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Wolbachia Influences the Maternal Transmission of the gypsy Endogenous Retrovirus in Drosophila melanogaster

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ABSTRACT The endosymbiotic bacteria of the genus Wolbachia are present in most insects and are maternally transmitted through the germline. Moreover, these intracellular bacteria exert antiviral activity against insect RNA viruses, as in Drosophila melanogaster, which could explain the prevalence of Wolbachia bacteria in natural populations. Wolbachia is maternally transmitted in D. melanogaster through a mechanism that involves distribution at the posterior pole of mature oocytes and then incorporation into the pole cells of the embryos. In parallel, maternal transmission of several endogenous retroviruses is well documented in D. melanogaster. Notably, gypsy retrovirus is expressed in permissive follicle cells and transferred to the oocyte and then to the offspring by integrating into their genomes. Here, we show that the presence of Wolbachia wMel reduces the rate of gypsy insertion into the ovo gene. However, the presence of Wolbachia does not modify the expression levels of gypsy RNA and envelope glycoprotein from either permissive or restrictive ovaries. Moreover, Wolbachia affects the pattern of distribution of the retroviral particles and the gypsy envelope protein in permissive follicle cells. Altogether, our results enlarge the knowledge of the antiviral activity of Wolbachia to include reducing the maternal transmission of endogenous retroviruses in D. melanogaster.

IMPORTANT Animals have established complex relationships with bacteria and viruses that spread horizontally among individuals or are vertically transmitted, i.e., from parents to offspring. It is well established that members of the genus Wolbachia, maternally inherited symbiotic bacteria present mainly in arthropods, reduce the replication of several RNA viruses transmitted horizontally. Here, we demonstrate for the first time that Wolbachia diminishes the maternal transmission of gypsy, an endogenous retrovirus in Drosophila melanogaster. We hypothesize that gypsy cannot efficiently integrate into the germ cells of offspring during embryonic development in the presence of Wolbachia because both are competitors for localization to the posterior pole of the egg. More generally, it would be of interest to analyze the influence of Wolbachia on vertically transmitted exogenous viruses, such as some arboviruses.

P{}rokaryotic organisms are present in many eukaryotic species and can establish symbiotic relationships with their hosts that can range from detrimental to beneficial. In recent years, studies have shown that insect-endosymbiotic bacteria replicate within eukaryotic cells and are maternally transmitted. Among them, the genus Wolbachia is present in all insect orders, and its within-species propagation is optimized due to a biased efficient transmission through infected female oocytes. Interestingly, it has been shown that Wolbachia confers protection against several RNA viruses in insects (1–3), including arboviruses present in transmission vectors such as Aedes mosquitoes, and so gives the opportunity to improve arbovirus control in natural populations of vectors (4). The cellular and evolutionary characteristics of Wolbachia and its variants (wMel, wMelCS, and wMelPop) in Drosophila species are well documented, thanks to the powerful genetic model Drosophila melanogaster (5, 6). One important point concerns the host mechanisms hijacked by Wolbachia for its maternal transmission: Wolbachia localizes at the posterior pole of mature oocytes through an active mechanism that relies mostly on microtubules and pole plasm (7, 8). This polarized concentration ensures that Wolbachia is incorporated into the pole cells of the embryos, in order to be maternally transmitted. Wolbachia bacteria from Drosophila melanogaster (wMel) also show a strong tropism for the somatic stem-cell niche (SSCN) and are therefore present in the somatic follicle cells covering the germline at the early stage of oogenesis (9). Interestingly, horizontal transmission of Wolbachia can also occur within and between Drosophila species, and the results of experimental infections of D. melanogaster by microinjection of Wolbachia-infected hemolymph demonstrate the capacity of Wolbachia to enter the SSCN and, later, the follicle cells surrounding the germline (10). Follicle cell-to-oocyte transcytosis is not restricted to bacteria and cellular proteins; it has been shown that several Drosophila endogenous retroviruses (ERVs), including the gypsy retroelement, are maternally trans-
mitted to the next generation. *gypsy* is an active endogenous retrovirus present in several strains of *Drosophila melanogaster*. Its 7.5-kb genome contains three open reading frames similar to the *gag*, *pol*, and *env* genes present in vertebrate retroviruses. The *gypsy* RNAs and proteins are mainly expressed in the ovaries of permissive females at stages 8 to 10 (11–13). Females are permissive if they are defective for the production of specific P-element-induced wimpy testis (Piwi)-interacting RNAs (piRNAs) that are able to target *gypsy* RNAs (14–16). It was shown that these piRNAs are encoded by the X-linked *flamenco* locus, which has two classes of alleles, *flamP* (permissive) and *flamR* (restrictive) (15, 17). The integration of *gypsy* occurs only into the germline of *flamP/flamP* females lacking *gypsy* piRNAs. *gypsy*, like other ERVs, is expressed in the follicular cells of permissive females and integrates into the nuclei of the offspring, suggesting that there is a transfer from follicle cells to oocytes (11, 13, 18, 19). Moreover, it has been demonstrated that the trafficking of the endogenous retrovirus ZAM relies on the transport of vitellogenin (20). There is experimental evidence to indicate that the *gypsy* endogenous retrovirus is also horizontally transmitted and then integrates into the chromosomes of the offspring by virtue of a strong tropism to the germline (18). Our results indicate that the frequency of *gypsy* insertion-induced *ovo* mutants is decreased in the presence of *wMel*, suggesting a new role for this endosymbiont in the control of endogenous retroviruses.

