

Passage of *Wolbachia pipientis* through Mutant *Drosophila melanogaster* Induces Phenotypic and Genomic Changes

Irene L. G. Newton, Kathy B. Sheehan

Department of Biology, Indiana University, Bloomington, Indiana, USA

Wolbachia pipientis is a nearly ubiquitous, maternally transmitted bacterium that infects the germ line of insect hosts. Estimates are that *Wolbachia* infects 40 to 60% of insect species on the planet, making it one of the most prevalent infections on Earth. However, we know surprisingly little about the molecular mechanisms used by *Wolbachia* to infect its hosts. We passaged *Wolbachia* through normally restrictive *Drosophila melanogaster* hosts, bottlenecking *Wolbachia* through stochastic segregation while simultaneously selecting for mutants that could recolonize these previously restrictive hosts. Here, we show that *Wolbachia* alters its behavior when passaged through heterozygous mutant flies. After only three generations, *Wolbachia* was able to colonize the previously restrictive hosts at control titers. Additionally, the *Wolbachia* organisms passaged through heterozygous mutant *D. melanogaster* alter their pattern of tissue-specific Wsp protein production, suggesting a behavioral response to the host genotype. Using whole-genome resequencing, we identified the mutations accumulated by these lineages of *Wolbachia* and confirmed the existence and persistence of the mutations through clone library Sanger sequencing. Our results suggest that *Wolbachia* can quickly adapt to new host contexts, with genomic mutants arising after only two generations.

Wolbachia pipientis is an obligate intracellular member of *Alphaproteobacteria* that forms symbioses with an extremely broad array of hosts (1). *Wolbachia* infections are considered a “pandemic” in insects, and estimates suggest that upwards of 40% of insect species are infected by the bacterial parasite (2, 3). *Wolbachia* is well known for reproductive effects induced in the host, which range from the exotic (male killing) to the most common of reproductive effects: cytoplasmic incompatibility (CI) (1). In CI, infected females can breed with either infected or uninfected males, but uninfected females cannot breed with infected males. This results in a drop in fecundity for uninfected females, allowing *Wolbachia* to spread effectively through new insect populations via maternal transmission (1, 4).

Because *Wolbachia* is obligately intracellular and not genetically tractable, the research community has turned to genomic sequencing to identify potential mechanisms behind host interaction. Even closely related *Wolbachia* strains (based on multilocus [MLST] profiles) exhibit significant genomic diversity (5). That is, between related hosts, *Wolbachia* strains can differ substantially in terms of both genomic content and divergence of orthologous genes (6). In an attempt to identify the loci involved in the induction of different reproductive phenotypes, *Wolbachia* strains that infect different hosts (7) or cause different reproductive phenotypes (8) have been compared, and the loci have been correlated with those phenotypes. In order to pinpoint the mechanism behind pathogen blocking, *Wolbachia* variants that are more or less pathogenic or protect the host against viruses to a lesser or greater extent have been sequenced (9). Additionally, even individual *Wolbachia* strains within individual hosts may not exist as single, clonal populations (10). The significance of this interhost diversity is still unclear, although different *Wolbachia* genotypes might be important during various aspects of host infection. For example, low-titer *Wolbachia* variants within individual hosts may become prominent in the context of colonization of new hosts (11), suggesting that *Wolbachia pipientis* may exist as a diverse quasi-species within a single host. All of these studies have identified interesting loci for future work and have revealed much about the

basic evolutionary processes for *Wolbachia*. However, due to the lack of a genetic system in *Wolbachia*, researchers have been unable to directly confirm the hypothesis that specific loci are responsible for the phenotypes.

Although *Wolbachia pipientis* is not yet genetically tractable, the bacteria infect the model organism *Drosophila melanogaster*, which is amenable to genetic manipulation. *Wolbachia* relies on an ability to target the germ line and replicate within it in order to persist between generations. *Wolbachia* has no free-living counterpart, as far as we know, and therefore the ecology of this bacterium is the host environment. In this study, we knocked down *Wolbachia*’s titer in *Drosophila melanogaster* by introducing *Wolbachia* into a host background that substantially reduces bacterial titer. We therefore both increased the stochastic segregation of *Wolbachia* and imposed a selective pressure on these *Wolbachia* sp. strains to adapt to a new ecological context in order to persist in the flies. Below, we characterize the passaged *Wolbachia* populations through molecular assays and show that after only 3 generations, *Wolbachia* has phenotypically adapted to a new, mutant host context.

