Wolbachia suppresses cell fusing agent virus in mosquito cells

Guagmei Zhang, Kayvan Etebari and Sassan Asgari

Australian Infectious Disease Research Centre, School of Biological Sciences, The University of Queensland, Brisbane, QLD 4072, Australia

The genus Flavivirus contains a large number of positive-sense ssRNA viruses. While some are transmitted by mosquitoes or other arthropods and are pathogenic to humans and animals (e.g. dengue and Zika viruses), some are insect-specific and do not replicate in vertebrate cells. These are known as insect-specific flaviviruses (ISFs). Cell fusing agent virus (CFAV) was the first described ISF, which was detected in an Aedes aegypti cell line, Aag2. Here, we investigated the effect of Wolbachia, a widespread endosymbiont of many insect species, that is known to block replication of several pathogenic flaviviruses, on CFAV. Our results demonstrated that, in mosquito cells, Wolbachia vastly suppresses replication of CFAV, with significantly less CFAV viral interfering small RNAs produced in the cells. However, removal of Wolbachia with tetracycline led to increased CFAV replication. These results suggest that Wolbachia is also able to suppress an ISF.

Flaviviruses are a diverse group of positive-sense ssRNA viruses that are mostly transmitted by mosquitoes or other blood-feeding arthropods (e.g. ticks) causing devastating diseases in animals and humans such as dengue fever, encephalitis, Zika-associated syndromes, yellow fever and paralysis (Roby et al., 2012). In addition to flaviviruses pathogenic to vertebrates, a number of insect-specific flaviviruses (ISFs) have been described from mosquitoes that do not replicate in vertebrate cells but specifically in insect hosts (Blitvich & Firth, 2015). These are believed to be vertically transmitted as they are not infectious by oral feeding (Bolling et al., 2012; Cook et al., 2012; Lutomiah et al., 2007; Saiyasombat et al., 2011). Interestingly, a number of them have been shown to negatively affect replication of medically important flaviviruses in co-infected or superinfected mosquitoes (Bolling et al., 2012; Goenaga et al., 2014; Hall-Mendelin et al., 2016; Hobson-Peters et al., 2013; Kenney et al., 2014).

Due to lack of effective vaccines or drugs to most medically important flaviviruses, huge efforts have concentrated on vector population control or reduction of vector competence in major vectors of these diseases. A successful approach has been the utilization of Wolbachia. Wolbachia pipientis is a Gram-negative endosymbiotic bacterium present in about 40–65 % of insect species, in addition to other arthropods and nematodes (Hilgenboecker et al., 2008; Jeyaprakash & Hoy, 2000). Amazingly, when present in an insect, in the majority of cases, it blocks replication of viruses (reviewed by Johnson, 2015). However, some of the major mosquito vectors are devoid of Wolbachia, but when transfected with Wolbachia from another insect host, virus protection is conferred (Moreira et al., 2009; Xi et al., 2005). For example, in Aedes aegypti mosquitoes stably transfected with Wolbachia, replication of several medically important arboviruses, replication of several medically important arboviruses, such as dengue virus, Zika virus, Chikungunya virus and West Nile virus (WNV), is blocked (Bian et al., 2010; Dutra et al., 2016; Hussain et al., 2013; Moreira et al., 2009). Here, we investigated the effect of Wolbachia on an ISF, cell fusing agent virus (CFAV), in the A. aegypti Aag2 cell line persistently infected with the virus.

Reverse transcription (RT)-PCR was carried out on RNA extracted from Aag2 cells, and Aag2 cells infected with wMelPop strain of Wolbachia (aag2.wMelPop-CLA) (Frentiu et al., 2010) using CFAV NS5 gene-specific primers (forward: 5'-GGCCACATCTGGGCRRTNWCGCTTNGC-3'; reverse: 5'-GGGCAAGTARBMACTTATGCVTTGAACAC-3'). Total RNA from mosquito cells was isolated using Trizol and subsequently incubated with DNase I at 37 °C for 10 min followed by heat inactivation at 75 °C for 10 min. The first-strand cDNA was synthesized by RT with CFAV-specific or oligo(dT) (for ribosomal protein S17 control, RPS17) primers. In each RT reaction, approximately 2 µg of total RNA was used as template in a volume of 20 µl. Amplification was performed at 95 °C for 1 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s, 68 °C for 1 min and a final extension at 68 °C for 5 min. Results...
showed a CFAV-specific band in Aag2 cells only, but not in aag2.wMelPop-CLA cells (Fig. 1a; Aag2 and Pop). We also tested RNA samples extracted from A. aegypti mosquitoes infected with wMelPop and those treated with tetracycline but did not find any CFAV infection in the mosquitoes (Fig. 1a; Wol− and Wol+).

