Applied and Environmental Microbiology, May 2009, p. 3373–3376 Vol. 75, No. 10
0099-2240/09/$08.00+0 doi:10.1128/AEM.00207-09
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The Virulent *Wolbachia* Strain wMelPop Efficiently Establishes Somatic Infections in the Malaria Vector *Anopheles gambiae*▽

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Received 29 January 2009/Accepted 20 March 2009

***Wolbachia pipientis* bacteria are maternally inherited endosymbionts that are of interest to control the *Anopheles* mosquito vectors of malaria. *Wolbachia* does not infect *Anopheles* mosquitoes in nature, although cultured *Anopheles* cells can be infected. Here, we show that the virulent *Wolbachia* strain wMelPop can survive and replicate when injected into female *Anopheles gambiae* adults, but the somatic infections established are avirulent. These in vivo data suggest that stable *Wolbachia* infections of *Anopheles* may be possible.**

Infecting up to 500 million people per year (with almost 3 million annual deaths), malaria is the most important vector-borne disease in the world (8, 30, 31, 32). The *Plasmodium* parasites that cause the disease are transmitted to humans by the bite of *Anopheles* mosquitoes, with *Anopheles gambiae* being the principle vector in sub-Saharan Africa (6). Malaria control is limited by the lack of a vaccine and by parasite and mosquito evolution of drug and insecticide resistance (9, 28, 31). In light of these problems, there has been a recent concerted effort to develop innovative methods for malaria control based on the genetic modification of *Anopheles* mosquitoes (transgenesis) or their associated symbiotic microorganisms (paratransgenesis) (5, 10, 11, 13, 15, 23, 25, 27, 36, 37).

In many mosquitoes, *Wolbachia pipientis* symbionts are the causative agents of cytoplasmic incompatibility, a phenomenon where matings between uninfected females and infected males have reduced egg hatch, while matings in the reciprocal cross are fertile. In a mixed population, infected females have a reproductive advantage which can allow *Wolbachia* to increase rapidly in frequency due to maternal inheritance. The propensity of *Wolbachia* to "drive" through populations has been investigated using mathematical models and has been validated by both laboratory and field investigations (20, 21, 33, 34, 35, 36).

There are three scenarios currently envisioned to use *Wolbachia* as part of a malaria control strategy: (i) use *Wolbachia* spread to "drive" refractory transgenes into *Anopheles* populations, converting the mosquito population into one that cannot maintain transmission of the malaria parasites (18, 21, 24, 29, 33, 36); (ii) release *Wolbachia*-infected males into uninfected *Anopheles* populations to reduce population sizes through cytoplasmic incompatibility, similar to the sterile insect technique but without exposing males to radiation or chemical sterilants that could lower their fitness (2, 4, 37); and (iii) release mosquitoes infected with pathogenic or virulent *Wolbachia* strains that shorten mosquito life span. Pathogens must pass through an extrinsic incubation period in the vector before they are able to be transmitted. By shortening mosquito life span, it is theoretically possible to reduce the number of mosquitoes that live through the extrinsic incubation period and become infectious (14, 15, 17, 20, 24).

All of the above strategies require the stable transfer of *Wolbachia* into *Anopheles*. *Wolbachia* symbionts are common in mosquitoes, but no infections have ever been identified in any species of *Anopheles* (12, 22, 26). The negative infection status of natural *Anopheles* populations offers good potential for *Wolbachia*-based malaria control strategies, since preexisting infections can complicate the behavior of introduced infections (33). However, the absence of natural infections in anophelines has led some to suggest that *Anopheles* mosquitoes may be physiologically/genetically incapable of harboring *Wolbachia* infections (1, 29). If this hypothesis is true, then *Wolbachia*-based malaria control strategies are likely doomed to fail. In vitro studies demonstrated that cultured immunocompetent *Anopheles gambiae* cell lines (Sua5B and Moss55) are fully competent to harbor infections of distinct *Wolbachia* strains (the "A" supergroup strains wRi and wMelPop from *Drosophila simulans* and *Drosophila melanogaster* and the "B" supergroup strain wAlbB from *Aedes albopictus*) (16, 18). Some cultured infections reached very high levels where 100% of cells were infected at extremely high levels (wAlbB in Sua5B and wMelPop in Moss55) (16, 18), while other strains were eventually eliminated from the cells (wRi in Sua5B cells) (24). The combined results of these experiments, using multiple *Wolbachia* strains and multiple *Anopheles* cell lines, indicate that there is no intrinsic genetic block to *Wolbachia* infection in *Anopheles* cells, although certain strains of *Wolbachia* may be more likely to colonize *Anopheles* than others.