MATERIALS AND METHODS

***Drosophila* stocks, crosses, and phenotypic assays.** Standard methods were used for all crosses and culturing. The following stocks were obtained

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Address correspondence to Irene L. G. Newton, irnewton@indiana.edu.

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from the Bloomington *Drosophila* Stock Center (BDSC) at Indiana University (<http://flystocks.bio.indiana.edu/>): stock number 145, which carries *w*¹, was used as the *Wolbachia*-infected control line. Stock number 25211, an OreR-derived strain, was used as an uninfected control. One *chickadee* mutant stock was used in this study. The *chic*²²¹ *cn*¹/CyO; *ry*⁵⁰⁶ flies carry a null allele resulting from the deletion of 5' noncoding and some *chic*-coding sequences (12). *Wolbachia* infection status for stocks acquired from the BDSC was determined via PCR and Western blotting targeting the gene *wsp* or its product (see below). *Wolbachia* bacteria were introduced into the heterozygous mutant backgrounds through crosses between *w*¹ females (stock 145) and heterozygous males (mutant/CyO). *Wolbachia* was passaged in this heterozygous background for three generations, and our *Wolbachia*-infected lines were genotyped before and after introgression into the mutant/CyO background (see "Bioinformatic analysis of *Wolbachia* resequenced genomes" below). To clear flies of *Wolbachia* infection, they were raised for two generations on conventional fly media containing 0.25 mg/ml tetracycline (as described in reference 13). After treatment, flies were reared in bottles previously occupied by uninfected male *Drosophila melanogaster* flies in order to repopulate the microbiome. All flies were examined for *Wolbachia* infection and age matched in order to avoid confounding correlations between fly age and *Wolbachia* titer. We performed single-pair fly crosses in order to count the number of progeny produced. Parental flies were allowed to lay eggs in vials for 3 days and then were transferred 3 times to new vials. Progeny were counted 12 to 16 days posteclosion.

Western blots. Western blots were used to characterize expression of the *Wolbachia* surface protein in flies. Flies were ground in 1.5-ml centrifuge tubes using an electric hand drill and disposable pestle in lysis buffer: 150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl (pH 8) containing Halt protease inhibitor cocktail (Thermo Scientific), and 5 mM EDTA. The lysates were centrifuged for 1 min at 8,000 × *g* to pellet debris. Samples were heated for 5 min at 95°C in Laemmli sample buffer containing 5% β-mercaptoethanol (Bio-Rad) prior to SDS-PAGE electrophoresis. Proteins were separated on 4 to 20% Tris-glycine NB precast gels (NuSep) in 1× Tris-glycine-SDS running buffer (Bio-Rad) and transferred to a polyvinylidene difluoride (PVDF) membrane in Tris-glycine transfer buffer with 15% methanol at 40 V on ice for 3 to 4 h. The membrane was blocked for 5 min in Starting Block T20 (TBS) blocking buffer (Thermo Scientific), followed by incubation in primary antibody (for 1 h at room temperature [RT] or overnight [O/N] at 4°C) according to standard protocols. SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) was used according to the manufacturer's instructions to detect horseradish peroxidase (HRP) (after incubation with secondary antibodies) on the immunoblots. Blots were reprobated after stripping in 100 mM glycine–0.1% NP-40–1% SDS (pH 2) for 1 h at RT and then O/N at 4°C. A PageRuler prestained protein ladder (Thermo Scientific) was used as a molecular mass marker. The following antibody was obtained through BEI Resources, NIAID, NIH: monoclonal anti-*Wolbachia* surface protein (WSP), NR-31029; it was used at a dilution of 1:1,000. Additional antibodies were anti-actin monoclonal antibody (clone C4) at 1:10,000 (Seven Hills Bioreagents) and secondary antibodies HRP enzyme conjugates (Invitrogen) at 1:5,000.