To determine whether Wolbachia suppresses CFAV, we treated a flask of aag2.wMelPop-CLA cells with 1 µg ml⁻¹ tetracycline (Tet) for three passages (P1–P3). Removal of Wolbachia by tetracycline was confirmed by quantitative PCR (qPCR) with WSP (Wolbachia surface protein)-specific primers (Fig. 1b, upper panel). For qPCR, total genomic DNA was extracted from cells and qPCR was carried out in three technical replicates using wsp-specific primers (forward: 5′-ATCTTTTATAGCTGGTGGTGGT-3′ and reverse: 5′-GGAGTGATAGGCATATCTTCAAT-3′) by using Platinum SYBR Green Mix (Invitrogen) with 20 ng of total genomic DNA in a Rotor-Gene thermal cycler (QIAGEN) under the following conditions: 95 °C for 5 min then 40 cycles of 95 °C for 10 s, 56 °C for 15 s and 72 °C for 20 s, followed by the melting curve (68–95 °C). The RPS17 gene was used for normalizing data. Removal of Wolbachia was also confirmed by Western blot using polyclonal antibodies specific to WSP raised in rabbit (Fig. 1b, lower panel). Interestingly, in Tet-cured cells (P3), the CFAV levels determined by RT-qPCR were quite high but hardly detectable in aag2.wMelPop-CLA cells (Fig. 1c), which suggested that Wolbachia wMelPop-CLA strain strongly suppresses the replication of CFAV in aag2.wMelPop-CLA cells. The qPCR primers for CFAV detection were CFAV-qF 5′-CTGATG TGGGTCAAGCTCCTT-3′ and CFAV-qR 5′-CACAAGGG TAGCGAGAGACA-3′ as described previously (McFarlane et al., 2014). The reverse primer was used for the RT of DNase I-treated RNA extracted from the cells using Super-Script III (Invitrogen). RPS17 gene was used for normalizing data. We also repeated this experiment a few months later and found that CFAV again started to appear after four passages of tetracycline treatment (Fig. S1a, available in the online Supplementary Material). Interestingly, we happened to have a stock of aag2.wMelPop-CLA cells with lower Wolbachia density (Fig. S1b). When those cells were tested by RT-qPCR after treatment with tetracycline for three

![Graph](image)

**Fig. 1.** CFAV is suppressed by Wolbachia in aag2.wMelPop-CLA cells. (a) RT-PCR analysis was performed using total RNA extracted from Aag2 and aag2.wMelPop-CLA cells (Pop) using CFAV NS5-specific primers. RPS17 gene was used as a control to show the integrity of RNA, and the wsp gene was used to confirm Wolbachia infection. (b) Quantitative PCR analysis was performed using DNA extracted from aag2.wMelPop-CLA (Pop) cells, and tetracycline-treated (Tet) aag2.wMelPop-CLA cells for three passages (P1–P3) with wsp-specific primers in three technical replicates (upper panel). Western blot was performed by using a polyclonal antibody to the WSP protein (1 : 4000) probing the proteins from aag2.wMelPop-CLA and tetracycline-treated aag2.wMelPop-CLA cells (lower panel). aag2.wMelPop-CLA cells were treated with 1 µg ml⁻¹ tetracycline for three passages. (c) Quantitative RT-PCR analysis of Pop, P3 tetracycline-treated aag2.wMelPop-CLA cells and untreated Aag2 cells using CFAV-specific primers with three technical replicates. Different letters in (b) and (c) indicate statistically significant differences (a and b at P<0.0001, and b and c at P<0.01).
passages, we found that CFAV was already present in the cells in the first passage (Fig. S1a). This indicates that CFAV has not been completely cleared from aag2.wMelPop-CLA in our hands and its levels are affected by Wolbachia density.

