 4). However, the transmission efficiency of AegW was $\approx 40\%$ after G_6 (Fig. 1), which, in the simulation model, would predict a decrease in infection frequency to nearly 0 within five gener-

ations (Fig. 3, line 2). All three previous predictions were based on higher fecundity of AegW. However, Xi *et al.* (22) found that their transinfected lines had equal fecundity to control lines, and including this in the simulation indicates that replacement does not occur for both 10% and 30% initial AegW under equal fecundity and that AegW frequency decreases until it remains fixed at nearly 0 (Fig. 3, lines 1 and 5). So, according to our simulations, AegW will invade and replace uninfected populations if AegW has higher fecundity at 85% transmission efficiency and 51% relative hatch rate starting with at least 10% initial AegW. The prediction that AegW will not completely replace an uninfected population is in contrast to that of Xi *et al.* (22), but it does provide important information that there is a potential to use *Wolbachia* as a gene driver in vector populations. Incomplete CI of AegW is a major barrier in promoting the spread of AegW in uninfected populations. However, Xi *et al.* (22) were able to demonstrate complete CI and the invasion and establishment of CI-inducing *Wolbachia* in a laboratory population of this mosquito. A recent increase in the infection rate of AegW up to 81.8% in G_{26} indicates the possibility that this transinfected colony could reach high *Wolbachia* density, high maternal transmission, and complete CI in the higher generations, which requires further investigation.

In this work, double infections of *Wolbachia* (*w*AlbA and *w*AlbB strains) were established and caused incomplete CI at G_5 and G_6 of transinfected *Ae. aegypti*. These results were different from the work of Xi *et al.* (22), whose single infection, *w*AlbB, was established and caused complete CI, although the same *Wolbachia* strains were transferred into the same host species. The lower infection rate of *w*AlbA relative to *w*AlbB is used to explain the establishment of a single infection in Xi *et al.* (22). Concentrating *Wolbachia* numbers during extraction before direct microinjection may be a key to the establishment of double infections because the numbers of *w*AlbA are lower than *w*AlbB in *Ae. albopictus* (27). In addition, the lumen space of the adult for loading *Wolbachia* is bigger than the egg;
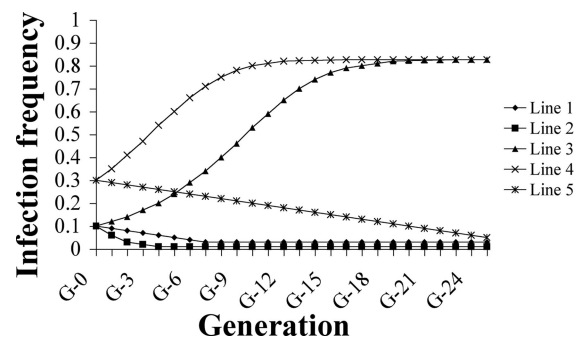
Fig. 3. Simulated changes in *Wolbachia* infection frequency. All simulations were generated by using the Dobson-specific *Ae. albopictus* model (26). Fecundity effect by *Wolbachia*, transmission efficiency, and initial infection rate was as follows for each line: line 1, 1.0, 0.85, and 0.1; line 2, 0.71, 0.4, and 0.1; line 3, 0.71, 0.85, and 0.1; line 4, 0.71, 0.85, and 0.3; line 5, 1, 0.85, and 0.3. Relative hatch rate was 0.51.

therefore, more *Wolbachia* numbers can be injected into the adult, providing higher potential of establishment of double infections than the egg. The random establishment of *Wolbachia* in different tissues during G_0 would result in low infection of *Wolbachia* in reproductive tissues and thus incomplete transmission of *Wolbachia* in the early generation. In contrast, Xi *et al.* (22) used syncytial embryonic microinjection, which would result in codevelopment of *Wolbachia* and pole cells forming the germ-line tissues and thus a higher establishment of *Wolbachia* within reproductive tissues. However, alone this is unlikely to explain the incomplete CI expression observed in our work compared with the complete CI expression reported by Xi *et al.* (22) because from G_1 onward the transinfected *Wolbachia* reside within the syncytial egg in both cases and can replicate to the controlled density in each developing tissue type. Thus, the possibility of some additional host factors suppressing the replication of *Wolbachia* within our AegW lines, but not Xi *et al.* (22), remains to be clarified.