DNA extractions and PCRs. Quantitative PCR was used to calculate the relative titers of *Wolbachia* in our passaged stocks and in the ancestral line. DNA was extracted from flies by utilizing the Qiagen DNeasy blood and tissue kit (Qiagen) according to directions. DNAs were quantified using absorbance at 260 nm with an Epoch spectrophotometer (Biotek). Quantitative PCR was performed to detect the *Wolbachia* titer (with reference to the host) using an Applied Biosystems StepOne real-time PCR system and SybrGreen chemistry (Applied Biosystems). We used *wsp* primers for *Wolbachia* (forward, CATTGGTGTGGTGTGGTG; reverse, ACCGAAATAACGAGCTCCAG) and Rpl32 primers for the host (forward, CCGCTTCAAGGGACAGTATC; reverse, CAATCTCCTTGC GCTCTTG) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Reactions were performed in a 96-well plate and

TABLE 1 Comparison of titers of *Wolbachia* bacteria found in F₁ *chic*²²¹/+ *Drosophila* female backgrounds and in comparable control (wild-type) flies^a

<i>Drosophila</i> background	Relative quantity of gene:		Pfaffl ratio compared to control	Relative ratio of expression
	<i>wsp</i>	<i>rpl32</i>		
Control (stock 145)	13.98	15.57		4.37
F ₁ <i>chic</i> ²²¹ /+	18.98	19.61	0.33	1.55

^a Relative quantity of the *Wolbachia* specific gene (*wsp*) compared to the host gene reference (*rpl32*) across five individual mutant flies and five individual control flies (stock 145, from which the infection within *chic*²²¹/+ was derived). The relative ratio of *wsp* expression is statistically significantly reduced in the heterozygous mutant background (*t* = 15.586, *df* = 6, *P* < 0.001).

calibration standards used in every run to calculate primer efficiencies. These efficiencies, along with the threshold cycle (*C*_T) values generated by the machine, were used to calculate the relative amounts of *Wolbachia* using the ΔΔ*C*_T (Livak) and Pfaffl (14) methods.

Wolbachia enrichment and processing for Illumina library generation. A simple *Wolbachia* enrichment protocol was used. First, 20 dissected ovaries were homogenized in GNE buffer (50 mM glycerophosphate, 10 mM NaF, 1.5 mM EGTA; pH 7.6 [with HCl]) with added dithiothreitol (DTT) (final concentration, 2 mM) using a glass dounce homogenizer on ice. These homogenates were filtered (passage through a 10-μm filter) and centrifuged at 16,000 × *g* for 20 min at 4°C. Samples were preserved in RNAlater solution and frozen at –80°C before DNA extraction. Genomic DNA was extracted using a Qiagen DNeasy kit, and the genetic material from each lineage was used for library generation with the Nextera DNA sample preparation kits and pooled for multiplex sequencing on a MiSeq sequencer (paired-end [PE], 300-bp reads).

Bioinformatic analysis of *Wolbachia* resequenced genomes. We created a total of 10 libraries for this project: 1 for each of the passaged lines (in the mutant/CyO or the OreR background for 3 generations) and 2 for the original, ancestral line (GenBank SRA accession number SRP049613). Between 1 million and 2 million reads were achieved for each sample (see Table S1 in the supplemental material), and roughly 5% of the reads mapped to the *Wolbachia* wMel genome, resulting in between 20× and 60× coverage for each line. We utilized the bioinformatics pipeline Breseq (15) to identify polymorphisms and predict mutations in each resequenced genome. This pipeline is based on bowtie mapping to a reference sequence, for which we used the sequenced wMel genome (GenBank accession number NC_002978.6). Importantly, we required that indels be supported by reads in both directions, that each position be covered by 20 reads, that each single nucleotide polymorphism (SNP) investigated appear at >8% frequency in the population, and that these SNPs not appear in our ancestral sequence. Genetic mutations identified in this fashion were validated using Sanger sequencing. Primers were designed to flank two SNPs in particular (FicF, CTCGTACCACTGCGCAA; FicR, GCACT TTATTGAAATTTGACCTAT; WD0633F, CTGTATCCTGACATACAG GAT; WD0633R, AATATTAGACTCAATATTGCG), and the resulting amplicons were cloned into pCR-TOPOII blunt cloning vectors. Twenty-four clones from each amplicon pool were sequenced, and SNPs were manually verified by examining ab1 sequence files using 4 Peaks software.

