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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 w*Ri 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

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Generation	Tested females lineage	Father	%Triple infection; n
G_0	HouR-1F	HT1	100% ; 1
G_1	1 2	HT1	100% ; 2
G_2	1…10 1…11	HT1	100% ; 21
G_3	1…10	HT1	100% ; 10
G_4	1 2 3 4	HT1	100% ; 4
G_5	1 2 3 1 2 3 1 2 1…18	HT1	100% ; 26
G_6	CI and fecundity tests	HouR (G_6)	nd
G_7	1…23	HT1	96% ; 23*
G_8	1…16	HT1	100% ; 16
G_{13}	1…10	HouR (G_{13})	100% ; 10

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 G_7 female, only the *w*AlbA and *w*AlbB infections were detected by the PCR assay (i.e., *w*Ri infection was not detected).

natural infection types. Previously, *Wolbachia* strain *w*Ri 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 modification/rescue mechanism(s) of the *w*Ri infection is known to differ from those of the naturally occurring infection types. Therefore, we hypothesized that individuals harboring the combined *w*Ri, *w*AlbA, and *w*AlbB infections would be unidirectionally incompatible with the naturally infected mosquitoes.

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.

MATERIALS AND METHODS

Insect strains. Two mosquito strains were used in experiments: the wild-type *A. albopictus* "Hou" strain double infected with *Wolbachia* types *w*AlbA and *w*AlbB (24) and the aposymbiotic HT1 strain, generated by treatment of the Hou strain with tetracycline (6). Mosquitoes were maintained in 30- by 30- by 30-cm cages at 28 ± 2°C and 75% ± 10% relative humidity with a photoperiod of 18 h of light and 6 h of darkness. Adult mosquitoes were continuously provided 10% sucrose as a carbohydrate source, and a blood meal was given once a week with anesthetized mice (IACUC no. 00905A2005). *Drosophila simulans* Riverside embryos, naturally infected with *Wolbachia* strain *w*Ri (31), were used as the *Wolbachia* donor. Fly rearing and egg collection were performed as previously described (30).

Embryonic microinjection. Embryos were collected, prepared, and microinjected as previously described (29, 30). Injection was performed using an IM 300 microinjector (Narishige Scientific, Tokyo, Japan). Injection needles were prepared using quartz glass capillaries with an outer diameter (OD) of 1.00 mm, an inner diameter (ID) of 0.70 mm, and a length of 7.5 cm (QF100-70-7.5; Sutter Instrument Co., Novato, CA) and a P-2000 micropipette puller (Sutter Instrument Co., Novato, CA). Needles were beveled at a 15° angle using a micropipette beveler, model BV-10 (Sutter Instrument Co., Novato, CA). Microinjection was done using an Olympus IX70 inverted microscope (Olympus Co., Tokyo, Japan) at ×200 magnification. One of the resulting triple-infected *A. albopictus* lines was named HouR (derived from the Hou recipient strain and the *Drosophila simulans* Riverside donor strain).

Rearing and selection of microinjected lines. Females of the parent generation (G_0) were isolated as virgins and were mated with HT1 males (Fig. 1). After oviposition, G_0 females and males were assayed for infection by using PCR. Daughters (G_1) from triple-infected G_0 females were isolated as virgins and were outcrossed with HT1 males. All ovipositing females were tested for *Wolbachia* infection by PCR. Subsequently, PCR-guided selection was performed for 5 generations (i.e., G_1 to G_5). At G_6, individuals were combined and used for CI crosses. In the G_7 and G_8 generations, the progeny from positive mothers were pooled, and selection continued. After G_8, the HouR strain was closed (i.e., not outcrossed with HT1 males, but crossed with HouR males), and PCR was used to monitor the frequency of infection periodically through generations.