**RESULTS**

The *wMel* variant is present in the *gypsy*-rich *Drosophila melanogaster* strain N271. We investigated Wolbachia’s distribution pattern in N271 permissive ovaries using fluorescence *in situ* hybridization. *Wolbachia* was observed in the germarium and mainly in the posterior pole of the stage 10 oocyte, as previously described (Fig. 1A and B) (7, 9, 10). Ultrastructural electron microscopy (EM) analysis of permissive ovarian late egg chambers enabled us to identify at the posterior pole of the oocyte several *wMel* cells showing the typical morphology of *Wolbachia*, i.e., a

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**FIG 1** Characterization of Wolbachia in the N271 strain. (A) Schematic representation of *Drosophila melanogaster* ovaries, stages of oogenesis (germarium [g] and stages S2 to S14), and enlarged view of S10 egg chamber. (B) Detection of Wolbachia by *in situ* hybridization. Blue, DAPI; red, rhodamine-labeled probe against *Wolbachia* 16S DNA. Scale bars = 50 μm. Top, early-stage egg chambers infected with *Wolbachia*; bottom, stage 10 egg chamber. (C) Observation by electron microscopy of *Wolbachia* (black arrows) in the cytoplasm of a follicle cell (FC) (left, scale bar = 1 μm) and at the posterior pole of the oocyte (Oo) surrounded by follicle cells (right, scale bar = 0.5 μm). (D) Relative mtDNA COI cycle threshold values (Ct) after normalization with *rp49*. NS, no statistically significant difference; *wMel*−, tetracycline-treated flies; *wMel*+, untreated flies. Horizontal bars represent medians.
cline treatment, using primers specific for the cytochrome-
relative mtDNA threshold cycle (CT) of the cytochrome subunit I (COI) gene. Our results indicate that the average (qPCR) on medium after treatment (21). We performed quantitative PCR (qPCR) on mtDNA density in flies raised for two generations on standard shown that tetracycline increased the mitochondrial DNA density.

The three-layered envelope surrounding a matrix of moderate density (Fig. 1C, right). Altogether, our data suggest that the N271 females contain a consistent level of Wolbachia variant wMel bacteria in follicle cells and in the oocytes of late-stage egg chambers. In order to compare genetically identical females with or without Wolbachia (denoted as wMel+ and wMel- females, respectively), we treated N271 individuals with tetracycline as previously described (3). wMel was undetectable either by PCR (see Fig. S1 in the supplemental material) or by in situ hybridization in flies treated with tetracycline to be wMel- (Fig. 1B). Moreover, we estimated the levels of mitochondria in wMel+ (untreated) and wMel- permissive females, as it was shown that tetracycline increased the mitochondrial DNA (mtDNA) density in flies raised for two generations on standard medium after treatment (21). We performed quantitative PCR (qPCR) on wMel+ and wMel- permissive females after tetracycline treatment, using primers specific for the cytochrome-c oxidase subunit I (COI) gene. Our results indicate that the average relative mtDNA threshold cycle (Ct) values are not different for wMel+ and wMel- flies (Fig. 1D).