RESULTS

Heterozygous profilin flies harbor low-titer *Wolbachia* infections. *Wolbachia pipientis* is reduced in titer when colonizing *Drosophila melanogaster* harboring single copies of mutant profilin (Table 1). We crossed infected, wild-type flies (stock number 145) to *chic*²²¹/CyO males and collected F₁ female progeny of the *chic*²²¹/+ genetic background. Compared to control flies of the

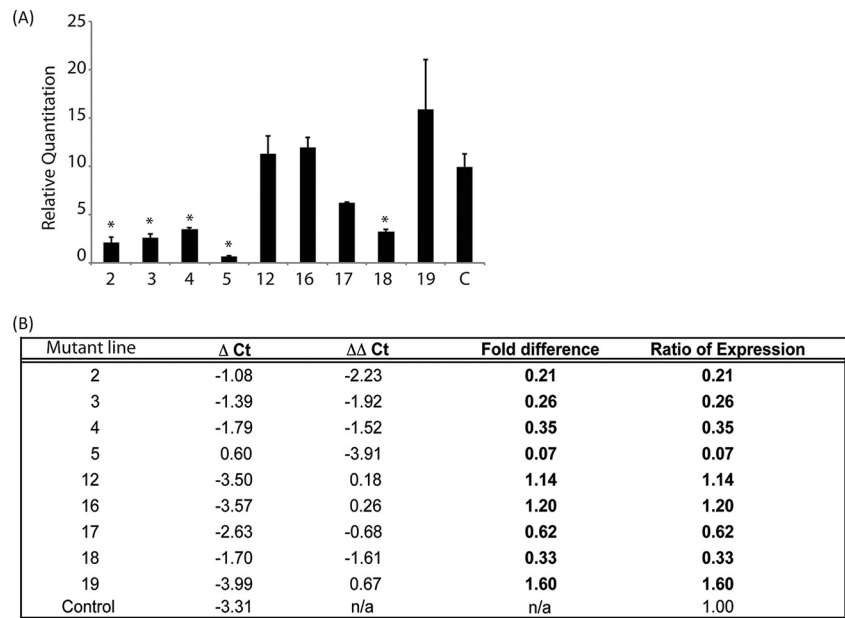


FIG 1 *Wolbachia* bacteria passed within *chic*²²¹/+ backgrounds are altered in titer compared to their progenitor. (A) Relative quantity of the *Wolbachia* specific gene (*wsp*) compared to the host gene reference (*rpl32*) across mutant lines and the control line (stock 145, labeled “C”). Statistically significant deviations from control are shown by an asterisk (*). (B) Quantitative PCR statistics based on relative quantitation of *wsp* compared to *rpl32* calculated for each of the mutant lines and the control flies. Flies were age matched and reared simultaneously.

same age, these heterozygous *chic*²²¹/+ flies harbor one-third of the *Wolbachia* titer (Table 1).

Sequential passage of *Wolbachia* within profilin mutant (*chic*²²¹/+) and control backgrounds. We wanted to determine if *chic*²²¹/+ hosts would induce phenotypic changes in *Wolbachia* populations. We therefore established 20 single fly crosses between uninfected *chic*²²¹/*Cyo* (stock 4892) male flies and infected *w*¹ females (stock 145). As a control for the effect of bottlenecking *Wolbachia* in single fly lines, we also established 20 single fly crosses between uninfected wild-type males (stock 25211) and infected *w*¹ females (stock 145). We allowed these single-pair matings to produce F₁ progeny, after which females were dissected and their ovaries preserved for subsequent DNA extraction. We then selected a single, straight-winged female from each of the mutant lines (*chic*²²¹/+) and mated her with the parental uninfected *chic*²²¹/*Cyo* male flies. For our control lines, we selected a single female and mated her with the uninfected control male stock (25211). We continued the passage of *Wolbachia* through these host backgrounds (either *chic*²²¹/+ or control) for three generations. Each of the mutant and control lines was tested for a *Wolbachia* infection. At that point, we had a total of 9 surviving mutant lines infected with *Wolbachia*, and we kept 9 control lines.

