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The Small Interfering RNA Pathway Is Not Essential for *Wolbachia*-Mediated Antiviral Protection in *Drosophila melanogaster*

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***Wolbachia pipientis* delays RNA virus-induced mortality in *Drosophila* spp. We investigated whether *Wolbachia*-mediated protection was dependent on the small interfering RNA (siRNA) pathway, a key antiviral defense. Compared to *Wolbachia*-free flies, virus-induced mortality was delayed in *Wolbachia*-infected flies with loss-of-function of siRNA pathway components, indicating that *Wolbachia*-mediated protection functions in the absence of the canonical siRNA pathway.**

Viruses, as obligate intracellular pathogens, rely on their hosts for proliferation, and the interaction is therefore a balance between the virus and the host. However, recently it has become increasingly clear that in addition to the contributions of the host and the parasite, other microbes within the host organism can also affect the outcome of infection (reviewed in reference 3). An example of this is the antiviral effect observed in insects, which is mediated by infection with the endosymbiotic bacterium *Wolbachia pipientis* (8, 23).

The presence of *Wolbachia* protects *Drosophila* spp. from virus-induced mortality (8, 21, 23). *Wolbachia* is an obligate intracellular, Gram-negative bacterium that is maternally inherited (reviewed in references 18, 22, and 26). *Wolbachia* is estimated to infect up to 70% of all insect species as well as filarial nematodes and crustacean and mite species (10, 12). Compared to *Wolbachia*-free flies, *Drosophila melanogaster* flies infected with *Wolbachia* have delayed virus-induced mortality when injected with diverse single-stranded RNA viruses, including the dicistroviruses *Drosophila* C virus (DCV) and cricket paralysis virus (CrPV) or the nodavirus Flock House virus (FHV) (8, 23). *Wolbachia*-infected *D. melanogaster* flies also accumulated lower titers of DCV and Nora virus than their *Wolbachia*-free counterparts (8, 23). However, delayed virus-induced mortality can occur without decreased virus accumulation (21, 23). To date, *Wolbachia*-mediated protection in *D. melanogaster* has only been observed against RNA viruses. The presence of *Wolbachia* did not affect either accumulation of or mortality induced by the double-stranded DNA (dsDNA) virus insect iridescent virus 6 (IIV-6) in *D. melanogaster* (23).

The *Wolbachia*-mediated antiviral effect is not specific to *D. melanogaster*. Similar antiviral protection is observed in *Drosophila simulans* as well as in mosquitoes (1, 15, 19, 21). Artificial infection of *Aedes aegypti* with *Wolbachia* inhibits infection by dengue virus and Chikungunya virus as well as the malaria parasite *Plasmodium gallinaceum*, the bacterium *Erwinia carotovora*, and filarial nematodes (1, 11, 14, 15, 19). While in the mosquito *Culex quinquefasciatus*, natural *Wolbachia* infection has a minor impact on West Nile virus (WNV) infection (7). Interestingly, *Wolbachia*-infected *Drosophila* is not protected against infection with pathogenic Gram-negative bacteria, including *Erwinia carotovora* (27), indicating that the mechanism of antiviral protection is independent of the mechanism of antibacterial protection. Despite being an increasingly observed phenomenon, the mechanism by which *Wolbachia* infection mediates an antiviral effect against RNA viruses is currently unknown.

In insects, RNA interference (RNAi) is a key host viral defense pathway. *Drosophila melanogaster* has three major RNAi pathways: the siRNA pathway, which has been found to be important for the control of virus infection; the microRNA (miRNA) pathway, which primarily regulates host gene expression; and the Piwi-associated RNA (piRNA) pathway, which is involved in controlling germ line mobile genetic elements (6). The siRNA pathway inhibits viral replication by sequence-specific degradation of the viral RNA. In *Drosophila*, double-stranded RNA (dsRNA) is first recognized and digested by the RNase III enzyme Dicer-2 (Dcr-2) into short interfering RNAs (siRNA) (6). Dicer-2 forms a heterodimer with a dsRNA-binding protein (dsRBP) called R2D2. R2D2 is required for loading the siRNA into the RNA-induced silencing complex (RISC). One siRNA strand is then used as a template for Argonaute2 (AGO2) to cleave complementary, single-stranded RNA (6). *Drosophila* lines with loss-of-function or null mutations for some components of the siRNA pathway have previously been shown to increase susceptibility to RNA virus infections (5, 24, 25).

