Artificial Triple *Wolbachia* Infection in *Aedes albopictus* Yields a New Pattern of Unidirectional Cytoplasmic Incompatibility

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Obligately intracellular *Wolbachia* bacteria infect numerous invertebrates and often manipulate host reproduction to facilitate the spread of infection. An example of reproductive manipulation is *Wolbachia*-induced cytoplasmic incompatibility (CI), which occurs commonly in insects. This CI has been the focus both of basic scientific studies of naturally occurring invasion events and of applied investigations on the use of *Wolbachia* as a vehicle to drive desired genotypes into insect populations (“gene drive” or “population replacement” strategies). The latter application requires an ability to generate artificial infections that cause a pattern of unidirectional incompatibility with the targeted host population. A suggested target of population replacement strategies is the mosquito *Aedes albopictus* (Asian tiger mosquito), an important invasive pest and disease vector. *Aedes albopictus* individuals are naturally “superinfected” with two *Wolbachia* types: *w*AlbA and *w*AlbB. Thus, generating a strain that is unidirectionally incompatible with field populations requires the introduction of an additional infection into the preexisting superinfection. Although prior reports demonstrate an ability to transfer *Wolbachia* infections to *A. albopictus* artificially, including both intra- and interspecific *Wolbachia* transfers, previous efforts have not generated a strain capable of invading natural populations. Here we describe the generation of a stable triple infection by introducing *Wolbachia* wRi from *Drosophila simulans* into a naturally superinfected *A. albopictus* strain. The triple-infected strain displays a pattern of unidirectional incompatibility with the naturally infected strain. This unidirectional CI, combined with a high fidelity of maternal inheritance and low fecundity effects, suggests that the artificial cytotype could serve as an appropriate vehicle for gene drive.

*Wolbachia* spp. are maternally inherited, obligately intracellular bacteria that commonly infect invertebrates, including ~20% of insect species (2). A hypothesized explanation for the evolutionary success of *Wolbachia* is its ability to affect host reproduction; cytoplasmic incompatibility (CI) is one of the most widely reported effects (25). Unidirectional CI can occur when the *Wolbachia* infection type present in a male differs from that in his mate. Although the precise mechanism is unknown, a lock/key model has been proposed in which the *Wolbachia* infection modifies the sperm during spermatogenesis (27). If the male inseminates a female lacking a compatible *Wolbachia* type, the modified sperm fail to achieve karyogamy. In contrast, “rescue” of the modified sperm occurs in embryos from females infected with compatible *Wolbachia* types. Thus, in populations that include both infected and uninfected individuals, *Wolbachia*-infected females can mate successfully with all males in the population. In contrast, uninfected females can reproduce successfully only with uninfected males. This pattern of unidirectional CI allows *Wolbachia* to spread rapidly through host populations.

Previous studies of insects with multiple *Wolbachia* types have demonstrated that unidirectional CI can be additive (4, 5). Multiple *Wolbachia* infection types within an individual male may independently modify sperm, requiring a similar combination of infection types in female mates for compatibility. Additive unidirectional CI can result in repeated population replacement events, in which single- or double-infection cytotypes are replaced by a *Wolbachia* “superinfection” (i.e., individuals harboring two or more infections).

The concept of population replacement has attracted attention for its potential applications. A frequently referenced strategy is based on the replacement of natural populations with modified populations that are refractory to disease transmission (1, 4, 8, 12, 22). A *Wolbachia*-based population replacement strategy requires the generation of artificial infection types that differ from those of the targeted populations. *Aedes albopictus* (Skuse) (Diptera: Culicidae), the Asian tiger mosquito, is native to Asia and is a globally invasive insect. Examples of introduction and establishment include North and South America (11), and recent invasions have extended to Africa, Australia, and Europe (9). In addition to being an invasive pest, this mosquito is an aggressive daytime human biter and has been implicated as a vector of animal (20) and human (11) disease. Recent reports have highlighted its role as a primary vector during recent chikungunya virus epidemics (17, 21).

