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Group B *Wolbachia* Strain-Dependent Inhibition of Arboviruses

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Mosquito-borne viruses, including Zika virus (ZIKV) and dengue virus (DENV), are global threats that continue to infect millions annually. Historically, efforts to combat the spread of these diseases have sought to eradicate the mosquito population. This has had limited success. Recent efforts to combat the spread of these diseases have targeted the mosquito population and the mosquito's ability to transmit viruses by altering the mosquito's microbiome. The introduction of particular strains of *Wolbachia* bacteria into mosquitos suppresses viral growth and blocks disease transmission. This novel strategy is being tested worldwide to reduce DENV and has early indications of success. The *Wolbachia* genus comprised divergent strains that are divided in major phylogenetic clades termed supergroups. All *Wolbachia* field trials currently utilize supergroup A *Wolbachia* in *Aedes aegypti* mosquitos to limit virus transmission. Here we discuss our studies of *Wolbachia* strains not yet used in virus control strategies but that show strong potential to reduce ZIKV replication. These strains are important opportunities in the search for novel tools to reduce the levels of mosquito-borne viruses and provide additional models for mechanistic studies.

Keywords: arbovirus, *Wolbachia*, vector control, emerging viruses, Zika virus, dengue virus

Introduction

Mosquito-transmitted viruses are a global concern due to increasing incidence and geographic range. Although these viruses have been identified for decades, we still lack proper treatment and control. Dengue virus (DENV) cases have doubled every decade since 1900 and expanded geographically such that four DENV serotypes can be found throughout Europe, Asia, Africa, and the Americas (Messina *et al.*, 2014; Sharp *et al.*, 2017). Likewise, Chikungunya virus (CHIKV) has caused a greater rather than diminishing threat over time (Pybus *et al.*, 2015). Recently, Zika virus (ZIKV) has shown us just how quickly a new outbreak of mosquito-transmitted disease can spread. ZIKV was introduced into Brazil in 2013 (Faria *et al.*, 2016) and is now endemic throughout the Americas causing devastating birth defects (Fauci and Morens, 2016). DENV, CHIKV, and ZIKV are all transmitted by *Aedes* sp. mosquitos, including *Aedes aegypti* and *Aedes albopictus*. Our inability to stop the spread of these diseases emphasizes the need to control the disease vector in addition to virus-specific efforts.

Common Mosquito Control Strategies

Targeting the mosquito vector by decreasing the mosquito population decreases disease spread without the timely cost of vaccination, appealing to the immediate need to stop these viruses. Strategies to reduce total mosquito populations include the use of insecticides or sterile insect technique (SIT) (Fig. 1). Insecticides are efficient chemicals to kill larvae and adult mosquitos but have high environmental and health costs. They also require regular innovation to counterbalance the emergence of resistance in insects [reviewed by Liu (2015) and Moyes *et al.* (2017)]. SIT sterilizes males or limits the development of offspring by irradiating males (Rodriguez *et al.*, 2013; Yamada *et al.*, 2014; Dandalo *et al.*, 2017) or genetically modifies reproductive genes (Catteruccia *et al.*, 2009), respectively. SIT requires costly annual release of sterile males to prevent populations from rebounding, suggesting alternative sustainable strategies are needed.

Wolbachia-Based Vector Control Approaches

Recent novel control strategies have focused on a self-sustaining method using the bacteria *Wolbachia pipientis* to restrict virus transmission (Fig. 1). *Wolbachia*-infected female mosquitos have reduced capacity to transmit pathogens [reviewed by Caragata *et al.* (2016)]. *Wolbachia* are maternally transmitted from mother to offspring suggesting this strategy could be widely effective. Some mosquitos, including *A. albopictus* (Armbruster *et al.*, 2003), *A. fluviatilis*

