

## Host Adaptation of a *Wolbachia* Strain after Long-Term Serial Passage in Mosquito Cell Lines<sup>∇</sup>

Conor J. McMeniman,<sup>1</sup> Amanda M. Lane,<sup>1†</sup> Amy W. C. Fong,<sup>1</sup> Denis A. Voronin,<sup>2‡</sup>  
Iñaki Iturbe-Ormaetxe,<sup>1</sup> Ryuichi Yamada,<sup>1</sup> Elizabeth A. McGraw,<sup>1</sup> and Scott L. O'Neill<sup>1\*</sup>

School of Integrative Biology, The University of Queensland, Brisbane, Queensland 4072, Australia,<sup>1</sup> and Institute of Cytology and Genetics, Siberian Division of the Russian Academy of Sciences, pr. Akademika Lavrenteva 10, Novosibirsk 630090, Russia<sup>2</sup>

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**The horizontal transfer of the bacterium *Wolbachia pipientis* between invertebrate hosts hinges on the ability of *Wolbachia* to adapt to new intracellular environments. The experimental transfer of *Wolbachia* between distantly related host species often results in the loss of infection, presumably due to an inability of *Wolbachia* to adapt quickly to the new host. To examine the process of adaptation to a novel host, we transferred a life-shortening *Wolbachia* strain, wMelPop, from the fruit fly *Drosophila melanogaster* into a cell line derived from the mosquito *Aedes albopictus*. After long-term serial passage in this cell line, we transferred the mosquito-adapted wMelPop into cell lines derived from two other mosquito species, *Aedes aegypti* and *Anopheles gambiae*. After a prolonged period of serial passage in mosquito cell lines, wMelPop was reintroduced into its native host, *D. melanogaster*, by embryonic microinjection. The cell line-adapted wMelPop strains were characterized by a loss of infectivity when reintroduced into the original host, grew to decreased densities, and had reduced abilities to cause life-shortening infection and cytoplasmic incompatibility compared to the original strain. We interpret these shifts in phenotype as evidence for genetic adaptation to the mosquito intracellular environment. The use of cell lines to preadapt *Wolbachia* to novel hosts is suggested as a possible strategy to improve the success of transinfection in novel target insect species.**

*Wolbachia pipientis* is a maternally transmitted obligate intracellular bacterium that chronically infects thousands of insect species, as well as a range of other arthropods and filarial nematodes (13). *Wolbachia* bacteria can induce various reproductive abnormalities in hosts, such as cytoplasmic incompatibility (CI), that promote the bacteria's vertical transmission and spread (14). The discordance of host and *Wolbachia* phylogenies indicates that these bacteria have moved between host lineages on multiple occasions during their evolutionary history (3, 42, 48), although the mechanisms that facilitate the transfer of *Wolbachia* are not well understood. The success of such host shifts is inherently reliant on the ability of the bacteria to adapt to new intracellular environments.

The experimental transfer of *Wolbachia* between host species (transinfection) has proved technically challenging, and the success of such experiments is difficult to predict. Despite an increasing number of reports that document *Wolbachia* transinfection, many attempts to experimentally infect host species are unsuccessful due to poor maternal transmission rates in the novel host (40). In some cases, transferred strains are extremely stable and maternally inherited at very high rates. This situation occurs primarily when *Wolbachia* is transferred within or between closely related species in a family or

genus (6, 45, 47). In other cases, the infecting strain appears to be poorly adapted to its new host, showing fluctuating infection densities and various degrees of transovarial transmission. The result is often the loss of infection within a few host generations. Not surprisingly, *Wolbachia* infections tend to be more susceptible to loss when they have been transferred between phylogenetically distant hosts (17, 35). Similarly, those species that do not naturally harbor *Wolbachia* can be especially challenging to successfully transinfect (10, 36).

Understanding the process of *Wolbachia* adaptation to new hosts is central to gaining insight into the current distribution of *Wolbachia* bacteria among species and the evolutionary success of the genus. It may also facilitate the use of *Wolbachia* in an applied setting to introduce desirable traits into insect populations. For example, it has been proposed previously that the life-shortening *Wolbachia* strain wMelPop from the fruit fly *Drosophila melanogaster* might be introduced into populations of mosquito disease vectors in order to shift the population age structure and reduce pathogen transmission to humans (9, 38). The success of these strategies is predicated on the successful transfer of *Wolbachia* strains between host species in the laboratory.

To examine the process of *Wolbachia* adaptation to a new host, we transinfected a cell line from the mosquito *Aedes albopictus* with the wMelPop strain native to *D. melanogaster* (23) and maintained the line for ~240 serial passages. We then used the cell line-adapted wMelPop to infect both *Aedes aegypti* and *Anopheles gambiae* cell lines. Both lines were maintained for an additional 60 passages before *D. melanogaster* was transinfected with the *A. aegypti*-adapted *Wolbachia* by embryonic microinjection. We report on the phenotypic outcomes of the long-term serial passage of wMelPop in mosquito

\* Corresponding author. Mailing address: School of Integrative Biology, The University of Queensland, St. Lucia, Brisbane, QLD 4072, Australia. Phone: 61 7 3365 2471. Fax: 61 7 3346 9213. E-mail: scott.oneill@uq.edu.au.