The gypsy insertion rate decreases in the presence of Wolbachia. The fact that Wolbachia and gypsy are both vertically transmitted prompted us to test whether their mechanisms of transmission may interfere with each other. To address this question, we measured the rate of integration of gypsy into the genomes of offspring from permissive females in the presence or absence of wMel. The ovo gene is a hot spot for gypsy insertion (22) and can be used as a readout for gypsy transposition (11, 13, 14, 22). To estimate the rate of gypsy integration into ovo, permissive females were crossed with X-linked mutant ovoD1 males. As the ovoD1 allele is dominant, the ovoD1/ovo+ daughters are sterile, because ovarian development does not occur (23). However, several daughters with one functional ovary were observed due to gypsy insertion into the ovoD1 gene occurring after colonization of the gonads by germ cells (Fig. 2A). Hence, the percentage of daughters with restored fertility was positively related to the gypsy insertion rate. The percentages of ovo reversions were estimated in daughters from crosses between (i) wMel+ permissive females or (ii) wMel- permissive females with ovoD1 males (Table 1). The results indicated that the percentage of ovoD1 reversion was significantly higher in wMel+ than in wMel- females (P < 0.01) (Table 1). In order to check that tetracycline-sensitive commensal bacteria were not involved in this phenomenon, we restored the gut microbiota in wMel- permissive females (see Fig. S1 in the supplemental material) and concluded that the gut microbiota had no effect on the percentage of ovoD1 reversion. Indeed, the percentage of ovoD1 reversion was significantly lower in the progeny of wMel+ permissive females than in the progeny of wMel- permissive females and wMel- permissive females with restored microbiota (P < 0.05) (Table 1). There was no statistical difference between the percentage of ovoD1 reversion in the progeny of wMel- permissive

**FIG 2** Ovaries of the progeny of an ovoD1 reversion test and detection of gypsy insertion into ovo by PCR. (A) Ovaries from a sterile ovo+/ovo+ female, a revertant ovo/ovo+ female, and a wild-type ovo+/ovo+ female shown by phase-contrast microscopy (×50 magnification). (B) PCR detection of gypsy insertion into ovo in the two parental lines and samples of revertant F1 females. The schematic depicts the primers used (P1, P2, P3, and P4) and their localization in ovo and gypsy. Large arrows show gypsy long terminal repeats (LTR).
females with or without microbiota \((P > 0.05)\) (Table 1). As negative controls, we performed similar crosses using restrictive females in which the presence of fertile daughters might be due to mitotic crossovers generating \(ovo^+/ovo^+\) cells, as previously shown (17). In this case, the presence of bacteria had no influence on the very low percentage of \(ovo^\text{D}1\) reversion estimations \((P > 0.05)\) (Table 1). In order to determine whether \(ovo^\text{D}1\) reversion events resulted from \(gypsy\) insertions (denoted as \(ovo\)’ alleles), we performed PCR on DNA samples of a pool of revertant ovaries using primers specific for \(gypsy\) and \(ovo\), respectively, in the two \(gypsy\) orientations according to the method of Dej et al. (22). Indeed, we obtained several PCR products in the \(wMel^-\) and \(wMel^+\) revertant ovaries, meaning that multiple independent \(gypsy\) integrations were responsible for the reversion. No positive signal for \(gypsy\) insertion into \(ovo\) was observed in the two parental lines (Fig. 2B). In conclusion, we demonstrated that the presence of \(Wolbachia\) diminishes the rate of integration of \(gypsy\), which indicates for the first time cross talk between an endosymbiont and an ERV in \(Drosophila\).

**Wolbachia does not modify gypsy expression levels.** To assess whether the differential \(gypsy\) insertion rate is due to modulation of its expression induced by \(Wolbachia\), we first performed quantitative reverse transcription PCR \((qRT-PCR)\) to compare \(gypsy\) RNA levels (11) between \(wMel^+\) and \(wMel^-\) \(flamenco\) permissive and restrictive ovaries. We found that the relative \(gypsy\) RNA levels did not significantly differ between \(wMel^+\) and \(wMel^-\) permissive ovaries (Wilcoxon test, \(P = 0.439\)) (Fig. 3A), suggesting that \(Wolbachia\) does not interfere with the RNA transcription machinery. The \(gypsy\) RNA level was very low in restrictive ovaries, as expected (11). The presence of \(Wolbachia\) did not significantly modify the \(gypsy\) RNA level (Wilcoxon test, \(P = 0.093\)) (Fig. 3A), which suggests that \(Wolbachia\) does not interfere with the repression mechanism induced by \(flamenco\). The \(gypsy\) Env protein levels were also monitored by Western blotting in permissive and

![Diagram](image)

**FIG 3** Quantitative RT-PCR analysis and Western blot analysis of \(gypsy\) expression. (A) Relative \(C_p\) values for \(gypsy\) levels after normalization with \(rp49\); NS, no statistically significant difference. Thin horizontal bars represent medians. (B) Expression levels of \(gypsy\) envelope protein in permissive and restrictive ovaries with the presence or absence of \(wMel\) were analyzed using Western blotting. \(\alpha\)-Tubulin protein was used as a loading control. The 50-kDa band revealed by the Env antibody corresponds to the full-length envelope glycoprotein. Actual band sizes are indicated at the left.
restrictive ovaries. The presence of Wolbachia did not modify the Env protein level independently of the flamenco genotype, as shown by the results in Fig. 3B. Altogether, our results indicate that Wolbachia did not affect gypsy expression levels.