RNA interference is an efficient antiviral response in insects leading to the cleavage of the viral genome (if dsRNA) or viral dsRNA intermediates produced during viral replication to produce viral short interfering RNAs (vsiRNAs) (reviewed by Bronkhorst & van Rij, 2014). We investigated the small RNA profiles of CFAV vsiRNAs in Aag2 and aag2.wMelPop-CLA cells using deep sequencing data produced previously from the cytoplasmic and nuclear fractions of the cells accessible through GEO series accession number GSE55210 (Mayoral et al., 2014b). Small RNA reads were mapped to the CFAV genome (accession no. M91671) with strict mapping criteria (mismatch, insertion and deletion costs: 2 : 3 : 3, respectively) using CLC Genomic Workbench (version 7.5.1). Results demonstrated a large number of vsiRNAs that mapped to the CFAV genome in both Aag2 cellular fractions, but in comparison, there were drastically fewer vsiRNAs in both the cytoplasmic and nuclear fractions of aag2.wMelPop-CLA cells (Fig. 2a). The vsiRNAs were spread throughout the whole CFAV genome and mapped to both strands, with some hot spot regions being highly targeted (3500–4000 and >10,000 nt) and other regions being targeted less frequently (Fig. 2a). The majority of vsiRNAs were 21 nucleotides (typical siRNA size) in both the cytoplasmic and nuclear fractions of aag2.wMelPop-CLA cells (Fig. 2a, lower panel). These viral small RNAs exhibit the hallmarks of ping-pong cycle derived piRNAs, which are an over-representation of U in the first position and A in the tenth position (Fig. 2c). This phenomenon was previously reported for other arboviruses in Aag2 cells as well as C6/36 and U4.4 cell lines from Aedes albopictus (Vodovar et al., 2012). The absence of 21 nt vsiRNAs in Wolbachia-infected cells together with the results shown above suggest that CFAV replication is significantly inhibited in these cells but perhaps not completely cleared as some small RNAs still mapped to the viral genome. Based on these results, it also appears that RNA interference is not the mechanism underlying virus blocking by Wolbachia in mosquito cells, which was also shown in Drosophila melanogaster (Hedges et al., 2012).

CFAV was the first mosquito-only flavivirus discovered, and this was from an A. aegypti cell culture, Aag2 (Stollar & Thomas, 1975). Subsequently, several strains of the virus have also been detected in field-collected A. aegypti and other mosquito species (Cook et al., 2009; Espinoza-Gómez et al., 2011; Hoshino et al., 2007; Kihara et al., 2007; Sang et al., 2003; Yamanaka et al., 2013). CFAV has extensive similarities to medically important flaviviruses in terms of genome size, structure and gene order (Cammisa-Parks et al., 1992). With the application of novel advanced molecular tools for viral detection, there has been a dramatic increase in the isolation and characterization of mosquito-only viruses recently (Bolling et al., 2012; Calzolari et al., 2012; Cook et al., 2009; Crabtree et al., 2009; Hobson-Peters et al., 2013; Hoshino et al., 2007; Junglen et al., 2009; Nasar et al., 2015). The occurrence of these viruses in mosquitoes may affect the vector competence of mosquitoes in transmission of arboviruses that are pathogenic to vertebrate hosts. For example, it has been reported that Culex flavivirus suppressed dissemination of WNV at early stages of infection (7 days) in Culex pipiens mosquitoes, which may affect the intensity of enzootic transmission of WNV (Bolling et al., 2012, 2015). Hobson-Peters et al. (2013) reported that an ISF, Palm Creek virus, suppressed replication of WNV and Murray Valley encephalitis virus in mosquito cells, but did not suppress replication of the alphavirus Ross River virus. Kenney et al. (2014) described a new ISF, Nhumirim virus, that is more closely related to mosquito-borne flaviviruses but is only able to replicate in mosquito cell lines. The virus was shown to negatively affect replication of WNV, St. Louis encephalitis virus and Japanese encephalitis virus (Kenney et al., 2014). Nasar et al. (2015) reported that the ISF, Ellat virus, reduced the replication of several mosquito-borne alphaviruses such as Sindbis virus, eastern, western and Venezuelan equine encephalitis viruses and Chikungunya virus. In addition, it was reported that Culex flavivirus (Izabal) from Guatemala could significantly enhance the transmission rate of WNV in Culex quinquefasciatus from Honduras (Kent et al., 2010).