† Present address: School of Biological Sciences A08, University of Sydney, Sydney, NSW 2006, Australia.

‡ Present address: Écologie Microbienne UMR CNRS 5557, Université Claude Bernard Lyon 1, 69622 Villeurbanne, France.

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cell lines as demonstrated by direct comparisons of the growth kinetics, life-shortening abilities, and levels of CI expression of the cell line-adapted *wMelPop* and original *wMelPop* strains in *D. melanogaster*.

#### MATERIALS AND METHODS

**Cell lines and maintenance.** Three cell lines were used in this study: (i) Aa23.T, a tetracycline-treated cell line derived from *A. albopictus* embryos and referred to herein as Aa23 when infected with *Wolbachia* (27); (ii) RML-12, derived from *A. aegypti* larvae (19; C. E. Yunker, personal communication); and (iii) MOS-55, derived from *A. gambiae* larvae (20). All these cell lines were confirmed by PCR to be negative for *Wolbachia* infection prior to this study, as outlined below. Aa23.T and RML-12 cell lines were maintained in growth medium consisting of equal volumes of Mitsubishi-Maramorosch (24) [1 mM CaCl<sub>2</sub>, 0.2 mM MgCl<sub>2</sub>, 2.7 mM KCl, 120 mM NaCl, 1.4 mM NaHCO<sub>3</sub>, 1.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 22 mM D-(+)-glucose, 6.5 g of lactalbumin hydrolysate/liter, and 5.0 g of yeast extract/liter] and Schneider's insect medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum. MOS-55 was maintained in Schneider's insect medium supplemented with 20% heat-inactivated fetal bovine serum. Both media also contained penicillin (50 U/ml) and streptomycin (50 µg/ml). For routine maintenance, cells were grown in 25-cm<sup>2</sup> plastic tissue culture flasks containing 5 ml of medium at 26°C without CO<sub>2</sub> incubation. Cells were passaged every 3 to 4 days by vigorous shaking of the flask and the seeding of a new flask with 20% of the resuspended cells in 5 ml of fresh medium.

**Establishment of *wMelPop*-infected cell lines.** *wMelPop* was purified from *D. melanogaster* *w*<sup>1118</sup> embryos (23) and established in an uninfected *A. albopictus* cell line (Aa23.T) by using the shell vial technique (11). Embryos were collected every 45 min onto molasses agar plates covered with live yeast paste and dechlorinated using freshly prepared 50%-diluted bleach (2.1% sodium hypochlorite final concentration; White King, Victoria, Australia) for 2 min. Embryos were then rinsed several times in sterile distilled water, immersed in 70% ethanol for 15 s, and rinsed three times in sterile phosphate-buffered saline (PBS), pH 7.4. Approximately 20 mg of surface-sterilized embryos (~50 to 100 µl of packed embryos) was transferred into a mini Dounce tissue homogenizer (Wheaton) and suspended in 400 µl of PBS. Embryos were then homogenized for 2 to 3 min with a tight pestle. Two wells of 80% confluent Aa23.T cells in a 12-well cell culture plate prepared 24 h earlier were overlaid with 200 µl of homogenate each. The plate was centrifuged at 2,000 × *g* for 1 h at 15°C. Cells were then incubated at 26°C for 24 h, and the contents of each well were transferred into individual 25-cm<sup>2</sup> cell culture flasks with 5 ml of fresh medium. After a confluent monolayer had formed, cells were split 1:5 and passaged as usual.

To establish the infection in *A. aegypti* RML-12 and *A. gambiae* MOS-55 cell lines, *wMelPop* was purified from Aa23 cells as described below and introduced into these cell lines by using the shell vial technique.

**Characterization of *wMelPop* in cell lines.** *Wolbachia* infections in cell lines were characterized using (i) PCR screening and sequencing and (ii) electron microscopy. For each assay, naturally uninfected or tetracycline-cured derivatives of each cell line were used as negative controls.

(i) **PCR screening and sequencing.** To monitor the infection status of cells, DNA was extracted from cultures as described previously (11) and amplified using the general *Wolbachia* surface protein (*wsp*) gene primers 81F and 691R or the diagnostic *wsp* primer set for *wMelPop*, 308F and 691R (48). To confirm the presence of *wMelPop* in these three cell lines, fragments of the *Wolbachia* 16S rRNA and *wsp* genes were PCR amplified, cloned, and sequenced. DNA was extracted from cells using a DNeasy tissue kit (Qiagen) and amplified as described previously using the diagnostic primers 99F and 994R for the *Wolbachia* 16S rRNA gene (26) and the primers 81F and 691R for the *wsp* gene (48). Total DNA from cell lines was also PCR amplified using the general eubacterial 16S rRNA primers 10F/1507R (21) and 968F/R1401R (25). The resulting PCR products were cloned into the pGEM-T Easy vector (Promega), and for each product, four clones from each infected cell line were randomly picked and sequenced. The presence of *wMelPop* and no other contaminating bacteria in cell lines was verified by denaturing gradient gel electrophoresis using a general primer set targeting eubacterial 16S rRNA genes (F-968-GC and R-1401) (25) by methods described previously (29).