*Aedes albopictus* populations are naturally infected with two *Wolbachia* types: *w*AlbA and *w*AlbB (13, 24). Therefore, to employ *Wolbachia* as a vehicle for population replacement, an additional, incompatible infection must be introduced into the
natural infection types. Previously, Wolbachia strain wRi was successfully established in A. albopictus by microinjecting the cytoplasm of Drosophila simulans (Riverside) into the embryos of aposymbiotic (i.e., without Wolbachia) A. albopictus mosquitoes (28). As hypothesized, the resulting artificial infection displayed a pattern of bidirectional CI when these mosquitoes were crossed with the naturally double infected strain. Thus, the modulation of bidirectional CI when these mosquitoes were crossed with the naturally double infected strain. Therefore, we hypothesized that individuals harboring the combined wAlbA and wAlbB infections were detected by the PCR assay (i.e., wRi infection was not detected).

To develop a strain appropriate for an applied population replacement strategy, we have performed experiments to generate an artificial triple infection. Following embryonic microinjection, experiments were designed to examine individuals across generations for the hypothesized unidirectional CI pattern, to determine the stability and segregation of the different infection types, and to characterize the relative fitness of triple-infected individuals.

FIG. 1. Genealogy and transinfection rates following the generation of the A. albopictus HouR strain. n, number of females tested. The asterisk indicates that for one G7 female, only the wAlbA and wAlbB infections were detected by the PCR assay (i.e., wRi infection was not detected).

TABLE 1. Diagnostic primers for determining the Wolbachia type

<table>
<thead>
<tr>
<th>Wolbachia strain/phage</th>
<th>Gene</th>
<th>Primer</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Hybridization temp (°C)</th>
<th>Expected amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>wAlbA</td>
<td>wsp</td>
<td>328F</td>
<td>CCA GCA GAT ACT ATT GCG</td>
<td>55</td>
<td>379</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>691R</td>
<td>AAA AAT TAA ACG CTA CTC CA</td>
<td>55</td>
<td>501</td>
<td>32</td>
</tr>
<tr>
<td>wAlbB</td>
<td>wsp</td>
<td>183F</td>
<td>AAG GAA CCG AAG TTC ATG</td>
<td>55</td>
<td>501</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>691R</td>
<td>AAA AAT TAA ACG CTA CTC CA</td>
<td>55</td>
<td>501</td>
<td>32</td>
</tr>
<tr>
<td>wRi</td>
<td>wsp</td>
<td>169F</td>
<td>ATT GAA TAT AAA AAG GCC ACA GAC A</td>
<td>52</td>
<td>523</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>691R</td>
<td>AAA AAT TAA ACG CTA CTC CA</td>
<td>52</td>
<td>523</td>
<td>32</td>
</tr>
<tr>
<td>WO</td>
<td>orf7</td>
<td>WOF</td>
<td>CCC ACA TGA GCC AAT GAC GTC TG</td>
<td>57</td>
<td>353</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WOR</td>
<td>CGT CTC TGC TGC AAG TAA CTA CTC CAT TAA AAC</td>
<td>57</td>
<td>353</td>
<td>14</td>
</tr>
<tr>
<td>12S rRNA</td>
<td></td>
<td>12s A1</td>
<td>AAA CTA GGA TTA GAT ACC CTA TTA T</td>
<td>55</td>
<td>400</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12s B1</td>
<td>AAA GAC GAC GGG CGA TGT</td>
<td>55</td>
<td>400</td>
<td>16</td>
</tr>
</tbody>
</table>

* Control for template quality.
TABLE 2. Survival and infection levels of *A. albopictus* Hou strain mosquitoes injected as embryos with *Wolbachia* wRi from *D. simulans*

<table>
<thead>
<tr>
<th>Expt</th>
<th>G₀ survival</th>
<th>G₀ infection status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of larva/eggs (hatch rate [%])</td>
<td>No. of pupae/larvae (pupation rate [%])</td>
</tr>
<tr>
<td>1</td>
<td>8/170 (4.7)</td>
<td>3/8 (37.5)</td>
</tr>
<tr>
<td>2</td>
<td>14/241 (5.8)</td>
<td>14/14 (100.0)</td>
</tr>
</tbody>
</table>

*a* Coinfected with wAlbA, wAlbB, and wRi.

94°C; 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing (specific annealing temperatures and primer sequences are listed in Table 1), and 1 min of extension at 72°C, and an additional 10-min extension at 72°C.