In this study, we investigated the establishment of in vivo *Wolbachia* infections in *Anopheles gambiae* (Keele strain) mosquitoes by injection of the virulent *Wolbachia* strain wMelPop

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▽ Published ahead of print on 27 March 2009.

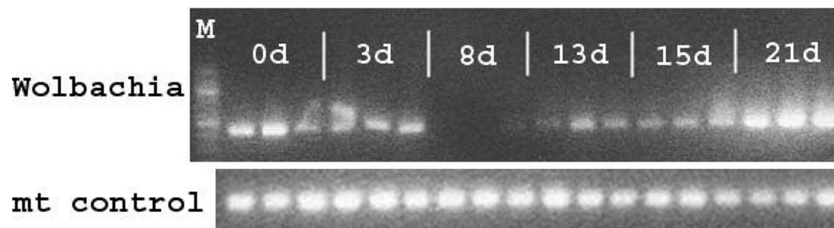

FIG. 1. Typical results using conventional PCR, showing changes in *Wolbachia* levels in injected adult *Anopheles gambiae* females at sequential time points postinjection. Results for mt control (host ND4 mitochondrial gene) indicate that PCR efficiency was approximately equal for all samples. M, 100-bp marker; d, days.

into the hemolymph of adult female mosquitoes. Approximately 200 adult mosquitoes were reared in 30-cm cube cages in a walk-in insectary held at 28°C and 80% relative humidity on a 12:12 h light/dark cycle. Mosquitoes were allowed access to a cotton wick soaked in 10% sucrose as a carbohydrate source. Adults were allowed to blood feed on an anesthetized mouse 5 days postemergence according to JHU animal use protocol MO-03H210. Two days after blood feeding, an oviposition substrate (consisting of a filter paper cone inside a 50-ml beaker half filled with water) was introduced into cages and removed the next day for egg collection. Approximately 250 eggs were placed into a 41- by 34- by 6-cm rearing tray half filled with distilled water and one pellet of dry cat food, with one additional pellet added to each tray daily after day 3. Larvae were removed, and tray water changed if polluted. Pupae were picked with an eyedropper, placed in a cup, and introduced into cages (~200 pupae/cage) to begin the next generation.

wMelPop was cultured in *Anopheles gambiae* Moss55 cells (16), purified, and assessed for viability as described previously (18, 19). Purified *Wolbachia* cells were suspended in culture medium and adjusted to a final concentration of 10^8 bacteria per ml. Using a calibrated glass capillary needle, amounts of 100 to 200 nl suspended *Wolbachia* cells were injected into the thorax of 2-day-old, cold-immobilized adult *Anopheles gambiae* females. Injected mosquitoes were held at 18°C for 5 days and then transferred to the 28°C insectary. Mosquitoes were allowed to blood feed on a mouse twice per week.

Mosquito genomic DNA was extracted by salt extraction/ethanol precipitation as described previously (21), quantified using a NanoDrop spectrophotometer, and adjusted to 20 ng/μl. *Wolbachia* infections in individual mosquitoes were detected by PCR amplification of a fragment of the *Wolbachia* 16S rRNA gene (440 bp) using primers WspecF and WspecR (18). As a control, we amplified a 400-bp fragment from the *Anopheles* mitochondrial NADH dehydrogenase subunit 4 gene (ND4) (18). Mosquitoes were assayed for *Wolbachia* infection by PCR at 6, 10, 20, or 30 days postinjection (p.i.). In a second experiment, mosquitoes were assayed at 0, 3, 8, 13, 15, and 21 days p.i. Amplified fragments were separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and viewed under UV light. Template DNA from infected and uninfected Moss55 cells was included as positive and negative controls.