(ii) **Electron microscopy.** Insect cells were washed in PBS and rapidly fixed with microwave processing in 2.5% glutaraldehyde solution containing 0.1% CaCl<sub>2</sub> and 1% sucrose in 0.1 M Na cacodylate, enrobed in 2% agarose, and postfixed in 1% osmium tetroxide in 0.1 M Na cacodylate buffer. Samples were then dehydrated in a sequence of increasing ethanol concentrations and, in a

final step, in acetone (100%) and then embedded in epoxy resin (Epon 812) by using microwave processing (12, 27). Ultrathin sections (50 to 80 nm) prepared on an Ultracut T ultramicrotome (Leica Inc.) were placed onto copper grids and stained with 2% uranyl acetate followed by Reynolds lead citrate. The sections were examined in a JEOL-1010 electron microscope operated at 80 kV.

**Purification of *Wolbachia* from cell culture for embryonic microinjection.** Insect cells from the confluent monolayers of two 175-cm<sup>2</sup> flasks were harvested and centrifuged in 50-ml conical flasks at 1,000 × *g* for 5 min at 4°C, and the cell culture medium was discarded. The cellular pellet was then washed in SPG buffer (218 mM sucrose, 3.8 mM KH<sub>2</sub>PO<sub>4</sub>, 7.2 mM K<sub>2</sub>HPO<sub>4</sub>, and 4.9 mM L-glutamate, pH 7.2), and the centrifugation and wash steps were repeated. After being washed, the pellet was resuspended in 5 ml of SPG buffer on ice and sonicated twice for 10 s at 12.5 W with a Fisher Scientific model 60 sonic dismembrator (3-mm microtip diameter) to lyse the cells. This suspension was then centrifuged at 1,000 × *g* for 5 min at 4°C to pellet cellular debris. The supernatant was passed through a 5-µm Acrodisc syringe filter (Pall Life Sciences), and the filtrate was collected in 1.5-ml microcentrifuge tubes. These tubes were centrifuged at 12,000 × *g* for 15 min at 4°C to pellet *Wolbachia* cells. The supernatant was discarded, and the pellets were combined and resuspended in 400 µl of SPG buffer and centrifuged at 300 × *g* for 5 min to remove any remaining debris (44). The supernatant was then transferred into a clean tube and stored on ice until being used for injection (<3 h).

**Embryonic microinjection.** Purified *Wolbachia* from RML-12 was microinjected into embryos of the *D. melanogaster* line *w*<sup>1118</sup>.T, which had previously been cured of infection by tetracycline treatment (23). Prior to microinjection, this line was confirmed to be free of *Wolbachia* by PCR using primers specific for the *wMelPop* IS5 repeat: IS5-FWD1 (5'-GTATCCAACAGATCTAAGC) and IS5-REV1 (5'-ATAACCCTACTCATAGCTAG). IS5 is a multicopy insertion element and, as such, is a much more sensitive target for determining infection status than single-copy genes such as *wsp*. For microinjection, early (preblastoderm)-stage embryos were collected every 30 min by using molasses agar plates with live yeast paste. Purified *Wolbachia* was microinjected into the posterior poles of embryos within 30 min of collection by standard techniques (1, 6, 44). After hatching, larvae were transferred into a standard cornmeal-based *Drosophila* rearing medium (2) and incubated at 24°C.

***Drosophila* rearing and PCR screening for infection status.** Virgin females resulting from injected embryos (generation 0 [G<sub>0</sub>]) were placed in vials with three *w*<sup>1118</sup>.T males to establish isofemale lines. After egg laying, G<sub>0</sub> females were sacrificed and DNA was extracted using the Holmes-Bonner DNA extraction protocol (16). *Wolbachia* was detected in samples using PCR primers specific for the IS5 repeat element in *wMelPop*. The quality of the insect DNA was assessed using the primer set 12SA1 and 12SB1, which amplifies the *D. melanogaster* 12S rRNA gene (26). The amplification of DNA was carried out in a 20-µl reaction volume which included 2.0 µl of 10× buffer (New England Biolabs, Beverly, MA), 25 µM deoxynucleoside triphosphates, 0.5 µM forward and reverse primers, 0.75 U of *Taq* polymerase (New England Biolabs, Beverly, MA), and 1.0 µl of the DNA template. PCR conditions were as follows: denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and a final 10-min extension step at 72°C.

To select for a stable infection, only offspring from females that tested positive for *Wolbachia* by PCR screening were used as parental stock. Each generation, 25 to 50 females from each line were isolated as virgins, placed into individual vials, and outcrossed with three *w*<sup>1118</sup>.T males. Females that tested negative for *Wolbachia* were discarded along with their progeny. This selection regime was maintained for three generations, after which the lines were closed. The two resulting lines, those carrying the *wMelPop*CLA-1 and *wMelPop*CLA-2 (*wMelPop* cell line adapted) strains, were then monitored periodically by PCR to confirm the infection status. The selection regime was again repeated at G<sub>14</sub> due to fluctuations in infection frequencies in both lines.