Passage of *Wolbachia* through mutant host backgrounds results in increased titer after only three generations. Pathogenic variants of *Wolbachia* tend to be of high titer compared to their less pathogenic relatives (16, 17). We therefore wondered if our passaged *Wolbachia* would colonize *Drosophila* at higher titer than expected. To characterize the titer of the *Wolbachia* strains in each of the passaged lines, we utilized quantitative PCR (qPCR). Flies were reared simultaneously and age matched in order to remove the confounding effects of host age on *Wolbachia* titer. The relative quantity of the *Wolbachia*-specific gene *wsp* was used to quantify the bacteria within each fly line (relative to the host gene

rpl32). We found that many of the passaged lines within the profilin heterozygous mutant background contained significantly fewer *wsp* copies than did the control flies; specifically, lines 2, 3, 4, 5, and 18 displayed less than one-half of the quantity of *Wolbachia* organisms expected based on the control ancestral line (Fig. 1). Because these *Drosophila* flies are carrying the *chic*²²¹ mutation and we have already shown that this mutation results in a decrease in *Wolbachia* titer, this finding was not surprising. However, we did identify four lines for which the quantity of *wsp* was not statistically significantly different from the control lines (lines 19, 17, 16, 12). In these four lines, the *Wolbachia* titer resembles that of the original, maternal line, although the flies carry the *chic*²²¹ mutation (Fig. 1). This result suggests that *Wolbachia* strains within the lines have adapted to a new ecological context and are now able to colonize the mutant host at the same titer as the wild type.

Passage of *Wolbachia* through mutant hosts induces changes in Wsp protein production. For heterozygous mutant flies, we sought to characterize *Wolbachia* Wsp protein production. *Wolbachia* surface protein (Wsp) is an antigen found on the surface of *Wolbachia* and shed into the blood of vertebrate hosts infected by *Wolbachia*-carrying filarial nematodes (18, 19). Although its function in the bacterium is currently unknown, antibodies to the protein have been successfully used to monitor bacterial titer (20). We initially used anti-Wsp to identify titer differences in our passaged lines. However, in passaged lines we detected an alteration in tissue-specific production of Wsp (Fig. 2). In control females harboring *w*Mel infections, production of Wsp is limited to nonovary tissues (i.e., carcasses postdissection). Although *Wolbachia* is known to heavily colonize the ovary in *Drosophila* (21–24), the bacteria do not heavily produce Wsp in that same tissue (Fig. 2A). The Wsp antibody, generated from nematode *Wolbachia*, may not be sensitive enough to detect very

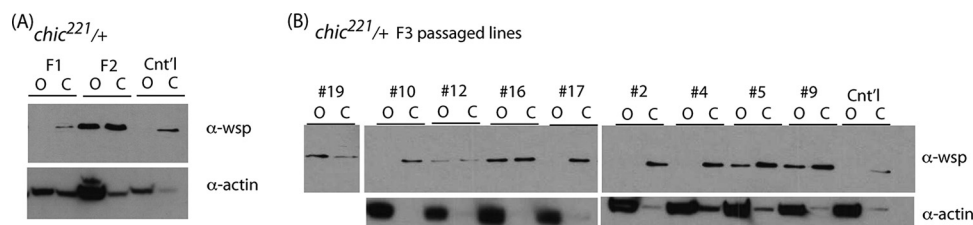


FIG 2 *Wolbachia* passaged within *chic*^{221/+} backgrounds displays altered expression of Wsp in the ovary. (A) Immunoblots of *Drosophila melanogaster* ovaries (O) and carcasses (C) from control and *chic*^{221/+} backgrounds, probed using an antibody against Wsp. (B) After passage for three generations through single fly crosses, some *Wolbachia* bacteria retain the expression of Wsp in the ovary while some revert to the control condition (expression detectable only in the carcass).