Since the siRNA pathway is a major antiviral defense of *Drosophila* and *Wolbachia* infection in *Drosophila* has been shown to have a protective affect against RNA viruses, we investigated whether the mechanism of *Wolbachia*-mediated protection was reliant on the siRNA pathway.

To assess whether the siRNA pathway is required for *Wolbachia*-mediated protection we used *Drosophila* strains with loss-of-function mutations in specific components involved in the siRNA pathway. All fly lines were maintained on standard cornmeal diet at a constant temperature of 25°C with a 12-h light/dark cycle. The lines used were the w^{1118} positive-control line and the $dcr\text{-}2^{L811fsX}$ (16), $r2d2^{1}$/*CyO* (Bloomington Stock Center, no. 8518) (17), and

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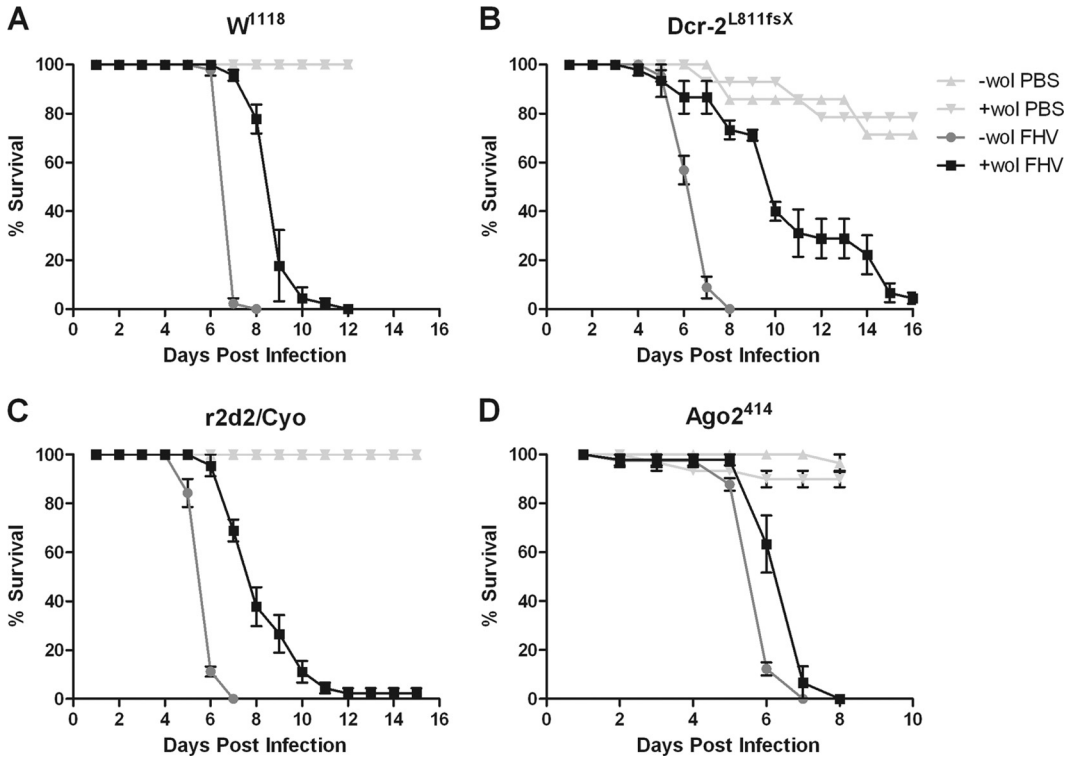

FIG 1 Effect of the presence (+wol) or absence (−wol) of *Wolbachia* on FHV-induced mortality in flies from the w^{1118} control line (A) and the *Dicer-2*^{L811fsX} (B), *r2d2*^{1}*/Cyo* (C), and *AGO2*^{414} (D) mutant lines. Adult flies 4 to 7 days old were injected with PBS as a negative control, or FHV, and their mortality was assayed. *Wolbachia*-mediated protection against FHV-induced mortality was statistically significant in w^{1118} flies and the siRNA mutants ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, and $P = 0.001$, respectively). Bars indicate standard errors. Each experiment was repeated in triplicate, and representative graphs are shown.