**Life span assays.** The life spans of *wMelPop*CLA-1-, *wMelPop*CLA-2-, and *wMelPop*-infected lines were compared to those of tetracycline-cured derivatives of each line created by the addition of tetracycline (0.3 mg/ml) to the adult diet according to standard methods (15). Treated flies were reared on tetracycline for two generations and then transferred to a normal diet for a minimum of five generations before being used in experiments. To reduce the effects of genetic drift that may have occurred in these lines during tetracycline treatment, 100 females from each fly line (including infected lines) were backcrossed with 100 males from the same *w*<sup>1118</sup>.T stock line and the progeny were combined to form the next generation. This procedure was repeated for five generations (G<sub>23</sub> to G<sub>28</sub>). Longevity assays were then conducted at G<sub>31</sub>, G<sub>33</sub>, and G<sub>35</sub>. To control for any crowding effects or size variability, the larval density in each stock bottle used to obtain flies was standardized (200 larvae/bottle) prior to longevity assays.

Stock bottles were kept at 24°C until adult eclosion 9 to 10 days later, when flies were sexed as virgins and separated. In each assay, six sets of 20 flies for each sex were maintained at 29°C in standard cornmeal food vials without additional live yeast. Each day, the number of new deaths was recorded. Flies were moved into fresh food vials every 5 days. Survival curves for the various treatment groups were compared using a mixed-effects Cox proportional hazard model of survival analysis with the kinship package of the R suite of statistical software ([www.r-project.org](http://www.r-project.org)).

**CI tests.** CI tests were conducted at G<sub>36</sub> and G<sub>38</sub> posttransinfection by using the previously backcrossed lines. To standardize rearing conditions for CI tests, larvae in fly stock bottles were grown under low-density conditions (150 to 200 larvae/bottle) at 24°C with a 12-h light-dark cycle. To obtain offspring for CI crosses, stock bottles were seeded with a set density of 200 eggs per 40 ml of food. After eclosion, flies were sexed, separated as virgins, and aged until the CI tests. Male flies were collected on day 2 of emergence and were used within 24 h of eclosion (33, 46). The female flies used were 5 to 7 days old. For each cross, single mating pairs ( $n = 40$ ) were introduced into plastic bottles with molasses plate lids. Pairs were given 24 h to mate, and then the males were removed and the females were allowed to lay eggs. Eggs were collected onto molasses agar plates dotted with a live yeast suspension every 24 h for 3 days. Females that laid <50 eggs total across the three plates were discarded from the experiment. The plates were then placed at 24°C for a further 36 to 48 h, and then the numbers of total and unhatched eggs were determined. The statistical significance of hatch rates for various crosses was determined using a Mann-Whitney U test. A Bonferroni correction was used to compensate for multiple comparisons.

**qPCR and density determination.** To examine if the density of *Wolbachia* bacteria in *D. melanogaster* had changed after long-term serial passage in mosquito cell lines, infection densities in head tissues of *w*<sup>1118</sup> flies carrying the *w*MelPopCLA-1, *w*MelPopCLA-2, or *w*MelPop strain were monitored over the life spans of the flies by quantitative PCR (qPCR). Heads were selected for qPCR as *w*MelPop infection densities had previously been shown to increase rapidly in nervous tissue with adult age (22, 23). The density of bacteria of the closely related nonvirulent *Wolbachia* strain *w*Mel was also examined after introgression for three generations from *yw*<sup>67c23</sup> into the *w*<sup>1118</sup> genetic background. qPCR assays were conducted at G<sub>46</sub> posttransinfection. Flies reared as described for life span assays were collected at 4-day intervals (from days 4 to 32 postemergence) until all the flies in a line were dead, and tissue samples were stored at -80°C before analysis. Total DNA was extracted from dissected head tissues by using the DNeasy tissue kit protocol (Qiagen). To estimate the relative abundance of *Wolbachia* bacteria in each sample, we compared the abundance of the single-copy *Wolbachia* ankyrin repeat gene WD\_0550 to that of the single-copy *D. melanogaster* gene *Act88F*. The forward primer 5'-CAGGAGTTGCTGTGGGTATATTAGC and the reverse primer 5'-TGCAGTAATGCAGTAGCGTAA were used to amplify a 74-bp amplicon from WD\_0550, and the forward primer 5'-ATCGAGCACGGCATCATCAC and the reverse primer 5'-CACGCGCAGCTCGTTGTA were used to amplify a 78-bp amplicon from *Act88F*. For each treatment, 12 biological replicates per time point were examined. For each sample, qPCR amplification of DNA was performed in triplicate using a Rotor-Gene 6000 system (Corbett Research, Australia). Amplification was carried out in a 10- $\mu$ l reaction volume which included 5  $\mu$ l of Platinum Sybr green I supermix (Invitrogen, CA), 1  $\mu$ M forward and reverse primers, and 1 ng of the DNA template. The PCR conditions were 50°C for 2 min; 95°C for 2 min; 40 cycles of 95°C for 5 s, 60°C for 5 s, and 72°C for 10 s; and a melting curve from 67 to 95°C. A standard calibrator was used to normalize between qPCR runs, and the specificity of PCR products was determined by melting-curve analysis. Crossing threshold ( $C_T$ ) and amplification efficiency values for each sample were calculated using Corbett Rotor-Gene (version 1.7.75) software. The relative abundance of *Wolbachia* bacteria in each sample was then determined using the method discussed by Pfaffl (28). Regression analysis was used to detect trends in the density of *Wolbachia* bacteria over the lifetimes of flies of individual lines. An analysis of covariance was then employed to examine the relationship between density and the covariates of fly age and bacterial strain. All abundance data were log transformed prior to analysis. A Bonferroni correction was used to compensate for multiple comparisons.