low quantities of protein expressed in this tissue. This tissue-specific protein production pattern also holds for F₁ progeny resulting from crosses between males harboring single mutations in the actin binding proteins villin and profilin and control females (Fig. 2A). However, after passage of *Wolbachia* through heterozygous profilin mutant (*chic*^{221/+}) hosts for two fly generations, *Wolbachia* strains that are able to colonize these hosts, regardless of titer, now alter the pattern of protein production and heavily express Wsp in the ovary (Fig. 2B).

***Wolbachia* lineages passaged through the *chic*^{221/+} host background are more pathogenic than their progenitor.** *Wolbachia* strains that are overly pathogenic can have effects on host life span (25) or host health (26). As a first step to identify the pathogenic effects of passaged *Wolbachia*, we examined the total number of progeny produced by each of the *Wolbachia*-infected *Drosophila* lines, comparing the lines in which *Wolbachia* was passaged through the mutant *chic*^{221/+} hosts to those passaged through control flies. The number of progeny produced by the mutant fly lineages harboring passaged *Wolbachia* was bimodal in distribution: three of the mutant lines produced very few progeny (mean number of progeny, 38), while seven of the mutant lines produced many progeny (mean, 177) and were statistically similar to the control lineages (mean, 168). However, averaged overall, the number of progeny produced by the passaged lines was not significantly less than the number produced by the control lines ($t = -1.407$, $df = 19$, $P = 0.176$).

This result suggests that the passaged *Wolbachia* strains from the mutant host backgrounds might induce a more severe reproductive phenotype. However, effects observed after passage may be due to either host genetic background (the effect of genetic drift combined with deleterious homozygous alleles) or the *Wolbachia* infection. In order to distinguish between these two possibilities, we selected the line with the most severe phenotype (mutant 5) and cleared it of a *Wolbachia* infection using tetracycline (for 2 generations). We then reexamined the number of progeny that result from the previously incompatible crosses using 20 single fly matings between passaged males (from the mutant 5 line) and uninfected females (25211) and compared the number to that obtained with the analogous cross using tetracycline-cleared mutant 5 males. We saw a fecundity defect to the passaged *Wolbachia* infection. Mutant 5 males cleared of the *Wolbachia* infection produced many more progeny than mutant 5 males carrying the passaged *Wolbachia* infection (80% more; $t = 3.064$, $df = 38$, $P = 0.004$; see Table S2 in the supplemental material). This difference in the number of progeny was primarily due to the smaller number of female offspring produced by mutant 5 males carrying the *Wolbachia* infection, whereas the number of male offspring was

not significantly different (for females, $t = 3.104$, $df = 38$, $P = 0.004$; for males, $t = 1.541$, $df = 38$, $P = 0.132$). This result suggests a genetic interaction between the *chic* null allele and *Wolbachia* that is currently being investigated. Therefore, the fecundity defects observed in the mutant 5 line were not a result of host loci alone but were influenced by the passaged *Wolbachia* infection.

Identification of the genomic loci in the passaged *Wolbachia* lines. To identify *Wolbachia* genomic differences that might exist in our passaged lines, we selected four profilin mutant lines (low-titer line 5 and high-titer lines 12, 19, and 16), four control lines (resulting from crosses between infected control females and uninfected wild-type OreR stocks), and two parental stocks (stock 145, the source of the *Wolbachia* infection) for Illumina library preparation and genomic resequencing (see Table S1 in the supplemental material). We chose these particular passaged lines because they displayed production of the Wsp protein in the host ovary (lines 5, 12, 19, and 16), induced fecundity defects in the host (line 5), and/or carried low-titer or control titer *Wolbachia* (low titer = 5; high titer = 12, 16, 19).