The wMel variant modifies the gypsy envelope distribution pattern. Whole-mount permissive ovaries with the presence (wMel+) or absence (wMel−) of Wolbachia were immunostained using an antibody raised against the gypsy Env protein (Fig. 4). gypsy envelope glycoprotein (Env) is mainly detected in the follicle cells of late egg chambers (stage 8 to 10) (11). In the permissive wMel+ follicle cells surrounding the posterior pole of the oocyte, gypsy Env displayed stick-shaped signals, as well as showing dot-shaped signals polarized at the apical pole (Fig. 4, bottom left). The wMel− permissive ovaries exhibited a different pattern: round gypsy Env staining was observed in the cytoplasm in a non-polarized manner (Fig. 4, bottom right). Interestingly, we did not observe any difference between wMel+ and wMel− restrictive egg chambers, i.e., gypsy Env was nearly absent in follicle cells whatever the Wolbachia status, meaning that neither tetracycline treatment nor the absence of wMel affected the flamenco restriction (Fig. 4).

Wolbachia affects the distribution of intracytoplasmic gypsy virus-like particles. As Wolbachia and gypsy are both maternally transmitted, we hypothesized that Wolbachia could interfere with gypsy within the oocyte and/or the follicle cells. To test this hypothesis, we investigated the gypsy distribution pattern in the presence or absence of wMel. We identified intracytoplasmic particles of about 50 nm that were present in follicle cells of permissive flies but absent in restrictive follicle cells (Fig. 5). We confirmed that these particles corresponded to gypsy by immunoelectron microscopy (immuno-EM) using an antibody raised against gypsy Env. We observed gold beads localized near the virus-like particles present along the plasma membrane (Fig. 5A). This observation fully agreed with the description of gypsy particles obtained previously by Lecher et al. (12) and strongly suggested that the particles were gypsy virus-like particles. Then, the distribution patterns of these particles were compared between wMel+ and wMel− permissive follicle cells. A major difference concerned the distribution of particles at the boundaries between two follicle cells: particles were scattered regularly at both sides of the junction between wMel− follicle cells (Fig. 5C), whereas they clustered asymmetrically, i.e., they were present in one cell and absent in its neighbor cell, at the junction between wMel+ follicle cells (Fig. 5D). We also noticed that the cell junctions close to the particles were tightly sealed along a straight line in wMel− follicle cells, which was never observed in wMel+ follicle cells (Fig. 5C). Moreover, we several times observed groups of particles in the cytoplasm of follicle cells, but these groups were systematically surrounded with a double membrane in wMel− females (Fig. 5E) but not in wMel+ females (Fig. 5F). Altogether, these observations indicate that the presence of wMel modifies the distribution of gypsy virus-like particles in follicle cells.

FIG 4  Immunostaining against gypsy envelope protein in permissive wMel+ and wMel− follicle cells and their respective restrictive counterparts (scale bar = 10 μm). Blue, DAPI; red, gypsy envelope. Top, schematic representation of a stage 10 egg chamber; the apical pole is boxed and corresponds to the region observed in confocal microscopy. Oo, oocyte. gypsy Env patterns differ between permissive wMel+ and permissive wMel− follicle cells, whereas Env is not detected in restrictive egg chambers whatever the Wolbachia status.
DISCUSSION

In this study, we show an effect of *Wolbachia* in reducing the rate of *gypsy* insertion into the *ovo* gene of the offspring of *Drosophila melanogaster* females permissive for *gypsy* expression. While *Wolbachia* is known to affect exogenous RNA virus replication, we show for the first time that it could also affect endogenous retroviruses. *Wolbachia* was horizontally transferred to *D. melanogaster* and then vertically transmitted (25). Like *Wolbachia*, *gypsy* has probably entered the genome of *D. melanogaster* recently, after its divergence from its sibling species, *D. simulans* (26). Colonization of the oocyte via follicle cells is the strategy used by several ERVs (11, 13, 20, 24). Toomey et al. have proposed that *Wolbachia* is delivered to the oocyte directly from the stem cell niches or indirectly through the somatic follicle cells (9). Conversely, it has been