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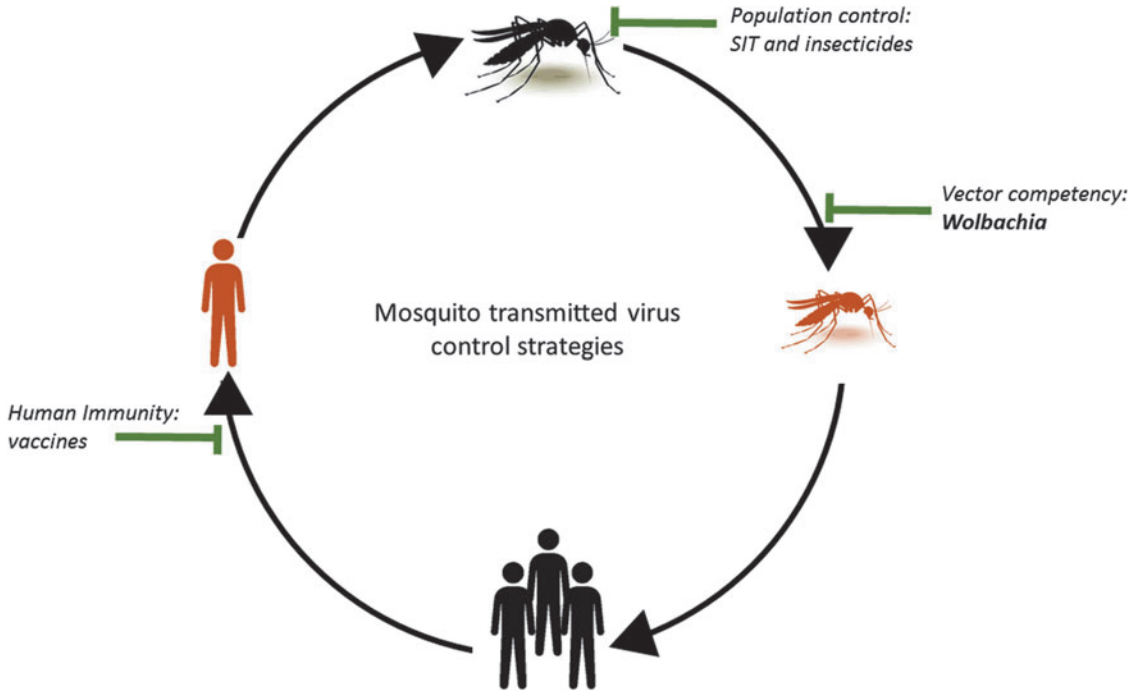

FIG. 1. Mosquito-transmitted virus control strategies target multiple stages of viral transmission: vaccination of human host, suppression of mosquito population (SIT—insecticides and transgenesis), and vector competency. Several strains of the intracellular bacteria *Wolbachia* suppress viral growth in mosquitos, blocking their competency to transmit several arboviruses, including DENV and more recently ZIKV. *Orange* indicates virus-infected mosquito or human. DENV, dengue virus; SIT, sterile insect technique; ZIKV, Zika virus.

(Baton *et al.*, 2013), and *Culex pipiens* (Hertig, 1936), are naturally infected with their own strains of *Wolbachia*. These native infections can limit virus replication (Glaser and Meola, 2010; Mousson *et al.*, 2012; Raquin *et al.*, 2015). However, *A. aegypti*, a prominent vector of DENV, CHIKV, and ZIKV is naturally devoid of *Wolbachia*. *w*Mel native to *Drosophila melanogaster* has been successfully introduced into *Aedes* sp. to limit virus replication (Fragkoudis *et al.*, 2009; Walker *et al.*, 2011; Joubert *et al.*, 2016). *w*Mel has been described to inhibit DENV (Walker *et al.*, 2011; Blagrove *et al.*, 2012; Ye *et al.*, 2015), ZIKV (Aliota *et al.*, 2016a; Dutra *et al.*, 2016), and CHIKV (Blagrove *et al.*, 2013; Aliota *et al.*, 2016b), demonstrating the broad reaching potential of *Wolbachia*-mediated virus control. As a result, *A. aegypti* mosquitos infected with *w*Mel are being released in a worldwide effort to control arboviruses.

Maternal transmission of *Wolbachia* drives an intimate relationship between *Wolbachia* and its host resulting in coevolution (Shaikevich and Zakharov, 2014) and promoting high diversity in *Wolbachia* phylogeny. The *w*Mel *Wolbachia* strain is part of a distinct clade termed supergroup A. Recent studies in *A. aegypti* mosquitos demonstrate that under natural cyclical heat stress, *w*Mel has reduced maternal transmission and cytoplasmic incompatibility, a form of reproductive manipulation that favors infected females (Ross *et al.*, 2017). This sensitivity of the *w*Mel strain to cyclical heat stress reduces the ability of this strain to integrate into large populations in certain regions and jeopardizes the success of utilizing this strain for vector control in *A. aegypti.* In contrast, maternal transmission of supergroup B *Wolbachia*, such as *w*AlbB, was unaffected by cycling temperatures suggesting that it or another *Wolbachia* strain from supergroup B would be better sustained in a mosquito population and more likely to succeed in *Wolbachia*-based interventions.