Wolbachia, as a naturally occurring endosymbiont in insects, has gained a substantial interest recently due to its typical property to block replication of RNA viruses and several other pathogens in infected insects (Bourtzis, 2008). However, they are mostly absent from several medically important mosquito species, including A. aegypti. In recent years, a number of different strains of Wolbachia have been successfully transinfected into various mosquito species such as A. aegypti, A. albopictus and Anopheles gambiae to block transmission of arboviruses and malaria (Bian et al., 2013; Blagrove et al., 2012; McMeniman et al., 2009; Xi et al., 2005). In particular, A. aegypti stably transinfected with Wolbachia wMel/wMelPop strongly suppresses replication of several arboviruses such as dengue, yellow fever, Zika and Chikungunya viruses (Bian et al., 2010; Dutra et al., 2016; Kambris et al., 2009; Moreira et al., 2009; Ye et al., 2015).

In our study, we found that CFAV was hardly detectable in aag2.wMelPop-CLA cells, which suggests that Wolbachia can strongly suppress the virus. This stably transinfected cell line was originally produced from an Aag2 cell line that was persistently infected with CFAV. It is commonly known that all the Aag2 cell lines are persistently infected with CFAV, which suggests that the original cell line established must have been from mosquitoes that had already been
infected with the virus. It is worth mentioning that \textit{w}Mel-Pop strain is known as an over-replicating strain of \textit{Wolbachia} (McMeniman & O’Neill, 2010; Min & Benzer, 1997) producing a very strong virus blocking effect (Moreira \textit{et al}., 2009). For example, in our hands, dengue virus was not detectable in aag2.\textit{w}MelPop-CLA cells infected with the virus when a sensitive method such as RT-qPCR was used (Hussain \textit{et al}., 2013). While this manuscript was under review, Schnettler \textit{et al}. (2016) also demonstrated that aag2.\textit{w}MelPop-CLA cells efficiently suppress acute and persist infections of CFAV. They also showed that Aag2 cells are persistently infected with another insect-specific virus (ISV), Phasi Charoen-like bunyavirus, but its replication was not inhibited by \textit{Wolbachia}, which suggests that

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{CFAV small RNA profiles in Aag2 and aag2.\textit{w}MelPop-CLA cells. (a) Distribution of small RNA reads from deep sequencing of the cytoplasmic and nuclear fractions of Aag2 and aag2.\textit{w}MelPop-CLA (Pop) cells that mapped across the CFAV genome. The dark and light red represent the reads mapped to the negative and positive strands, respectively. (b) Size distribution of CFAV vsiRNAs from the cytoplasmic and nuclear fractions of Aag2 and aag2.\textit{w}MelPop-CLA (Pop) cells. (c) Nucleotide distribution of reads mapped to CFAV. The bias for U in the first nucleotide position and that for A in the tenth nucleotide position of viral small RNAs is evident.}
\end{figure}
Wolbachia does not seem to inhibit all types of RNA viruses. Bunyaviruses are negative-sense RNA viruses, while all RNA viruses tested previously, by inhibition of Wolbachia, have been positive-sense viruses.

Interestingly, in our study, removal of Wolbachia by tetracycline treatment led to return of CFAV in aag2.wMelPop-CLA cells to high levels after three passages which was reproducible. However, Schnettler et al. did not detect CFAV after tetracycline treatment of the cells (Schnettler et al., 2016). It is known that Wolbachia density is important for virus blocking (Lu et al., 2012; Osborne et al., 2012). Therefore, this inconsistent observation could be due to differences in Wolbachia densities in the cells maintained in the two laboratories which may have led to total elimination of CFAV in one instance. Cell culture conditions are known to have significant impacts on Wolbachia density (Khoo et al., 2013). We also found that CFAV can be detected in aag2.wMelPop-CLA cells if Wolbachia density is low in the cells. The presence of some small RNA reads in aag2.wMelPop-CLA cells maintained in our laboratory (Mayoral et al., 2014a) also suggests that CFAV is not perhaps totally cleared from aag2.wMelPop-CLA cells (Fig. 2b).

Overall, our results and those of Schnettler et al. (2016) suggest that Wolbachia significantly blocks replication of CFAV and may even clear it from mosquito cells. An implication of inhibition of ISVs by Wolbachia is that introduction of Wolbachia into mosquito populations may affect the mosquito virome, although Wolbachia strains other than wMel-Pop, such as wAlb and wMel, which are not over replicators, may not have such extreme effects on ISVs. In addition, not all ISVs could be affected by Wolbachia (such as bunyaviruses). Relevant to this, application of ISVs as a novel control strategy may not be compatible with Wolbachia depending on the interaction of Wolbachia and the ISF being considered.

Acknowledgements

This project was funded by the Australian Research Council grant (ARC, DP150101782) and the National Health and Medical Research Council (NHMRC, APP1062983) to S. A.

References