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**FIG 5** Distribution of *gypsy* virus-like particles in permissive fly stage 10 egg chambers in the presence or absence of *Wolbachia*. Images show immuno-EM labeling (10-nm gold particles [black dots]) (A, B) of the *gypsy* envelope protein in stage 10 egg chambers from wMel+ permissive females (A), which is absent from restrictive females (B), as expected, and electron microscopy observations of cytoplasmic 50-nm particles (black arrows) in permissive wMel+ (D, F) follicle cells (FC) (scale bars = 0.2 μm). Note the asymmetrical clustering of viral particles at the plasma membrane (PM) of two follicle cells in panel D compared to their distribution in panel C and the double membrane surrounding viral particles in panel E compared to panel F.
proposed that Spiroplasma poulsonii, a natural endosymbiont of D. melanogaster, interacts with the host yolk machinery to pass between follicle cells and enter the oocyte (27). The similarity between the Wolbachia and gypsy transmission pathways prompted us to ask whether Wolbachia could affect the dynamics of gypsy transfer from follicle cells to the oocyte. Péllisson et al. (11) and Lécher et al. (12) have previously described distributions of gypsy Env and gypsy virus-like particles similar to those we observed in this study for the wMel− ovaries. Our results indicate that the presence of Wolbachia in the oocyte and/or follicle cells modifies the gypsy Env pattern of distribution and localization of gypsy viral particles in follicle cells, which could ultimately reduce the maternal transmission of gypsy. Immunofluorescence images suggest that, in wMel− permissive egg chambers, gypsy Env is “stuck” near the junctions between follicle cells and at the apical domain of the follicle cells. EM images indicate that the presence of gypsy viral particles at the tightly sealed junctions between follicle cells vanishes when wMel is present. The mechanism by which Wolbachia alters the distribution of gypsy in follicle cells is still unknown, and it could be worthwhile to further investigate the interaction between Wolbachia, gypsy, and cellular proteins involved in septate junctions. Another possibility worth investigating is whether a reciprocal influence of gypsy on Wolbachia maternal transmission could occur. We obtained data that indicated that the levels of Wolbachia maternally deposited in 0- to 2-h embryos are higher when the embryos are laid by permissive females than when they are laid by restrictive females (see Fig. S2 in the supplemental material). This result, which needs to be investigated further, corroborates the presence of interplay between gypsy and Wolbachia during maternal transmission. Our hypothesis is that Wolbachia could modify gypsy localization at junctions between follicle cells and at the apical domain, as it has previously been shown that bacteria and viruses can interact with junctions between epithelial cells (28–30). We therefore propose a model which takes into account previously proposed gypsy and Wolbachia transmission models (8, 9, 13). Wolbachia and gypsy share the same strategy, which is to localize at the posterior end of the oocyte and be taken up into the pole cells of the embryos when they bud (Fig. 6). In this model, Wolbachia exerts a repressive effect on the maternal transmission of gypsy: it modifies gypsy assembly and slows down follicle cell-to-oocyte transfer because of its presence in follicle cells and at the posterior pole of the oocyte, i.e., where the transfer occurs.

It was shown that Wolbachia manipulates a host miRNA in Aedes aegypti that decreases the expression of AdDnnmt-2, a methytransferase gene that is upregulated by dengue virus (31). In contrast, the small interfering RNA pathway seems not to be involved in the antiviral activity of Wolbachia in Drosophila melanogaster (32). We show here that Wolbachia does not modify gypsy RNA and protein expression levels in flamenco permissive and restrictive ovaries, suggesting that Wolbachia does not interfere with gypsy RNA and envelope levels in permissive ovaries or with the Piwi-mediated repression of gypsy by flamenco acting in restrictive ovaries. While the precise mechanism has not been elucidated, Wolbachia confers to the host a protective effect against gypsy integration. The antiviral protective effect of Wolbachia has been demonstrated for several exogenous viruses (33), and our results enlarge the spectrum of action of Wolbachia to include activity against endogenous retroviruses. The potential long-term consequence of a reduction of the endogenous retrovirus integration rate would be to confer a selective advantage to Wolbachia, increasing its frequency in natural populations. Furthermore, Wolbachia makes gypsy less harmful to the host, which may also contribute to the maintenance of gypsy and other retroelements that use the same road to the germline in D. melanogaster. Finally, it is notable that transovarial transmission has been demonstrated for several arboviruses and parasites (34, 35), and it would be worthwhile to investigate the effect of Wolbachia on their rates of maternal transmission.