Using strict thresholds, we were able to identify our lab strain (the ancestral *Wolbachia* strain used in our crosses) as a wMel type, not wMel CS (using markers found in reference 15). However, our lab strain contained a number of fixed or nearly fixed differences compared to the wMel sequence deposited in GenBank (9 in total) (Fig. 3). The majority of the differences (8/9) were indels of either a single nucleotide or a stretch of 9 bp (Fig. 3). Some of the changes are expected to impact gene function (causing frameshift mutations), and some occur within homopolymeric regions in the 5' end of the genes in question (4/9), suggesting that the changes may be important for gene regulation (e.g., phase variation).

We next focused on the mutations that occurred in *Wolbachia* within the profilin heterozygous mutant lines. The mutant lines contained the same 9 fixed differences found in the ancestral sequence, but in addition, each line was found to contain unique polymorphisms. These polymorphisms were covered by at least 20 reads and present at greater than 8% of the population of reads. Importantly, none of the new polymorphisms identified in each mutant line were shared between lines; that is, each line contained a unique set of polymorphisms. Some of the identified mutations were in intergenic regions (between *fabK* and WD1171 for mutant 5; between WD0756 and WD0757 for mutant 19), and although the mutations could potentially alter as-yet-unknown regulatory RNAs, they probably do not directly affect the encoded amino acids. Other identified mutations occurred within homopolymeric tracts (such as *efp*, WD0350 for mutant 19, WD0483 in mutant 16, and WD0625 in mutant 12) and could be important

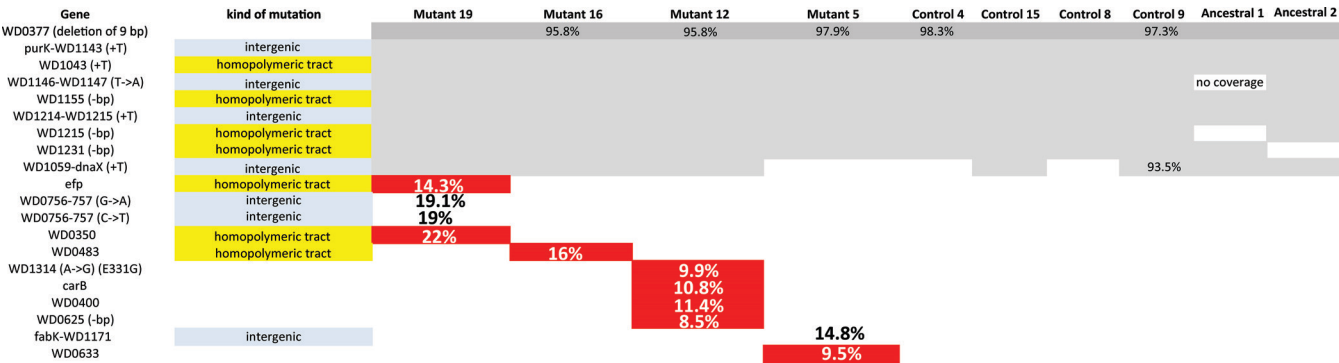


FIG 3 Mutations in passed *Wolbachia* within *chic*²²¹/+ background or control flies. Whole-genome sequencing using an Illumina MiSeq sequencer was used to identify individual mutations in each of 4 lines from *chic*²²¹/+ passed *Wolbachia* and compared to 4 control passed *Wolbachia* as well as the ancestral stock (sequenced twice). The identified loci affected by mutation are listed, and the presence of a mutation within each lineage is depicted. If the change is predicted to alter the amino acid encoded at that position, the mutation is colored in red. If the mutation was found in <100% of the mapped reads, the percentage is shown.

for gene regulation (e.g., phase variation). None of these mutations are in loci predicted to be essential. Four mutations occurred within coding regions and resulted in amino acid changes for mutant 12 and mutant 5. In mutant 12, we found changes in an ABC transporter (WD0400), the carbamoyl phosphate synthetase involved in production of arginine and pyrimidines (*carB*), and a filamentation induced by cyclic AMP (cAMP) protein (WD1314). In mutant 5, we found a single change in an ankyrin repeat containing prophage LambdaW1 protein: WD0633. We sought to confirm these nonsynonymous SNPs through PCR amplification of both WD1314 and WD0633 coupled to clone library and Sanger sequencing. We sequenced 24 clones from each of the amplified pools and recovered 1 or 2 clones with the SNP in question, supporting our observation that the *Wolbachia* populations do contain mutant alleles and that the observed changes were not Illumina sequencing errors.