**Cytoplasmic incompatibility and fitness assays.** The following 4 crosses (female × male) were set up with the triple-infected HouR line at G₀: Hou × HouR, HouR × Hou, HouR × HouR, and Hou × Hou. For each cross type, 10 virgin females and males (<48 h posteclosion) were mated. Five to six replicates were made for the four crosses. Mosquitoes were blood fed once a week, and oviposition papers were collected weekly. Three oviposition papers were collected and allowed to embryonate for 5 days prior to hatching. Egg hatch rates were measured 3 days after the oviposition papers were submerged in deoxy- genated water. The fecundity per female was estimated from the total number of eggs laid within 4 days by each group of females, divided by 10. For each replicate of each cross type, the hatch rate and fecundity were obtained by averaging the data from the three egg papers collected.

**Statistics.** The normality of the data was examined by a Kolmogorov-Smirnov normality test using StatView (SAS Institute, Cary, NC) software. Significant differences among mean egg hatch rates and among fecundity levels were tested by analysis of variance (ANOVA), followed by statistical comparison using a Scheffé test.

**RESULTS**

A triple *Wolbachia* infected strain of *A. albopictus* was generated by microinjecting embryos of the naturally double infected Hou strain with wRi-infected *D. simulans* (Riverside) embryos (Table 2). Triple-infected adults were obtained in each of two microinjection experiments. As in prior experiments (28), the survival of injected embryos (~ 5%) was the primary limitation. The rate of survival of larvae to the adult stage was relatively high, resulting in a total of seven females and seven males.

As illustrated in Fig. 2, DNA from both Hou (wild type: double infected) and triple-infected individuals was amplified by PCR assays with the 328F/691R and 183F/691R primer sets, indicating the presence of *Wolbachia* wAlbA and wAlbB. In contrast, PCR with the 169F/691R or WOF/WOR primer set yielded amplicons only for the triple-infected and *Drosophila simulans* Riverside individuals, indicating successful transfer of *Wolbachia* wRi into the Hou strain.

Of the 14 surviving G₀ adults, 2 (14.3%) triple-infected adult females were obtained (Table 2). The G₀ females were crossed with HT1 males, and the progeny were tested via PCR assay. In the two G₁ lines derived from one of the G₀ females, the triple infection was detected in all 21 G₁ individuals tested (Fig. 1). One line (named “HouR”) derived from the second infected female (G₁) was selected for subsequent characterization.

CROSSES BETWEEN ALL FOUR COMBINATIONS OF THE HOU AND HOU R STRAINS REVEALED A TYPICAL UNIDIRECTIONAL CI PATTERN (Table 3). Relative to the compatible crosses, the egg hatch rate was reduced by ~80% in crosses of HouR males with wild-type Hou females. In contrast, wild-type Hou males were compatible with HouR females, resulting in a mean hatch rate of 78%, similar to the egg hatch rate resulting from crosses between individuals with similar infection types. ANOVA indicated significant differences in egg hatch rates between the incompatible cross (Hou × HouR) and the three compatible crosses (*F* 79.97; *P* < 0.0001). The hatch rates of the three compatible crosses (HouR × Hou, HouR × HouR, and Hou × Hou) were not significantly different.

**DISCUSSION**

The results demonstrate that *A. albopictus* can stably support wRi from *D. simulans* as a superinfection, that the triple infection is unidirectionally incompatible with the wild-type infection, and that the triple infection is maternally transmitted at high rates. The results presented here are similar to prior
observations of the wRi single infection of A. albopictus, which also displayed stability and high levels of maternal inheritance (28). The results are consistent with the hypothesis that wRi causes CI when introduced into naturally double infected populations. In a prior examination of the wRi single infection of A. albopictus, the HTR strain ( singly infected with wRi) failed to rescue the Wolbachia-induced sperm modification caused by either wAlbA, wAlbB, or their combination (28). Furthermore, infection with wAlbA or wAlbB, or superinfection with wAlbA and wAlbB, failed to rescue sperm modification in crosses with wRi males. Thus, wRi displays bidirectional cytoplasmic incompatibility with the wAlbA and wAlbB infection types. Here we have observed that wRi continues to induce CI when coinfecting the HouR strain along with the wAlbA and wAlbB infection types. The CI level resulting in the Hou × HouR crosses (hatch rate, ~16%) is similar to the CI level observed with the HTR strain (hatch rate, ~14%) (28). Thus, coinfection with the three Wolbachia types did not have a measurable effect on the CI level induced by wRi infection, relative to that with wRi alone.