MATERIALS AND METHODS

Drosophila strains. Drosophila melanogaster strain N271 (14) was a gift from A. Péllisson (CNRS, France). It contains several active gypsy copies and a permissive flamenco allele (flam®). This strain segregates homozygous permissive (flam®/flam®) or restrictive (flam®/FM7) females for gypsy expression. The ovar® strain N376 has been previously described (23). This strain is maintained by crossing females with attached X chromosomes to ovar® males. All flies were reared on standard corn medium at 25°C.

In order to remove Wolbachia, the flies were grown during three generations on standard agarose medium containing 0.25 mg/ml tetracycline (Sigma-Aldrich). The strains were screened for the presence of Wolbachia using the Wolbachia 16S primers F (5’ TTGTAGCCTCCTGCTATGTTAT
AATCT 3') and R (5' GATAAGTCTAGTATGTTTACGTG 3'). We also used the Wolbachia wsp primers F (5'-TGGTCCATAAAGTGAAGAAAC 3') and R (5' AAAATTTAAGCCTACTCCA 3') for Wolbachia variant determination as described previously (6). rp49 gene amplification was used as a control for the PCR (36). Some Wolbachia-free flies were also maintained on standard medium that was inoculated with commensal bacteria (5). Bacterial universal 16S primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' GGTACCTTGTGACTGGCAT 3') were used as described previously (37) to detect bacteria. Treated flies were maintained on standard medium for at least three generations before experiments were performed. Concerning the ovo<sup>p</sup> test (see below), the crossing experiments were performed seven generations after treatment.

Fluorescence microscopy. Ovaries were processed and stained using standard immunofluorescence techniques with antibodies (7). All flies were 1.5 to 2 days old at the time of dissection. The antibodies used were rabbit polyclonal antibody against gypsy envelope (anti-ET78P antibody) (38) and Alexa Fluor 633 mouse anti-rabbit antibody (Invitrogen). Samples were then rinsed in phosphate-buffered saline (PBS) and mounted on a glass slide with the 4′,6-diamidino-2-phenylindole (DAPI) containing 10 ng of salmon sperm DNA, 1% poly(A), 250 mg·ml<sup>-1</sup> dextran sulfate; 250 mg·ml<sup>-1</sup> poly(A), 250 mg·ml<sup>-1</sup> salmon sperm DNA, 250 mg·ml<sup>-1</sup> RNA, 0.1 M dithiothreitol (DTT), 0.5% Denhardt's solution containing 10 ng of Wolbachia DNA probes W2 (5′ CTCTCTGTGAGTACCGTATTGCCGTTAAACAT 3′) and W03 (5′ TCTCTATCTCCCTTTCGAA 3′) that were 5′-end labeled with rhodamine (4). Samples were washed twice in 1× SSC–10 mM DTT at 55°C for 15 min. Samples were then rinsed in PBS and mounted on a glass slide with the DAPI-containing mounting medium Vectashield (Clinscience). A dozen egg chambers from three different immunostaining experiments were observed for each condition.

For in situ hybridization with Wolbachia DNA probes, ovaries were fixed for 20 min in 4% formaldehyde and heptane, postfixed for 10 min in 4% formaldehyde, and then washed once with PBS. Samples were incubated at 4°C for 4 h in 0.5 M NaCl plus 0.015 M sodium citrate, 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 200 mg·liter<sup>-1</sup> dextran sulfate; 250 mg·ml<sup>-1</sup> poly(A), 250 mg·ml<sup>-1</sup> salmon sperm DNA, 250 mg·ml<sup>-1</sup> RNA, 0.1 M dithiothreitol (DTT), 0.5% Denhardt's solution containing 10 mg of Wolbachia DNA probes W2 (5′ CTCTCTGTGAGTACCGTATTGCCGTTAAACAT 3′) and W03 (5′ TCTCTATCTCCCTTTCGAA 3′) that were 5′-end labeled with rhodamine (4). Samples were washed twice in 1× SSC–10 mM DTT and twice in 0.5× SSC–10 mM DTT at 55°C for 15 min. Samples were then rinsed in PBS and mounted on a glass slide with the DAPI-containing mounting medium Vectashield (Clinscience). All ovaries were analyzed with an SP5 confocal microscope (Leica).

Electron microscopy. Ovaries from 1.5- to 2-day-old flies were dissected in PBS and then fixed in 2% glutaraldehyde for 1 h at 4°C. Then, pieces were postfixed with 2% OsO<sub>4</sub> at 4°C. Ultrathin sections (approximately 70 nm thick) were cut on a Reichert ultracut E (Leica) ultramicrotome, mounted on 200-mesh copper grids, treated three times for 10 min in absolute ethanol. Impregnation was performed with equal parts of Epon A and Epon B plus DMP30 (1.7%). Ultrathin sections from three different immunostaining experiments were observed for each condition.