This study clearly shows that crosses of AegW males with uninfected *Ae. aegypti* females result in significantly lower hatch rate than other crosses and that CI expression is not affected by multiple matings. The level of CI expression corresponds with *Wolbachia* copy number within the AegW host. To develop optimum usage of *Wolbachia* as a gene driver, the isofemale lines of *Wolbachia*-double-infected AegW have been repeatedly selected for each generation to increase the level of maternal transmission and the number of *Wolbachia*, which will probably, then, lead to high levels of CI expression. This study demonstrates that there is a potential for using *Wolbachia* as a gene-driving system. By integrating *Wolbachia* with transgenic mosquitoes (2–5) it may be possible to manipulate vector populations genetically (13, 18). *Wolbachia* is not the only choice for driving genes into vector populations (12, 28), but this study demonstrates that it is one possible option.

Materials and Methods

***Wolbachia* Detection and DNA Analysis.** DNA was extracted from individual eggs, larvae, pupae, adults, or tissues by using the Holmes–Bonner method (29). PCR was performed as described (23). PCR products were diluted 500-fold and reamplified by using the previous profile (23) except for the modified 52°C annealing temperature and internal primers (151F, 5′-TGG TTA CAA AAT GGA CGA CA; 599R, 5′-CAC CAA CAG TGC TGT AAA GAA C) to increase sensitivity and specificity. Mitochondrial 12S rDNA primers were used as a quality control for DNA extraction (30). PCR products were resolved by 1% (wt/vol) agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. Desirable products were ligated into pGEM-T vectors (Promega, Madison, WI). At least three clones were purified and sequenced on an automated sequencer (Applied Biosystems, Foster City, CA). The *wsp* sequences were aligned by using a cluster algorithm followed by manual modification based on amino acid translation (DNASTAR, Madison, WI).

***Wolbachia* Microinjection.** *Wolbachia* were crudely purified from ovaries of 2-week-old *Ae. albopictus* double-infected with *w*AlbA and *w*AlbB strains. The ovaries were dissected under

PBS and homogenized in homogenizing buffer (31). The homogenate was filtered through a 0.95-μm pore size and centrifuged at $200 \times g$ for 5 min to remove cellular debris. Then the suspension containing *Wolbachia* was pelleted at $4,000 \times g$ for 10 min. The pellet was resuspended in homogenizing buffer. Twenty-four-hour-old *Ae. aegypti* recipients were knocked down and microinjected directly in the region between the posterior pronotum and the sternopleuron by glass needles with manually cut tips. Virgin females were mated to uninfected males the next day to establish isofemale lines.

Quantitative Real-Time PCR. DNA was extracted as previously described. A real-time quantitative PCR assay based on a single-copy gene (*wsp*) encoding a surface protein of *Wolbachia* was used to determine *Wolbachia* copy number in the hosts. Primers were designed specifically to detect *w*AlbA (GF and AR) and *w*AlbB (GF and BR) strains and amplified 124- to 250-bp regions of the *wsp* gene (GF, 5′-GGT TTT GCT GGT CAA GTA A; AR, 5′-GCA TCT TTG GTA ACT ACT TTT; BR, 5′-GCT GTA AAG AAC GTT GAT C). A specific TaqMan probe (5′-FAM TGT TAG TTA TGA TGT AAC TCC AGA A-TAMRA) for the central region of the PCR product was designed and used to measure the amount of *Wolbachia*. PCR was performed under the following conditions: 15 min at 95°C, then 45 cycles of 94°C for 1 min, 50°C for 1 min, and 60°C for 1 min. The PCR included a Probe PCR Master Mix (Qiagen, Hilden, Germany), 0.4 μM concentration of each primer, 0.2 μM TaqMan probe, and 2 μl of template DNA made up to a 25-μl total volume. A standard curve was constructed by using *w*AlbA and *w*AlbB amplicons that had been previously cloned into pGEM-T vector (Promega), linearized with NcoI, and quantified as template. Three replicates were performed and averaged for each sample. Strain-specific primers to *w*AlbA and *w*AlbB were applied to all samples, and then total *Wolbachia* copy number was calculated by integrating both numbers. Host cell number was measured based on a single-copy gene (defensin) encoding an insect immunity. Primers were designed specifically to defensin D of *Ae. albopictus* and defensin A of *Ae. aegypti* (Def-F, 5′-ATC ACT GGT GCT TAC CCA CAG G; Def-R2, 5′-GAC GCA CAC CTT CTT GGA GTT G). SYBR Green was used to measure the amount of host cell number under the following conditions: 15 min at 95°C, then 45 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The PCR included SYBR Green PCR Master Mix (Applied Biosystems), 0.5 μM concentration of each primer, and 2 μl of template DNA made up to a 25-μl total volume. A standard curve was constructed as described above. Three replicates were performed and averaged for each sample.