Infection status testing via PCR amplification. DNA was extracted from dissected adult ovaries by the STE method (16) or from whole adult mosquitoes. For the latter procedure, an individual adult mosquito was placed with a 2-mm-diameter autoclaved glass bead and 100 μl of squash buffer (10 mM Tris, 1 mM EDTA, 50 mM NaCl [pH 8.2]) in a 1.5-ml Eppendorf tube. Homogenization using a Mini-BeadBeater (Glen Mills Inc., Clifton, NJ) was followed by incubation at 100°C for 5 min. Samples were then held on ice for 2 min before centrifugation at maximum speed (14,000 rpm) for 5 min using an Eppendorf centrifuge, model 5415C (Brinkmann Instruments, Westbury, NY). The supernatant was transferred to a fresh Eppendorf tube for PCR analysis or storage at −20°C.

Adults putatively infected with *Wolbachia* were screened by type-specific PCR amplification (Table 1). PCR amplification was performed in a PTC-200 thermal cycler (MJ Research Inc., Waltham, MA) in a final volume of 20 μl containing 0.25 mM deoxynucleoside triphosphate (dNTP) mixture, 0.5 μM each primer, 1.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (New England Biolabs Inc., Ipswich, MA), and 1 μl DNA template. The PCR conditions were as follows: 4 min at

TABLE 1. Diagnostic primers for determining the *Wolbachia* type

Wolbachia strain/phage	Gene	Primer	Primer sequence (5′ to 3′)	Hybridization temp (°C)	Expected amplicon size (bp)	Reference
*w*AlbA	*wsp*	328F	CCA GCA GAT ACT ATT GCG	55	379	32
		691R	AAA AAT TAA ACG CTA CTC CA			
*w*AlbB	*wsp*	183F	AAG GAA CCG AAG TTC ATG	55	501	32
		691R	AAA AAT TAA ACG CTA CTC CA			
*w*Ri	*wsp*	169F	ATT GAA TAT AAA AAG GCC ACA GAC A	52	523	32
		691R	AAA AAT TAA ACG CTA CTC CA			
WO	*orf7*	WOF	CCC ACA TGA GCC AAT GAC GTC TG	57	353	14
		WOR	CGT TCG CTC TGC AAG TAA CTC CAT TAA AAC			
	12S rRNA[a]	12s A1	AAA CTA GGA TTA GAT ACC CTA TTA T	55	400	16
		12s B1	AAG AGC GAC GGG CGA TGT GT			

[a] 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*

Expt	G_0 survival				G_0 infection status			
					Female		Male	
	No. of larvae/eggs (hatch rate [%])	No. of pupae/larvae (pupation rate [%])	No. of adults/pupae (eclosion rate [%])	No. of females/total (sex ratio [%])	No. of samples tested	% Triple infected[a]	No. of samples tested	% Triple infected[a]
1	8/170 (4.7)	3/8 (37.5)	3/3 (100.0)	1/3 (33.3)	1	100	2	0
2	14/241 (5.8)	14/14 (100.0)	11/14 (78.6)	6/11 (54.5)	6	16.7	5	0

[a] Coinfected with *w*AlbA, *w*AlbB, and *w*Ri.

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 G_6: 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 deoxygenated 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 cytoplasm from *w*Ri-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* *w*AlbA and *w*AlbB. 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* *w*Ri into the Hou strain.

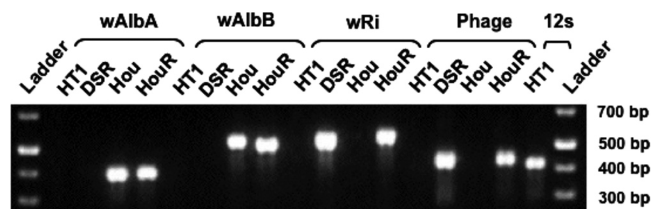

FIG. 2. PCR assays of the triple-infected *A. albopictus* HouR line using primers specific for the *w*AlbA, *w*AlbB, and *w*Ri strains and bacteriophage WO. 12S rRNA primers were used to check the DNA template quality of the negative control (HT1). DSR, *Drosophila simulans* Riverside.

Of the 14 surviving G_0 adults, 2 (14.3%) triple-infected adult females were obtained (Table 2). The G_0 females were outcrossed with HT1 males, and the progeny were tested via PCR assay. In the two G_1 lines derived from one of the G_0 females, the triple infection was detected in all 21 G_2 individuals tested (Fig. 1). One line (named “HouR”) derived from the second infected female (G_1) was selected for subsequent characterization.