For quantitative real-time PCR, total RNA from dissected Drosophila ovaries was isolated using Nucleospin RNA (Macherey-Nagel) following the manufacturer's protocol. Then, 1 μg of total RNA was reverse transcribed using the Omniscript reverse transcription kit (Qiagen) with oligo(dt) primers (Invitrogen). Roche Universal SYBR green mix (Roche) and StepOnePlus (Applied biosystem) were used for quantitative RT-PCR to amplify a gypsy envelope fragment gene with gypsy primers F (5′ GGTACCTTGTGACTGGCAT 3′) and R (5′ TCTCTATCTCCCTTTCG 3′). The changes in cycle threshold (ΔC<sub>T</sub>) values were calculated within the log-linear phase of the amplification curve with StepOne Plus software, version 2.2.2 (Applied Biosystems). Quantification was normalized to that of the mRNA encoding the endogenous ribosomal protein Rp49, which was amplified using the rp49 primers F (5′ CGGATCGATA TGCTAAAGCTTG 3′) and R (5′ GGCGCCTTGTGACCTGTA 3′). Statistical analyses were performed in R (http://www.R-project.org).

Quantitative PCR. For mitochondrial DNA density quantification, total DNA was extracted from wMel<sup>−</sup> and wMel<sup>+</sup> permissive N271 ovaries. Quantitative PCRs were performed with 30 ng of total DNA as described previously (21), using the following primers specific for the Dro sophila melanogaster cytochrome-c oxidase subunit I (COI) gene: F (5′ GCTCTGTATAGCTTCCTGCCAG 3′) and R (5′ CATGACATTTGCCGATAA 3′). Three independent DNA extractions were performed for each condition, and quantitative PCR assays were done in triplicate.

Wolbachia quantitative PCR was performed as previously described (39). DNA from 30 0- to 2-h embryos from wMel<sup>−</sup> permissive or restrictive N271 females was extracted as described previously (21). Quantitative PCR was done with 60 ng of total embryonic DNA. Three independent DNA extractions were performed for each condition (permissive/restrictive), and quantitative PCR assays were done in triplicate.

Protein extraction and Western blot analysis. Ovaries from 25 flies were dissected in cold PBS and then squashed in 50 μl of lysis buffer (Thermo) with protease inhibitor (Roche). Protein extracts were mixed with 2× Laemmli buffer (Sigma-Aldrich) and loaded on a 12% acrylamide gel. The same quantity of each sample was loaded twice in the same gel. Protein was transferred to nitrocellulose membranes and used for Western blot analysis as described previously (19). The membrane was cut into two pieces containing exactly the same samples. One piece was incubated with the rabbit polyclonal anti-gypsy envelope antibody E78P (38) and the other with rabbit anti-α-tubulin antibody (Abi12546; Abcam), and both were revealed by a horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody (A6154; Sigma-Aldrich) and SuperSignal (Pierce), following the manufacturer’s instructions.

The ovo<sup>p</sup> reversion assay. The ovo<sup>p</sup> test is a genetic assay for gypsy transposition that has been described previously (36). Briefly, the X-linked ovo gene is involved in ovarian maturation, and the dominant-negative mutation ovo<sup>p</sup> results in sterile females with no functional ovaries. The ovo locus is a hot spot for gypsy insertion, and the insertion of gypsy into the ovo<sup>p</sup> allele (denoted as an ovo<sup>alle</sup> allele) of a heterozygous female prevents the production of the repressor Ovd1 protein. The ovo<sup>p</sup> females are then fertile, and most of them carry only one ovary because gypsy integration happens in a late stage of germline development. The ovaries of five revertants were pooled, and the DNA was extracted using the Nucleospin tissue XS kit (Macherey-Nagel). The presence of gypsy in ovo was checked by PCR as described previously (24), using primers P1 (5′ CAACATGACCAGGACCGTCATAAA 3′), P2 (5′ CTCCCGCTCTGGGCGCTCTTCTTCTT 3′), P3 (5′ CTTTGCGCAAAATATGCGA TG 3′), and P4 (5′ CGGGTTTTTACGGCGCACAAGG 3′) (Fig. 4).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01529-14/-/DCSupplemental.

Figure S1, EPS file, 0.1 MB.
Figure S2, EPS file, 0.1 MB.

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REFERENCES

1. Teixeira I, Ferreira Á, Ashburner M. 2008. The bacterial symbiont Wolbachia induces resistance to RNA viral infections in Drosophila melanogaster. PLoS Biol. 6:e2. http://dx.doi.org/10.1371/journal.pbio.0060002.