DISCUSSION

Because *Wolbachia* bacteria are obligately intracellular, their ecology is their host. Here we manipulate *Wolbachia*'s ecology by introducing the bacteria into a host background that substantially reduces titer. The rationale is that this background imposes significant selective pressure on the *Wolbachia* to adapt to a new ecological context in order to persist in the fly lines and also that this severe bottleneck will increase the stochastic segregation of *Wolbachia* each generation. We characterized the passed *Wolbachia* organisms in four important ways: (i) through examination of titer using qPCR, (ii) through crosses to identify potential pathogenic effects of the passed *Wolbachia* such as effects on fecundity, (iii) through quantification of Wsp protein production, and (iv) through genomic resequencing.

Wolbachia bacteria that are passed through restrictive hosts alter their titer compared to their progenitor. In the majority of our passed lines, we observed low-titer *Wolbachia* infections, as would be expected given the *Wolbachia* transmission defect induced by the *chic*²²¹ mutation. However, in four of the passed lines, we saw *Wolbachia* titers return to control values, suggesting that the bacteria are able to adapt to a new host context in only three generations. Others have selected for *Wolbachia* titer increases and decreases over short time scales (5 generations) and have also found the bacterial parasite to be particularly plastic with regard to this parameter (27). Also, *Wolbachia* lineages passed

through the *chic*²²¹ heterozygous mutant hosts exhibit other phenotypic differences compared to the ancestral strain. Although the function of Wsp is not yet known, it is curious that the passed *Wolbachia* alters the production of Wsp in the ovary, the reproductive tissue on which the bacteria depend for maternal transmission.

Wolbachia passed through *chic*²²¹ heterozygous mutant hosts also exhibited genomic changes. We found mutations at significant frequencies (>8%) in each of our mutant lines and confirmed the presence of two particular nonsynonymous SNPs for two of the mutant lines, showing that the Illumina resequencing correctly revealed the mutations and that they were not sequencing errors. We think it is premature to propose that these specific mutations are selected for in direct response to the host actin phenotype. However, it is curious that one of the passed lines (mutant 12), which regained its wild-type titer, exhibited a mutation in a protein known in other systems to modify eukaryotic actin (WD1314, annotated as Fic) (Fig. 3). In sum, our results show that *Wolbachia* is able to adapt to a new host context on a short time scale.

What does *Wolbachia* heterogeneity mean for the host?

Wolbachia genetic variability within single hosts has been noted before. For example, Schneider et al. identified diverse haplotypes of *Wolbachia* within several insect hosts (11). Interestingly, in that study, the authors were able to shift the prevalence of different haplotypes upon transfer and maintenance in new hosts for over 100 generations (11), suggesting that *Wolbachia* haplotypes can shift in frequency depending on the host context. Similar to our results here, many of these mutations were found to be frameshift mutations and *de novo* stops (11). However, in that study, no specific phenotypic effect was attributed to the *Wolbachia* micro-heterogeneity. In our work, we were able to reveal mutations in *Wolbachia* in a very short number of generations (only 3). The mutations that we identified appear to be between 8.5 and 22% penetrant; the *Wolbachia* strains within these hosts are a pool of genetic haplotypes and do not exist as clonal isolates. We did not identify any of the SNPs in the mutant lines in the ancestral stock. However, it is possible that we lack the resolution necessary to identify rare mutants in the ancestral line. We cannot be certain if the SNPs are derived from an existing pool of genetic heterogeneity or are *de novo* mutations.

Here, we establish a straightforward method to generate populations of mutants in *Wolbachia*, an obligate intracellular parasite for which there is no genetic system. By passaging *Wolbachia* lineages through a host background that severely reduces titer, we enrich for or generate *Wolbachia* mutants. Utilizing this approach, which is analogous to forward genetics in other systems, it may be possible to learn about the biology of *Wolbachia* infection in a targeted and mechanistic manner.

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