AGO2^{414} (20) siRNA mutant lines. To generate *w*Mel-infected balancer lines, *w*Mel-infected w^{1118} (28) females were crossed with *CyO/Gla* or *TM6B/TM3 Sb* males. *w*Mel-infected *CyO/+* females were crossed with *w*Mel-infected *Gla/+* males to establish the *w*Mel-infected *CyO/Gla* line. *w*Mel-infected *TM6B/+* females were crossed with *w*Mel-infected *TM3 Sb/+* males to establish the *w*Mel-infected *TM6B/TM3 Sb* line. To generate *w*Mel-infected siRNA mutants, females of *w*Mel-infected balancer lines (*CyO/Gla* or *TM6B/TM3 Sb* flies) were crossed with males of siRNA mutants. *w*Mel-infected *AGO2*^{414} and *dcr-2*^{L811fsX} flies were maintained and used for experiments as homozygotes. The *w*Mel-infected *r2d2* mutant was maintained and used in experiments as heterozygotes balanced over the *CyO* chromosome due to high mortality of homozygotes. All *Wolbachia*-infected fly lines were treated with 0.03% tetracycline to cure the *Wolbachia* infection. Following the tetracycline treatment, flies were held for more than five generations to recover before the absence of *Wolbachia* was confirmed by PCR, and then the flies were used for experiments. All stocks were cleared of possible DCV contamination by bleach treatment of eggs, as previously described (21).

The siRNA mutant lines, in the presence and absence of *Wolbachia* infection, were injected with FHV, and their mortality rates were compared (Fig. 1). For survival assays, the flies used were male and 4 to 7 days old. Flies were anesthetized with carbon dioxide, and 50.6 nl of virus or phosphate-buffered saline (PBS) for controls was injected into the upper lateral part of the abdomen by using needles pulled from borosilicate glass capillaries and a Nanoject II microinjector. PBS was injected as a negative control. Virus samples prepared as previously described (9, 13) were diluted in PBS, and approximately 100 infectious units (IU) of DCV or 300 IU of FHV was injected into each fly. For each fly line assayed, three groups of 15 flies were injected with virus, one group of 15 flies was injected with PBS, and mortality was recorded daily. Mortality that occurred within 1 day of injection was deemed to be due to needlestick injury. Each experiment was then repeated three times using independent cohorts of male flies and once using female flies. The graphs shown represent results from a single male fly experiment; similar results were observed across all four experiments (data not shown). The data were analyzed using the log-rank (Mantal-Cox) test on Kaplan-Meier survival curves (GraphPad Prism 5).

Wolbachia-mediated delay of FHV-induced mortality was present in the positive-control flies (w^{1118}), as has previously been shown (8, 23). Likewise in the presence of *Wolbachia*, mortality was delayed in flies from the three siRNA mutant lines compared to *Wolbachia*-free flies from the same lines (Fig. 1). The continuing presence of *Wolbachia*-mediated protection in all siRNA mutants indicated that *Wolbachia*-mediated protection can function in the absence of the canonical siRNA pathway.

Antiviral protection mediated by *Wolbachia* in *D. melanogaster* has previously been shown for a number of RNA viruses, including DCV (8, 23). To confirm that *Wolbachia*-mediated protection is independent of the host's siRNA pathway in general, not just in the case of FHV infection, mortality assays of DCV-infected siRNA mutants, with and without *Wolbachia*, were undertaken (Fig. 2). For the w^{1118} line and both the *r2d2* and *AGO2* mutant