Most studies of *Wolbachia* suppression of ZIKV have previously been limited to supergroup A's *w*Mel. However, recent work by Schultz *et al.* (2017) assessed supergroup B *Wolbachia* strain's potential to limit ZIKV. Two supergroup B *Wolbachia* *w*AlbB and *w*Stri in *A. albopictus* cells were shown to reduce ZIKV growth. *w*AlbB, a native infection of these cells isolated from *A. albopictus* mosquitos, limited African ZIKV growth by 90% and Puerto Rican ZIKV growth by 99.9%. *w*Stri, a strain isolated from *Laodelphax striatellus* (a leafhopper) and infected into *A. albopictus* cells to form a non-native infection reduced African and Puerto Rican ZIKV by greater than 99.99% below the limit of detection (Fig. 2). It was previously shown that introduction of a non-native *Wolbachia* infection promotes a stronger antiviral response (Bian *et al.*, 2013). Furthermore, in *A. albopictus* mosquitos, *w*AlbB causes a moderate inhibition of DENV (Lu *et al.*, 2012; Mousson *et al.*, 2012), CHIKV (Raquin *et al.*, 2015), and a pronounced repression of DENV in its non-native host: *A. aegypti* (Bian *et al.*, 2010; Joubert *et al.*, 2016). Thus, increased viral protection by *w*Stri may be because it is not native to *A. albopictus*.

Wolbachia density is also important to sustained virus protection in mosquitos. *w*AlbB repression of DENV has previously been shown to be dependent on *w*AlbB density (Lu *et al.*, 2012). *w*Stri-infected cells carried two to three times more *Wolbachia*- than *w*AlbB-infected cells (Schultz

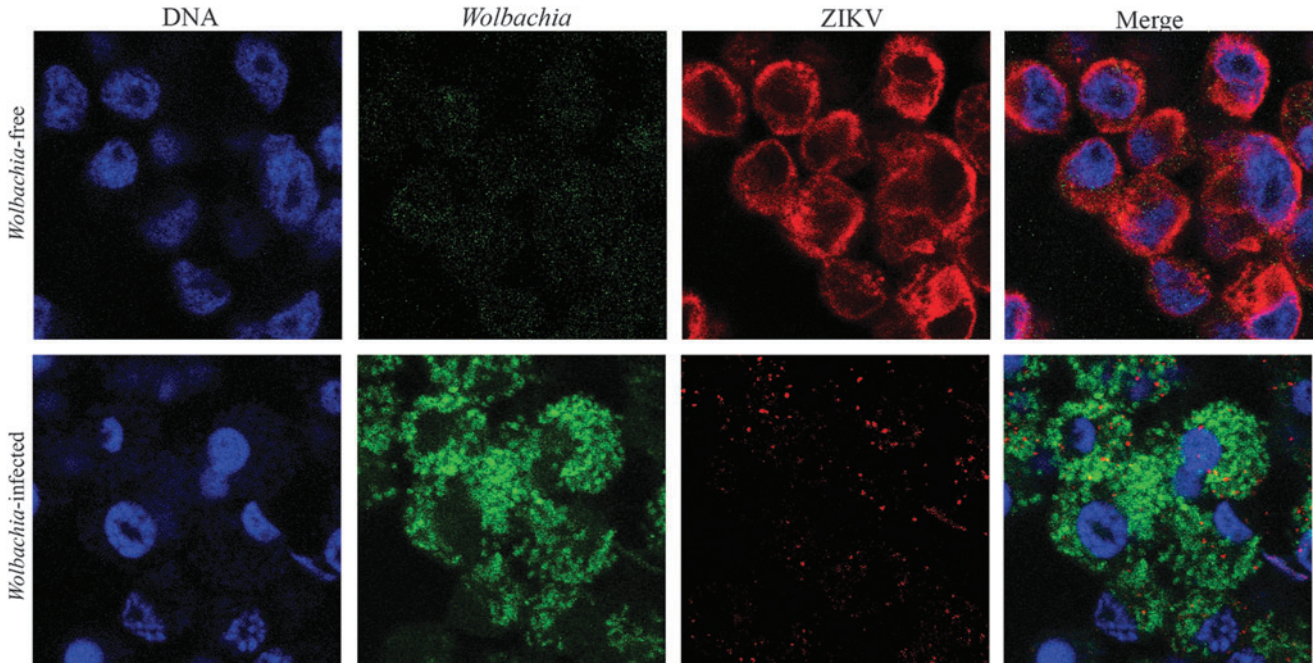

FIG. 2. *Wolbachia* *w*Stri-infected cells are resistant to ZIKV infection. *Aedes* mosquito cells infected with *Wolbachia* bacteria (stained in *green*) do not support ZIKV growth (shown in *red*).

et al., 2017). Reduced *w*Stri density resulted in increased viral growth suggesting a *Wolbachia* density-specific repression of ZIKV. Low *Wolbachia* titers have been suggested to be problematic in whole mosquitos, and superinfection of mosquitos with *w*Mel and *w*AlbB has been shown to sustain overall *Wolbachia* titers in *A. aegypti* mosquitos while repressing DENV (Joubert *et al.*, 2016). Further studies should investigate *w*Stri concentration in *A. aegypti* mosquitos and if superinfection with *w*Stri exacerbates antiviral phenotypes.