## RESULTS

Several initial attempts to establish *w*MelPop in the *A. albopictus* embryonic cell line Aa23 were unsuccessful. Typically, infection was lost after several passages or lines were discontinued due to a complete loss of confluence or growth of

mosquito cells. This situation mirrors that observed when *w*MelPop purified from *Drosophila* is injected into mosquitoes, with large fluctuations in infection density eventually leading to the loss of infection (E. A. McGraw and S. L. O'Neill, unpublished data). In total, only 2 (3%) of 68 independent attempts to establish the *w*MelPop infection in Aa23 cells were successful.

Once established in Aa23, *w*MelPop was serially passed for 237 passages (~2.5 years) before being transferred into the *A. aegypti* cell line RML-12 and the *A. gambiae* cell line MOS-55. The stable establishment of *w*MelPop in these two cell lines occurred much more easily than the initial infection of Aa23, with two of two independent attempts for each cell line yielding stable *w*MelPop infections. Partial sequences of the *Wolbachia* 16S rRNA and *wsp* genes from the three cell lines used were all identical to the sequence from *w*MelPop, confirming that infections were not the result of contamination with other strains. Infection in mosquito cells was also confirmed using transmission electron microscopy (TEM). TEM micrographs of the three infected mosquito cell lines show that representative cells from each line were heavily infected with *w*MelPop (Fig. 1).

*w*MelPop was purified from the *A. aegypti* RML-12 cell line and reintroduced into its native host, *D. melanogaster w*<sup>1118</sup>, which had been previously cured of its natural *w*MelPop infection by tetracycline treatment. At the time of reintroduction, *w*MelPop had been maintained for over 3 years outside its native host, through 237 passages in Aa23 cells and 60 passages in RML-12 cells. In total, 446 embryos were microinjected, giving rise to 108 G<sub>0</sub> larvae (24% hatch rate). All 10 surviving G<sub>0</sub> females were PCR positive for *Wolbachia*. Of these, eight produced offspring and two produced PCR-positive G<sub>1</sub> isofemale lines. The *Wolbachia* strains in these two independent isofemale lines were named *w*MelPopCLA-1 and *w*MelPopCLA-2.

The infection frequencies in *w*MelPopCLA-infected lines were then monitored periodically over time (Fig. 2). Both *w*MelPopCLA strains were initially observed to display variable maternal transmission rates in the original *Drosophila* host, reflected in fluctuating infection frequencies in the absence of experimental selection. During an initial period of experimental selection for increased infection (G<sub>1</sub> to G<sub>3</sub> posttransinfection), infection frequencies as detected by PCR were observed to increase in both the *w*MelPopCLA-1-infected line (58 to 87%) and the *w*MelPopCLA-2-infected line (55 to 100%). In the absence of experimental selection from G<sub>4</sub> onwards, infection frequencies in both lines initially were stable or fluctuated but then rapidly decreased such that by G<sub>14</sub> posttransinfection only 32% of individuals carrying *w*MelPopCLA-1 and 24% of those carrying *w*MelPopCLA-2 remained infected. Selection was repeated again at G<sub>14</sub>, and after one additional generation, infection frequencies in both lines increased to 100% and remained fixed until G<sub>46</sub>, when last assayed.

To assess the effect of continuous cell line culture on the ability of the *Wolbachia* strain *w*MelPop to colonize *Drosophila*, we compared infection densities in flies that contained *w*MelPopCLA strains with those in flies carrying the original *w*MelPop strain by qPCR. Since it is known that *w*MelPop densities increase rapidly in adult flies held at 29°C, we as-