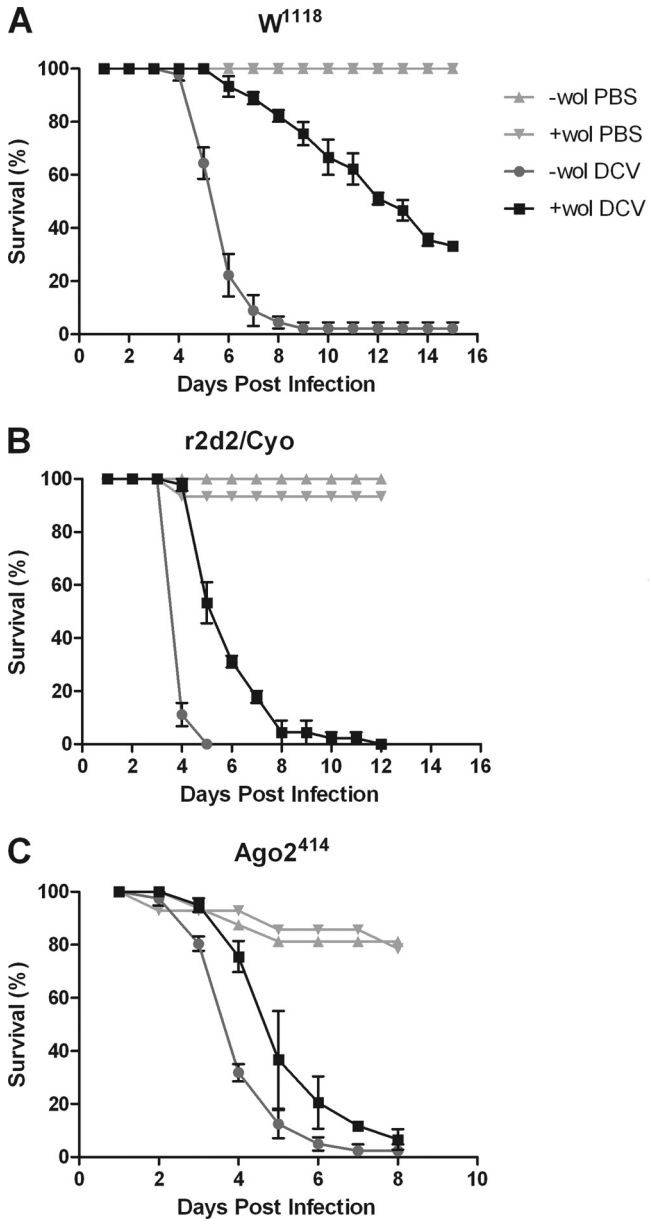

FIG 2 Effect of the presence (+wol) or absence (−wol) of *Wolbachia* on DCV-induced mortality in flies from the w^{1118} control line (A) and the $r2d2^{1}$/*Cyo* (B) and $AGO2^{414}$ (C) mutant lines. Adult flies 4 to 7 days old were injected with PBS as a negative control, or DCV, and their mortality was assayed. *Wolbachia*-mediated protection against DCV-induced mortality was statistically significant in w^{1118}, $r2d2^{1}$/*Cy*O, and $AGO2^{414}$ flies ($P < 0.0001$, $P < 0.0001$, and $P = 0.001$, respectively). Bars indicate standard errors. Each experiment was repeated in triplicate, and representative graphs are shown.

lines, mortality in *Wolbachia*-infected flies was delayed in comparison to that in paired *Wolbachia*-free flies, indicating that *Wolbachia* was mediating antiviral protection in these flies. The experiments presented do not exclude an auxiliary role for siRNA in *Wolbachia*-mediated protection; however, they indicate that *Wolbachia*-mediated protection against RNA viruses is not dependent on the canonical siRNA pathway in *Drosophila*. These findings are consistent with previous studies that showed that *Wolbachia* inhibits dengue virus replication in the *A. albopictus*-derived C6/36 cell line, which has a disfunctional antiviral RNA interference pathway (2, 4), and *AGO-2* mutant flies are more resistant to WNV infection when infected with *Wolbachia* (7).

There are many other possible mechanisms for *Wolbachia*-mediated protection, including competition for cellular components or upregulation of other immune pathways. There is some evidence for the involvement of immune genes in mosquitoes (1, 15, 19). Unlike in the artificially infected mosquitoes, *Drosophila* flies with natural *Wolbachia* infection are not protected against bacterial infection, and there is no upregulation of the Imd or Toll immune pathways (27). There may be differences in *Wolbachia*-host interactions between recently introduced *Wolbachia* infections and those where the host and *Wolbachia* have been evolving together for some time. In addition there are likely to be different mechanisms behind *Wolbachia*-mediated protection against viruses, bacteria, and eukaryotic protists.

This study investigated whether *Wolbachia*-mediated protection against viruses is dependent on the siRNA pathway, a key antiviral response in *Drosophila* and other insects. The results demonstrate that the canonical siRNA pathway is not essential for *Wolbachia*-mediated protection.

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