Of the 122 HouR mosquitoes assayed by PCR, only 1 male failed to demonstrate a triple Wolbachia infection. wAlbB and wRi were detected in this male (i.e., the natural wAlbA infection was not detected). This could represent an artifact of the PCR assay (i.e., false negative for the wAlbA infection). Alternatively, loss of the wAlbA infection could reflect the previously reported lower density of wAlbA versus wAlbB infections (7). The infrequent loss of wAlbA in males is not expected to impact Wolbachia infection dynamics substantially in A. albopictus, since males are a dead end for the maternally inherited infections.

Insects that are naturally infected with three Wolbachia types have been reported (26), and triple Wolbachia infected insects have been artificially generated by microinjecting a third strain into a double-infected Drosophila line (18). Mouton et al. (15) studied the regulation of Wolbachia strains in triple-, double-, and single-infected Leptopilina heterotoma (Thomson) (Hymenoptera: Cynipoidea: Eucoilidae) wasps and found that the total Wolbachia counts (total number of Wolbachia cells/wasp) are not at the same level for the different infection lines. Notably, the density (cell number per milligram fresh weight) of each Wolbachia strain remained unchanged in different infection lines; thus, the infection levels in the wasp strains were independent of co-occurring Wolbachia infections. Likewise, the artificial introduction of a third Wolbachia strain into double-infected D. simuliens resulted in an increase of the total Wolbachia density in the host (18). Therefore, the HouR strain provides an additional system for studying Wolbachia regulation.

The Wolbachia wRi strain is known to be infected by an active bacteriophage named WO (10, 14). In contrast, no phage has been described in association with Wolbachia wAlbA or wAlbB. During microinjection, phage WO was transferred together with Wolbachia wRi, and it can be detected within the triple-infected HouR mosquito strain (Fig. 2). This provides an opportunity for the study of the interaction of phage WO with the wAlbA and/or wAlbB infection. Specifically, the interaction among the phage, Wolbachia, and the insect host (14, 19) could be studied in HouR sublines that contain only wAlbA and/or wAlbB (e.g., generated from HouR treated with moderate antibiotic levels [3]).

The artificial strain resulting from this study displays stable triple Wolbachia infection and high maternal inheritance rates in A. albopictus with no observed fecundity effect. These features are consistent with the traits desired for an efficient population replacement strategy (23). Furthermore, the reduced hatch rate observed in crosses between naturally infected females and HouR males indicates a CI phenotype, suggesting that the HouR strain is potentially useful for field application.

ACKNOWLEDGMENTS
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REFERENCES

TABLE 3. Crossing results for the triple Wolbachia infected A. albopictus HouR line (G0)

<table>
<thead>
<tr>
<th>Expected CI type</th>
<th>Cross*</th>
<th>Infection types</th>
<th>Hatch rate (%)*</th>
<th>No. of eggs scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unidirectional CI</td>
<td>Hou × HouR</td>
<td>wAlbA, wAlbB</td>
<td>16.0 ± 9.3 A</td>
<td>5,153</td>
</tr>
<tr>
<td></td>
<td>HouR × Hou</td>
<td>wAlbA, wAlbB, wRi</td>
<td>78.1 ± 12.1 B</td>
<td>5,752</td>
</tr>
<tr>
<td>Compatible</td>
<td>HouR × HouR</td>
<td>wAlbA, wAlbB, wRi</td>
<td>75.9 ± 3.2 B</td>
<td>4,106</td>
</tr>
<tr>
<td></td>
<td>Hou × Hou</td>
<td>wAlbA, wAlbB</td>
<td>80.2 ± 8.2 B</td>
<td>7,736</td>
</tr>
</tbody>
</table>

* Female × male.

b Expressed as the mean for 5 to 6 replicates/cross type ± standard deviation. Different capital letters following the data indicate significant differences (P < 0.001).