1. Beaty, B. J. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 10295–10297.
2. Catteruccia, F., Noland, T., Loukeris, T. G., Blass, C., Savakis, C., Kafatos, F. C. & Crisanti, A. (2000) *Nature* **405,** 959–962.
3. Coates, C. J. (2000) *Nature* **405,** 900–901.
4. Ito, J., Ghosh, A., Moreira, L., Wimmer, E. A. & Jacobs-Lorena, M. (2002) *Nature* **417,** 452–455.
5. Coates, C. J., Jasinskiene, N., Miyashiro, L. & James, A. A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 3748–3751.
6. James, A. A. (2002) in *Insect Transgenesis: Methods and Applications*, eds. Handler, A. M. & James, A. A. (CRC, Boca Raton, FL), pp. 319–333.
7. Lo, N., Casiraghi, M., Salati, E., Bazzocchi, C. & Bandi, C. (2002) *Mol. Biol. Evol.* **19,** 341–346.
8. Werren, J. H., Windsor, D. & Guo, L. R. (1995) *Proc. R. Soc. London Ser. B* **262,** 197–204.
9. Jeyaprakash, A. & Hoy, M. A. (2000) *Insect Mol. Biol.* **9,** 393–405.
10. O'Neill, S. L., Hoffmann, A. A. & Werren, J. H. (1997) *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction* (Oxford Univ. Press, Oxford).
11. Tram, U. & Sullivan, W. (2002) *Science* **296,** 1124–1126.
12. Sinkins, S. P. (2004) *Insect Biochem. Mol. Biol.* **34,** 723–729.
13. Turelli, M. & Hoffmann, A. A. (1991) *Nature* **353,** 440–442.
14. Turelli, M. & Hoffmann, A. A. (1995) *Genetics* **140,** 1319–1338.
15. Gubler, D. J. (1989) in *The Arboviruses: Epidemiology and Ecology*, ed. Monath, T. P. (CRC, Boca Raton, FL), Vol. II, pp. 223–260.

16. Monath, T. P. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2395–2400.
17. Kittayapong, P., Baisley, K. J., Baimai, V. & O'Neill, S. L. (2000) *J. Med. Entomol.* **37,** 340–345.
18. Braig, H. R., Guzman, H., Tesh, R. B. & O'Neill, S. L. (1994) *Nature* **367,** 453–455.
19. Boyle, L., O'Neill, S. L., Robertson, H. M. & Karr, T. L. (1993) *Science* **260,** 1796–1799.
20. Kang, L., Ma, X., Cai, L., Liao, S., Sun, L., Zhu, H., Chen, X., Shen, D., Zhao, S. & Li, C. (2003) *Heredity* **90,** 71–76.
21. Sasaki, T. & Ishikawa, H. (2000) *Heredity* **85,** 130–135.
22. Xi, Z., Khoo, C. C. H. & Dobson, S. L. (2005) *Science* **310,** 326–328.
23. Zhou, W., Rousset, F. & O'Neill, S. L. (1998) *Proc. R. Soc. London Ser. B* **265,** 509–515.
24. Irvin, N., Hoddle, M. S., O'Brochta, D. A., Carey, B. & Atkinson, P. W. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 891–896.
25. McGraw, E. A., Merritt, D. J., Droller, J. N. & O'Neill, S. L. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 2918–2923.
26. Dobson, S. L., Marsland, E. J. & Rattanadechakul, W. (2002) *Genetics* **160,** 1087–1094.
27. Dutton, T. J. & Sinkins, S. P. (2004) *Insect Mol. Biol.* **13,** 317–322.
28. Enserink, M. (2000) *Science* **290,** 440–441.
29. Holmes, D. S. & Bonner, J. (1973) *Biochemistry* **12,** 2230–2338.
30. O'Neill, S. L., Giordano, R., Colbert, A. M. E., Karr, T. L. & Robertson, H. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 2699–2702.
31. Braig, H. R., Zhou, W., Dobson, S. L. & O'Neill, S. L. (1998) *J. Bacteriol.* **180,** 2373–2378.

MICROBIOLOGY