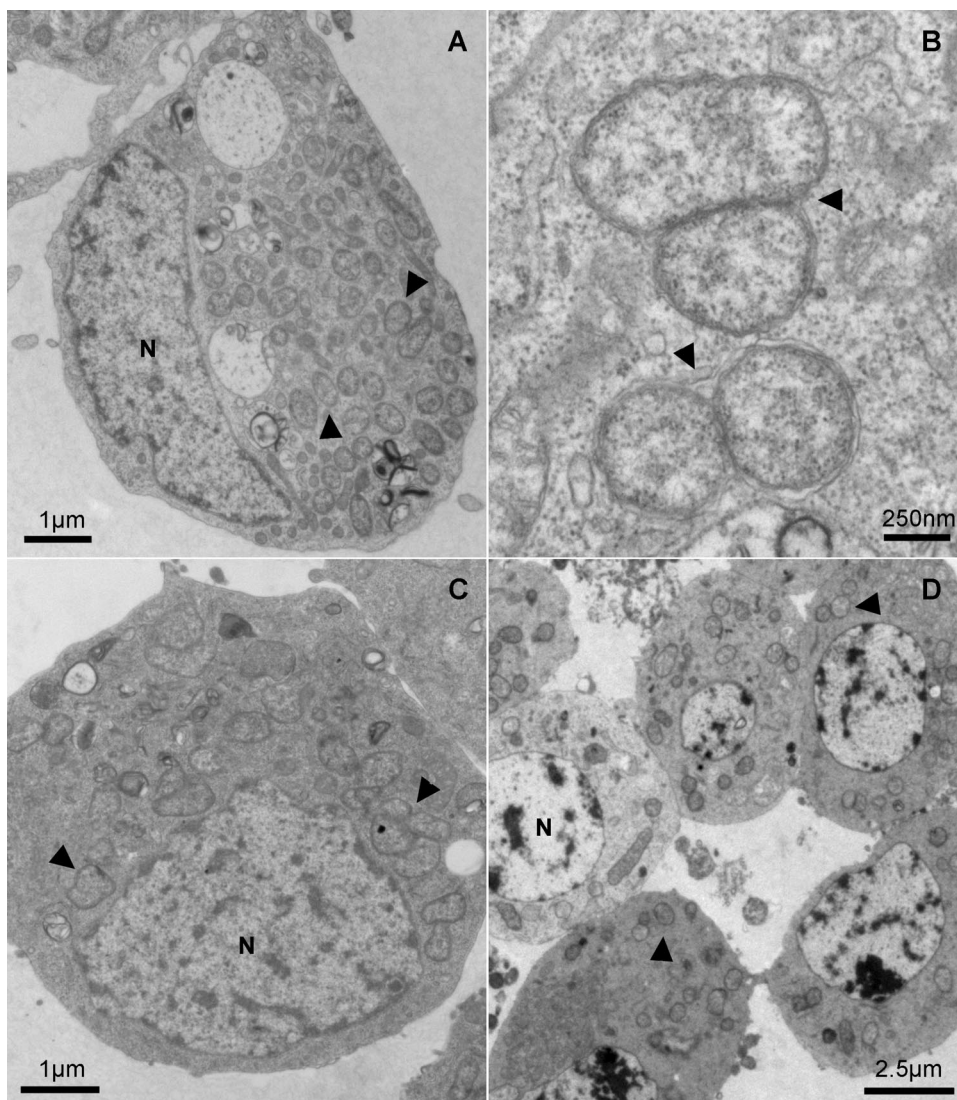


FIG. 1. Electron microscopy analysis of *wMelPop* in mosquito cell lines. (A) Low-magnification transmission electron micrograph showing a large number of *Wolbachia* bacteria (examples are marked with arrowheads) dispersed throughout the cytoplasm of an *A. aegypti* RML-12 cell. N, nucleus. (B) High-magnification micrograph of four *Wolbachia* cells presumably undergoing the process of cell division in RML-12 cells (arrowheads). (C) Low-magnification micrograph showing the presence of several *Wolbachia* bacteria in the cytoplasm of an *A. albopictus* Aa23 cell. (D) Cluster of *A. gambiae* MOS-55 cells each infected with multiple *Wolbachia* bacteria.

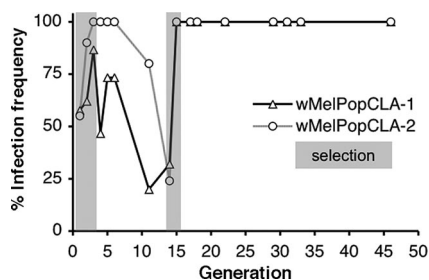


FIG. 2. Frequencies of *Wolbachia* infection in *D. melanogaster* *wMelPopCLA-1*- and *wMelPopCLA-2*-infected lines posttransfection ( $G_0$ ). Gray shaded regions represent periods of experimental selection for infection.

essed *Wolbachia* densities across the adult fly life span. As populations of flies aged, *Wolbachia* densities in head tissues of *wMelPop*-infected flies rapidly increased (Fig. 3). The densities of *Wolbachia* bacteria in *wMelPopCLA-1*- and *wMelPopCLA-2*-infected flies also increased as the flies aged, although these increases were noticeably less than those of the *wMelPop* densities. *Wolbachia* densities in *wMelPop*-infected flies were roughly fourfold higher than those in *wMelPopCLA-1*- or *wMelPopCLA-2*-infected flies at day 12 postemergence. Flies infected with the non-life-shortening *wMel* strain had the lowest levels of infection, which increased only slightly over the life spans of the flies. Overall, there were significant effects of fly age ( $F_{1,275} = 41.92$ ;  $P < 0.001$ ) and bacterial strain ( $F_{3,275} = 678.37$ ;  $P < 0.001$ ) on the *Wolbachia* density for all fly lines. This outcome was reflected by significant differences in the

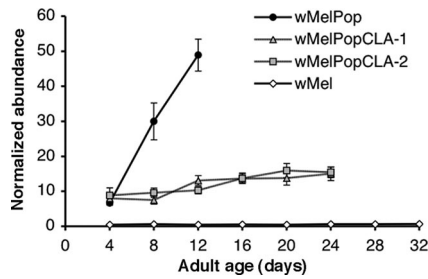


FIG. 3. Mean relative *Wolbachia* densities in fly heads ( $\pm$ SE;  $n = 12$  per datum point) as determined by real-time qPCR for four lines of infected flies collected at various ages over their life spans and maintained at 29°C. Fly samples were collected at 4-day intervals until the flies were dead.

effects of strain and age after pairwise comparisons between lines ( $P < 0.001$  for all comparisons), except for the *wMelPop* CLA-1- and *wMelPop*CLA-2-infected lines, for which strain effects were not significantly different from each other ( $F_{1,144} = 0.09$ ;  $P > 0.05$ ).