Crosses between all four combinations of the Hou and HouR 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.

No obvious impact of the triple infection on female fecundity was observed. Specifically, HouR and Hou females produced similar numbers of eggs when mated with Hou males (38.4 ± 11.3 and 51.6 ± 9.4 eggs/female, respectively).

Transmission efficiencies were monitored from G_1 to G_8 (Fig. 1). HouR females were randomly selected at each generation for PCR assays to determine the infection status. Triple infection was detected in 101/102 (99.0%) females tested. PCR assays failed to detect the *w*Ri infection in only one female (Fig. 1, G_7). At G_{13}, the maternal inheritance rate for triple infection was tested by PCR assays of 10 females and 10 males from five separate lines (i.e., 2 females and 2 males from each of five lines were assayed), and an overall 95% transmission rate of triple infection was observed: 100% for females and 90% for males. PCR failed to detect the *w*AlbA infection in one of 10 males tested (i.e., it detected *w*AlbB and *w*Ri infections only).

DISCUSSION

The results demonstrate that *A. albopictus* can stably support *w*Ri 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

TABLE 3. Crossing results for the triple *Wolbachia* infected *A. albopictus* HouR line (G_6)

Expected CI type	Cross[a]	Infection types		Hatch rate (%)[b]	No. of eggs scored
		Female	Male		
Unidirectional CI	Hou × HouR	*w*AlbA, *w*AlbB	*w*AlbA, *w*AlbB, *w*Ri	16.0 ± 9.3 A	5,153
	HouR × Hou	*w*AlbA, *w*AlbB, *w*Ri	*w*AlbA, *w*AlbB	78.1 ± 12.1 B	5,752
Compatible	HouR × HouR	*w*AlbA, *w*AlbB, *w*Ri	*w*AlbA, *w*AlbB, *w*Ri	75.9 ± 3.2 B	4,106
	Hou × Hou	*w*AlbA, *w*AlbB	*w*AlbA, *w*AlbB	80.2 ± 8.2 B	7,736

[a] 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 ($P < 0.001$).

observations of the *w*Ri single infection of *A. albopictus*, which also displayed stability and high levels of maternal inheritance (28). The results are consistent with the hypothesis that *w*Ri causes CI when introduced into naturally double infected populations. In a prior examination of the *w*Ri single infection of *A. albopictus*, the HTR strain (singly infected with *w*Ri) failed to rescue the *Wolbachia*-induced sperm modification caused by either *w*AlbA, *w*AlbB, or their combination (28). Furthermore, infection with *w*AlbA or *w*AlbB, or superinfection with *w*AlbA and *w*AlbB, failed to rescue sperm modification in crosses with *w*Ri males. Thus, *w*Ri displays bidirectional cytoplasmic incompatibility with the *w*AlbA and *w*AlbB infection types. Here we have observed that *w*Ri continues to induce CI when coinfecting the HouR strain along with the *w*AlbA and *w*AlbB 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 *w*Ri infection, relative to that with *w*Ri alone.

Of the 122 HouR mosquitoes assayed by PCR, only 1 male failed to demonstrate a triple *Wolbachia* infection. *w*AlbB and *w*Ri were detected in this male (i.e., the natural *w*AlbA infection was not detected). This could represent an artifact of the PCR assay (i.e., false negative for the *w*AlbA infection). Alternatively, loss of the *w*AlbA infection could reflect the previously reported lower density of *w*AlbA versus *w*AlbB infections (7). The infrequent loss of *w*AlbA 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. simulans* 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 w*AlbA or *w*AlbB. During microinjection, phage WO was transferred together with *Wolbachia w*Ri, 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 *w*AlbA and/or *w*AlbB infection. Specifically, the interaction among the phage, *Wolbachia*, and the insect host (14, 19) could be studied in HouR sublines that contain only *w*AlbA and/or *w*AlbB (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.

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