Quantitative PCR (qPCR) was used to determine if *Wolbachia* could survive and replicate in *Anopheles* by comparing normalized *Wolbachia* levels in individual mosquitoes at day 6 and day 30 p.i. The relative abundance of wMelPop bacteria in each mosquito was assessed by comparing the abundance of the single-copy *Wolbachia* ankyrin repeat gene WD_0550 (16) to that of the single-copy *Anopheles gambiae* ribosomal S7 gene (forward, 5′-TCC-TGG-AGC-TGG-AGA-TGA-AC-3′, and reverse, 5′-GAC-GGG-TCT-GTA-CCT-TCT-GG-3′). For each time point, 14 mosquitoes (biological replicates) were examined. Duplicate reactions were performed for every mosquito, and the results differed by less than 3%, demonstrating consistency of the assay. qPCR was performed using an ABI Prism 7300 detection system (Applied Biosystems) with a QuantiTect SYBR green PCR kit (Qiagen). Determinations of relative abundance of wMelPop in each mosquito and relative changes in wMelPop levels between time points, confidence interval estimation, and statistical analyses were carried out as described by Yuan et al. (38).

To test for virulence of wMelPop, 2-day-old adult female mosquitoes were injected with either wMelPop purified from cell culture or filtered lysate of uninfected Moss55 cells (control) and held at 18°C for 2 days as described above. Injected mosquitoes were held an additional 3 days at 28°C to control for mortality due to the injection procedure. At day 5 p.i., mosquitoes were moved into pint-sized cup cages for life table experiments. Approximately 25 mosquitoes were placed in each cage (two replicate control cages and three replicate *Wolbachia* treatment cages) and the entire experiment replicated two times, for a total of four control cages and six *Wolbachia* treatment cages. Mosquitoes were provided a cotton pad soaked in 10% sucrose but were not given access to blood. Dead mosquitoes were removed from each cage approximately every other day. For each experiment, mortality data were used to construct treatment-specific cohort life tables (3). Because the data did not conform to parametric assumptions, they were analyzed by the Mann-Whitney U test using STATVIEW (SAS Corporation).

In injected mosquitoes, *Wolbachia* bacteria were detectable by PCR at all time points, as follows [infection frequency (95% exact binomial confidence interval)]: day 6, 0.875 (0.617 to 0.985; $n = 16$); day 10, 0.75 (0.401 to 0.968; $n = 8$); day 20, 0.722 (0.465 to 0.903; $n = 18$); and day 30, 1.0 (0.794 to 1.0; $n = 13$). In further experiments, *Wolbachia* bacteria were easily detectable through day 3 p.i. but were weak or not detectable by conventional PCR by day 8 p.i. After 13 days p.i., *Wolbachia* bacteria were easily detectable again, and the bands increased in intensity for the remainder of the time series experiment (Fig. 1). This initial decrease, followed by an increase, in the apparent infection rate is possibly due to initial clearance of

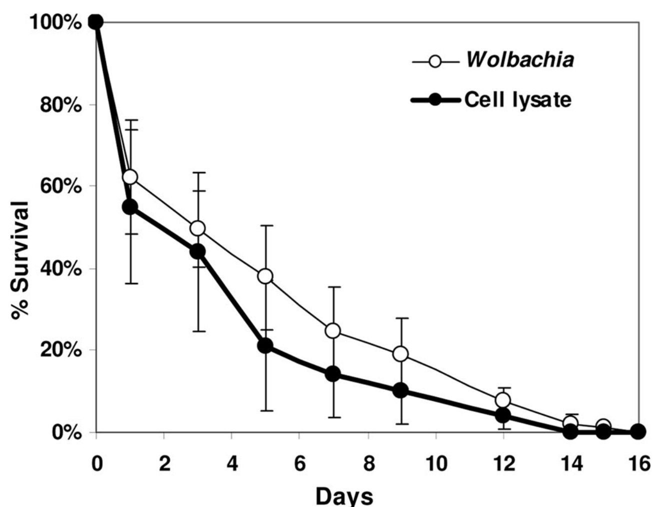

FIG. 2. Mean survival trajectories of wMelPop-injected versus cell lysate-injected *Anopheles gambiae* adult females. Survival trajectories do not differ significantly. Error bars show standard deviations.