To test whether the ability of *wMelPop* to induce life-shortening infection had changed during long-term serial passage, we conducted a series of longevity assays at  $G_{31}$ ,  $G_{33}$ , and  $G_{35}$  posttransfection. For these experiments, the survival rates of infected flies from the *wMelPop*CLA-1-, *wMelPop*CLA-2-, and *wMelPop*-carrying lines were compared with those of the corresponding uninfected tetracycline-treated derivatives maintained at 29°C. Survival curves for males and females of each treatment group were measured independently. In all assays, male and female flies from the *wMelPop*-infected line demonstrated more pronounced life span reductions than flies from the *wMelPop*CLA lines relative to the life spans of the tetracycline-treated controls (Fig. 4). The life spans of *wMelPop* CLA-1- and *wMelPop*CLA-2-infected lines appeared to be intermediate relative to those of the *wMelPop*-infected line but were shortened relative to those of the tetracycline-treated controls. For example, at  $G_{31}$  posttransfection, the mean time to death ( $\pm$  standard error [SE]) for *wMelPop*-infected females ( $9.8 \pm 0.1$  days) was noticeably shorter than that for *wMelPop*CLA-1-infected females ( $22.2 \pm 0.3$  days) or *wMelPop*CLA-2-infected females ( $23.4 \pm 0.3$  days). The mean time to death was increased for tetracycline-treated control lines, with the life spans of tetracycline-treated females derived from

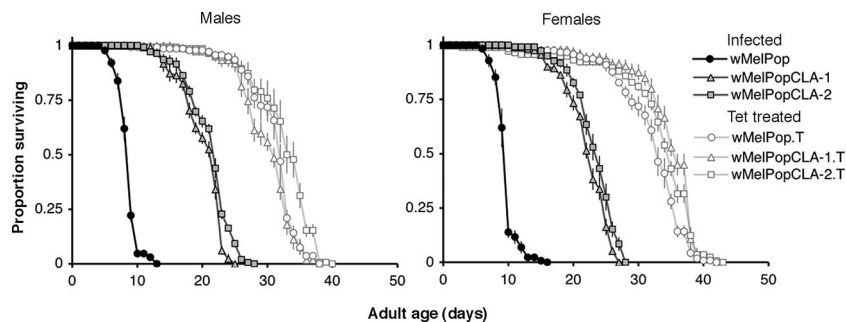


FIG. 4. Survival curves of populations of male and female flies from *wMelPop*- and *wMelPop*CLA-infected lines at  $G_{31}$  posttransfection. Shaded symbols represent infected flies, and unshaded symbols represent uninfected tetracycline (Tet)-treated counterparts. Error bars on curves represent SEs. Adult flies were maintained at 29°C.

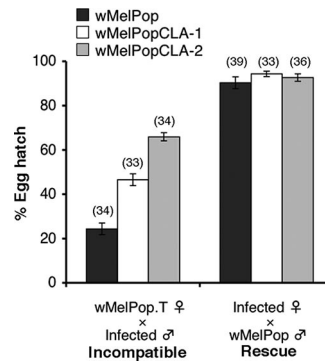


FIG. 5. Abilities of *wMelPop* and *wMelPop*CLA strains to induce and abolish CI. Shown are mean percentages of hatching eggs ( $\pm$ SE) for *wMelPop*.T females mated with infected males (incompatible cross) and infected females mated with *wMelPop*-infected males (rescue cross). Values in parentheses above error bars represent the numbers of replicate crosses.

the *wMelPop*-infected line (*wMelPop*.T females;  $32.1 \pm 0.5$  days), *wMelPop*CLA-1.T females ( $34.6 \pm 0.5$  days), and *wMelPop*CLA-2.T females ( $33.4 \pm 0.6$  days) all being longer than the life spans of infected counterparts. For females, the proportional hazard of death associated with carrying infection was significantly greater for individuals with *wMelPop* (relative risk ratio, 135.7; 95% confidence interval, 40.3 to 456.5) than for those carrying either *wMelPop*CLA-1 (relative risk ratio, 30.0; 95% confidence interval, 15.4 to 58.5) or *wMelPop*CLA-2 (relative risk ratio, 17.7; 95% confidence interval, 10.5 to 30.7) ( $P < 0.001$  for all comparisons to *wMelPop*-infected flies). The same trends were also observed for males. These results were consistent with those obtained from measurements at  $G_{33}$  and  $G_{35}$  posttransfection (data not shown).

In order to examine the effects of long-term cell culture on CI expression, we established test crosses between uninfected and infected flies and examined hatch rates of the resulting eggs. Results from incompatible test crosses indicated that *wMelPop*.T females mated with *wMelPop* males produced embryos with a mean hatch rate of 24%, which was significantly lower than that for embryos from the corresponding cross with *wMelPop*CLA-1-infected males or *wMelPop*CLA-2-infected males ( $P < 0.001$ ; Mann-Whitney) (Fig. 5). A statistically significant difference in the mean hatch rate for crosses with

wMelPopCLA-1-infected males and those with wMelPopCLA-2-infected males ( $P < 0.001$ ) was also observed. In rescue tests, mean hatch rates of embryos produced from crosses between wMelPop-infected males and wMelPop-infected females, wMelPopCLA-1-infected females, or wMelPopCLA-2-infected females were not significantly different from one another. Thus, wMelPopCLA strains had reduced abilities to induce CI compared to that of wMelPop. In contrast, the abilities of the cell-adapted strains to rescue an incompatible cross appeared to be unchanged.