Mechanistic Insights into *Wolbachia* Viral Suppression

The expansion of the repertoire of *Wolbachia* strains available in cell lines has allowed for the development of multiple *in vitro* models to investigate the mechanism of *Wolbachia*-mediated viral repression. Early viral inhibition by different *Wolbachia* strains has been shown for three viruses. Consistent with alphavirus studies of Semliki Forest virus by Rainey *et al.* (2016) and Sindbis virus by Bhattacharya *et al.* (2017) in *Drosophila* cells, Schultz *et al.* (2017) showed ZIKV repression occurs at or before viral replication in mosquito cells. These data move the field forward toward a molecular mechanism of viral repression, involving entry, viral translation, or genome replication.

Immune priming by *Wolbachia* may stimulate innate defense (*IMD*, *Toll*, and small interfering RNAs) to repress viral replication (Rancès *et al.*, 2012). Conflicting data negate (Rancès *et al.*, 2013) or implicate (Pan *et al.*, 2012) *IMD* and *Toll*-mediated virus protection by the stimulation of reactive oxygen species to repress virus growth. There is evidence that RNAi is not required for viral suppression (Hedges *et al.*, 2012), but it may participate in enhancing the antiviral response (Terradas *et al.*, 2017). Together, these studies show that innate immunity may promote protection from viruses but additional mechanisms are likely.

Competition between *Wolbachia* and the virus for host factors such as amino acids, cholesterol (Caragata *et al.*, 2014), and host lipids (Molloy *et al.*, 2016) may also facilitate viral inhibition. Cholesterol and lipids are important for virus entry, replication, and assembly (Stiasny *et al.*, 2003; Mazzon and Mercer, 2014), thus may be required by both organisms. In the model system, *D. melanogaster*, feeding flies with cholesterol has been shown to rescue Drosophila C virus (DCV) growth in the presence of *Wolbachia* implying that *Wolbachia* was sequestering cholesterol from DCV (Caragata *et al.*, 2013). Schultz *et al.* (2017) tested if cholesterol was also playing a role in *Wolbachia* suppression of ZIKV in mosquito cells. Cholesterol supplementation rescues ZIKV growth supporting this hypothesis. However, this rescue was incomplete, again suggesting multiple mechanisms of repression of viruses by *Wolbachia* working simultaneously.

A third mechanism proposed is by modulation of methylation patterns. *Wolbachia* disrupts global methylation of its host genome and RNA (Ye *et al.*, 2013). RNA methylation is a means to control viral translation and genome replication (Lichinchi *et al.*, 2016). *Dnmt2* is RNA methyltransferase dysregulated by *Wolbachia*. *Dnmt2* has been shown to be upregulated by *Wolbachia* to repress Sindbis virus (an alphavirus similar to CHIKV) growth (Bhattacharya *et al.*, 2017) in *D. melanogaster*. However, in *A. aegypti*, *Dnmt2* has been shown to be downregulated by *Wolbachia* limiting the growth of DENV (Zhang *et al.*, 2013). These contradicting results may be due to different methylation control of alphaviruses and flaviviruses suggesting a virus family-specific mechanism or due to different host organisms. Further studies are needed to elucidate the mechanisms of *Wolbachia*-mediated virus suppression.

Wolbachia release studies have shown promise in implementation (Hoffmann *et al.*, 2011). Release strategies are currently focusing on optimizing when and how many *Wolbachia*-infected mosquitos to release to successfully incorporate *Wolbachia* into a population. Using small-scale releases, optimal release quantities have been determined (Schmidt *et al.*, 2017). Future efforts should aim to test *Wolbachia*-mediated virus control by group B *Wolbachia*, specifically *w*Stri in *A. aegypti* cells and *in vivo*. Further *in vitro* and *in vivo* studies to delineate the multifaceted mechanisms of *Wolbachia*-mediated virus suppression with different *Wolbachia* strains and hosts are needed. This knowledge will aid in the development of novel strategies to reduce transmission of pathogens by insects. The release of *Wolbachia*-infected females is not yet approved in the United States. Field studies showing repression of *Wolbachia*-infected mosquitos to control DENV will inform the U.S. approval of virus control efforts.

Disclosure Statement

No competing financial interests exist.

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