some of the injected bacteria and then establishment of infection and bacterial replication. By qPCR, a highly statistically significant 42-fold increase in the normalized *Wolbachia* level was observed: on day 6 p.i., there were 23.7 *Wolbachia* genomes per host genome (95% confidence interval, 10.6 to 52.7; $n = 14$), and on day 30 p.i., there were 992 *Wolbachia* genomes per host genome (95% confidence interval, 433.6 to 2,267.4; $n = 14$) (Mann-Whitney U test, tied Z value $= -4.319$; $P < 0.0001$). Since *Wolbachia* cannot replicate in the extracellular environment (19), these results confirm that injected bacteria are able to infect cells, survive, and replicate in *Anopheles gambiae* in vivo.

wMelPop is a virulent *Wolbachia* strain that reduces the life span of its host by approximately 50%. While originally found and characterized in a laboratory colony of *Drosophila melanogaster*, it has similar pathogenic effects when artificially transferred into *Drosophila simulans* (14), and recently, the yellow fever mosquito *Aedes aegypti* (15). However, there was no statistically significant difference in survival trajectories between *Anopheles gambiae* mosquitoes injected with wMelPop and mosquitoes injected with filtered uninfected cell lysate (Mann-Whitney U test, tied Z value $= -1.799$; $P = 0.702$) (Fig. 2). Although wMelPop replicates to high levels in injected *Anopheles* (approximately 1,000 bacterial genomes per host genome), these levels do not seem to be associated with virulence. It is possible that the virulence of wMelPop has been attenuated during its culture in Moss55 cells, although during long-term culture in an *Aedes aegypti* cell line, wMelPop retained its virulent phenotype when reintroduced into either *Drosophila* or *Aedes aegypti* in vivo (15, 16). The specific mechanism of wMelPop virulence is not completely understood, but it seems that increased host mortality is not simply due to overreplication and high infection levels but rather to overreplication in and damage to specific host tissues, such as the brain and central nervous system (14, 15, 17). Investigation into the tissue localization of *Wolbachia* in injected *Anopheles* mosquitoes is currently ongoing, but in light of these results, it is reasonable to hypothesize that when injected into the hemolymph, *Wolbachia* bacteria reach high levels in some mosquito tissues but either do not infect or do not replicate in the *Anopheles* central nervous system. It remains to be seen whether vertically acquired infections will show virulence in *Anopheles gambiae*.

Previous studies showed that cultured *Anopheles gambiae* cells can be infected with *Wolbachia* (16, 18), but no data were available to assess whether the in vitro results could be extrapolated to *Anopheles* mosquitoes in vivo. The experiments outlined in this paper demonstrate that *Wolbachia* can infect *Anopheles* mosquitoes in vivo. However, for a *Wolbachia*-based malaria control strategy to be effective, simply infecting *Anopheles* by injection is not sufficient—the infection must be transmitted vertically to offspring. Stable (100%) vertical transmission of *Wolbachia* after injection into adults has been reported for *Drosophila melanogaster* (7). A similar phenomenon has been reported for *Aedes aegypti*, but transmission was unstable (approximately 40%) (27). Experiments to determine whether *Wolbachia* bacteria injected into the hemolymph of adult *Anopheles* will be transmitted vertically to offspring are ongoing, and if efficient vertical transmission of the symbionts can be established, *Wolbachia*-based strategies for malaria control should be possible.

This work was supported by NIH grant R21AI070178 and funds from the Bloomberg Family Foundation to J.L.R. C.J. was partially supported by the Calvin and Helen Lang Scholarship (JHSPH).

We thank Scott O'Neill for his kind gift of the wMelPop-infected Moss55 cell line.

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