## DISCUSSION

The use of an in vitro cell culture system provided an ideal means to examine the adaptation of *Wolbachia* to a novel host cell environment. This approach contrasts with the direct transfer of *Wolbachia* between insects, a method in which selective forces are presumably different and more complex and in which longer insect generation times, vertical transmission, and the labor-intensive nature of rearing live insects make selection for transinfected lines challenging.

The initial difficulty in establishing a wMelPop infection in the *A. albopictus* cell line Aa23 demonstrated that wMelPop was not naturally preadapted for growth in mosquito cells. Following the stable infection of Aa23 and serial passage for several years, wMelPop was successfully established in the RML-12 and MOS-55 cell lines from *A. aegypti* and *A. gambiae*, respectively, two species that are not naturally infected with *Wolbachia* (10, 18, 32, 34, 39). The transfer of wMelPop between Aa23 and these two mosquito cell lines occurred much more readily than the initial transfer from *D. melanogaster* to Aa23, potentially due to (i) the use of a higher infective dose of wMelPop purified from Aa23 for transfer than of that from *Drosophila* and/or (ii) a smaller divergence in intracellular environments among these mosquito cell lines than between those of *Drosophila* and Aa23. Our ability to establish stable wMelPop infections in MOS-55 was also consistent with previous reports that *Wolbachia* can be established in *A. gambiae* cell lines (31).

As observed by TEM, infection densities in the three cell lines, particularly in RML-12, closely resembled those previously described for somatic and nervous tissue in *D. melanogaster*, with individual mosquito cells heavily infected with bacteria (23). Whether wMelPop in Aa23, RML-12, and MOS-55 was exhibiting tropism for cell types similar to or different from *D. melanogaster* is unknown. The morphology of cells within Aa23 (derived from embryos) (27) and RML-12 and MOS-55 (derived from larvae) (C. E. Yunker, personal communication) (20) appeared to be heterogeneous, and the tissue-specific origin of cell lineages within these lines has not been identified.

When wMelPop was reintroduced into *Drosophila* after long-term serial passage in mosquito cell lines, initial establishment in wMelPopCLA-1- and wMelPopCLA-2-infected lines was problematic due to unstable fluctuations in infection frequency in the absence of selection. Unstable maternal transmission and variable infection frequencies are often commonly observed when *Wolbachia* is moved between distantly related hosts (17, 35, 40). After two rounds of selection for infection, populations progressed to fixation for infection and have remained stable (~2 years).

Longevity assays revealed that wMelPopCLA strains had become partially attenuated in virulence compared to the original wMelPop strain in *D. melanogaster*. This phenotypic shift in life-shortening ability may be related to the decreased replication rates of both wMelPopCLA strains in the head tissues of infected lines relative to that of wMelPop. The life-shortening phenotype of wMelPop is thought to result from pathology induced by the uncontrolled replication of bacteria in muscle and nervous tissue (22, 23). Therefore, it is likely that the decreased replication rates of the *Wolbachia* wMelPopCLA strains were directly correlated with decreased abilities to induce life shortening in the original host.

In addition to reductions in the abilities of cell line-adapted *Wolbachia* strains to induce life shortening, similar reductions in the abilities of these strains to induce CI were noted. Presumably, this phenotypic shift was also linked to the reduced replication rate of the cell line-adapted *Wolbachia* in its original host. In several insects, decreased *Wolbachia* densities in developing sperm cells have been correlated with decreased levels of CI expression (4, 5, 7, 8, 30, 41). It is also possible that the tropism of *Wolbachia* for different host cell types may have been altered during long-term passage in cell lines since CI induction was clearly distorted but CI rescue was not. This idea suggests that the densities of infecting bacteria in some tissues may be changed more dramatically than those in others.

In summary, the wMelPop strain was initially difficult to transfer into cell lines, but a small number of infected lines could eventually be established. The strain was subsequently much more easily transferred into cell lines derived from other mosquito species. The cell line-adapted *Wolbachia* displayed reduced infectivity and maternal transmission rates when injected back into its original host. It grew to lower densities and showed phenotypic shifts for both life shortening and CI expression. Taken together, our results provide evidence for the active genetic adaptation of wMelPop to mosquito cell lines during long-term serial passage.

Given that there is growing interest in the potential to use *Wolbachia* strains in an applied context (37, 45, 47), the preadaptation of strains to particular host intracellular environments may facilitate the subsequent transfer of these symbionts into hosts that are difficult to transinfect. Such an application may involve the use of life-shortening wMelPop adapted to mosquito cell lines as a source of material for experiments with the transinfection of *A. aegypti* and *Anopheles* mosquitoes, as part of an applied strategy to alter the mosquito population age structure to reduce the transmission of pathogens such as dengue virus and *Plasmodium* parasites to humans (9, 38). Furthermore, given the availability of genome sequence information for this bacterium (43), this system may allow for the underlying genetic mechanisms responsible for adaptation to novel hosts to be identified.

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