2. Hedges LM, Brownlie JC, O’Neill SL, Johnson KN. 2008. Wolbachia and virus protection in insects. Science 322:702. http://dx.doi.org/10.1126/science.1162418.

3. Shaw AE, Veronesi E, Maurin G, Ftaich N, Guigue F, Rixon F, Ratini M, Mertens P, Carpenter S, Palmarini M, Terzian C, Arnaud F. 2012. Drosophila melanogaster as a model organism for bluetongue virus replication in the nervous system. J. Virol. 86:9015–9024. http://dx.doi.org/10.1128/JVI.01311-12.

4. Mousson L, Zouache K, Arias-Goeta C, Raquin V, Mavingui P, Failloux AB. 2012. The native Wolbachia symbionts limit transmission of dengue virus in Aedes albopictus. PLoS Negl. Trop. Dis. 6:e1899. http://dx.doi.org/10.1371/journal.pntd.0001989.

5. Crostoe E, Marialva MSP, Esteves SS, Weinert LA, Martínez J, Jiggins FM. 2011. Wolbachia variates induce differential protection to viruses in Drosophila melanogaster: a phenotypic and phylogenomic analysis. PLoS Genet. 7:e1002367. http://dx.doi.org/10.1371/journal.pgen.1002367.

6. Riegler M, Sidhu M, Miller WJ, O’Neill SL. 2012. gypsy retroelement in the ovo locus. Nucleic Acids Res. 40:177–188. http://dx.doi.org/10.1093/nar/gnr105.

7. Song SU, Gerasimova T, Kurkulos M, Boeke JD, Corces VG. 2014. An Env-like protein encoded by a Drosophila retroelement: evidence that gypsy is an infectious retrovirus. Genes Dev. 28:1351–1366. http://dx.doi.org/10.1101/gad.253196.114.

8. Mardus M, Taddei AR, Arnaud F, Faye B, Fausto AM, Mazzini M, Giorgi F, Vaury C. 2006. Viral particles of the endogenous retrovirus ZAM from Drosophila melanogaster use a pre-existing endosome/exosome pathway for transfer to the oocyte. Ooytrovrilology 3:25. http://dx.doi.org/10.1186/1742-6004-3-25.

9. Ballard JW, Melvin RG. 2007. Tetracycline treatment influences mitochondrial metabolism and mtDNA density two generations after treatment with Drosophila. Insect Biochem. 37:799–802. http://dx.doi.org/10.1016/j.inbio.2006.12.002.

10. Shaw AE, Terzian C, Rebollo R, Burlet N, Essault C, Martinez S, Viginier B, Terzian C, Vieira F, Fabiet M. 2012. Tierant, a newly discovered active

ently an infectious retrovirus of Drosophila melanogaster. Proc. Natl. Acad. Sci. U. S. A. 91:1285–1289. http://dx.doi.org/10.1073/pnas.91.4.1285.

11. Song SU, Gerasimova T, Kurkulos M, Boeke JD, Corces VG. 1994. An Env-like protein encoded by a Drosophila retroelement: evidence that gypsy is an infectious retrovirus. Genes Dev. 28:1351–1366.

12. Hannon GJ. 1994. Mobilization of the gypsy retrotransposon in Drosophila melanogaster. Proc. Natl. Acad. Sci. U. S. A. 91:1285–1289. http://dx.doi.org/10.1073/pnas.91.4.1285.
endogenous retrovirus in *Drosophila simulans*. J. Virol. 86:3675–3681. http://dx.doi.org/10.1128/JVI.07146-11.

37. Chandler JA, Morgan Lang J, Bhatnagar S, Eisen JA, Kopp A. 2011. Bacterial communities of diverse *Drosophila* species: ecological context of a host–microbe model system. PLoS Genet. 7:e1002272. http://dx.doi.org/10.1371/journal.pgen.1002272.

38. Misseri Y, Cerutti M, Devauchelle G, Bucheton A, Terzian C. 2004. Analysis of the *Drosophila* gypsy endogenous retrovirus envelope glycoprotein. J. Gen. Virol. 85:3325–3331. http://dx.doi.org/10.1099/vir.0.79911-0.

39. Moreira LA, Ye YH, Turner K, Eyles DW, McGraw EA, O’Neill SL. 2011. The wMelPop strain of Wolbachia interferes with dopamine levels in *Aedes aegypti*. Parasit. Vectors 4:28. http://dx.doi.org/10.1186/1756-3305-4-28.