Parallel Infection of Japanese Encephalitis Virus and *Wolbachia* within Cells of Mosquito Salivary Glands

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ABSTRACT The endosymbiont *Wolbachia* usually causes cytoplasmic incompatibility in dipteran hosts, including mosquitoes. However, some important arbovirus-transmitting mosquitoes such as *Aedes aegypti* (L.) are not heritably infected by *Wolbachia*. In *Wolbachia*-harboring mosquito *Armigeres subalbatus* Coquillett, colocalization of *Wolbachia* and inoculated Japanese encephalitis virus (family *Flaviviridae*, genus *Flavivirus*, JEV) in salivary gland (SG) cells was shown by electron microscopy. The infection rate of JEV in SGs, detected with either immunofluorescent antibody test or reverse transcription-polymerase chain reaction, did not show significant differences between *Wolbachia*-infected and -free colonies. It is suggested that *Wolbachia* did not mediate resistance of SG cells to superinfection by JEV, although both microorganisms coexist in the same niche, i.e., the same SG cell. Therefore, a SG escape barrier may not be elevated due to *Wolbachia* infection, which presumably has no deleterious effects on vector competence in *Wolbachia*-harboring mosquitoes.

KEY WORDS *Armigeres subalbatus*, Japanese encephalitis virus, parallel infection, *Wolbachia*

Several mosquito species are known to harbor the endosymbiont *Wolbachia* in reproductive as well as somatic tissues (Dobson et al. 1999, Kittayapong et al. 2000, Tsai et al. 2004). *Wolbachia* is a monophyletic assemblage belonging to the alpha-subdivision of the Proteobacteria (Werren et al. 1995). The endosymbiotic infection usually causes cytoplasmic incompatibility in dipteran hosts, including mosquitoes (Werren 1997). Salivary glands (SGs) have been reported to be one target tissue for *Wolbachia* infection in susceptible mosquitoes (Tsai et al. 2004, Chen et al. 2005). SGs are also the destination of arboviruses during the journey of a virus in the host mosquito before transmission (Janzen et al. 1970, Whitfield et al. 1973, Chen et al. 1993).

The T1P1 strain of Japanese encephalitis virus (family *Flaviviridae*, genus *Flavivirus*, JEV) was recently isolated from *Armigeres subalbatus* Coquillett collected in an islet without paddy cultivation (Chen et al. 2000). This unconventional JEV vector was heritably infected with *Wolbachia* belonging to group A (Tsai et al. 2004). Infection of *Wolbachia* in SGs of *Ar. subalbatus* has been identified either by in situ hybridization or by electron microscopy (Chen et al. 2005).

Wolbachia infection is absent in many important disease-transmitting mosquitoes, including the Japanese encephalitis (JE) vector *Culex tritaeniorhynchus* Giles, the dengue vector *Aedes aegypti* (L.), and the malaria vectors (*Anopheles* spp.) (Kittayapong et al. 2000, Tsai et al. 2004). It becomes interesting whether the endosymbiont modulates transmission of pathogens carried by *Wolbachia*-harboring mosquitoes or other vectors (Brownstein et al. 2003). The relationships and mutual effects between the two microorganisms infecting mosquito SG cells in parallel are discussed in this report.

Materials and Methods

Viruses and Cell Culture. JE virus used in this study was the T1P1 strain that was previously isolated from field-caught *Ar. subalbatus* (Chen et al. 2000). The virus was propagated in C6/36 cells derived from *Aedes albopictus* (Skuse) and titrated in baby hamster kidney (BHK)-21 cells based on previous reports (Chiou and Chen 2001, Liu et al. 2004).

Mosquitoes. *Ar. subalbatus* originated from an islet where T1P1 JEV was isolated, and it was maintained in the laboratory as described previously (Chen et al. 2000). This unconventional JE vector was heritably infected with *Wolbachia* belonging to group A (Tsai et al. 2004). Throughout the study, the *Wolbachia*-free colony of *Ar. subalbatus* was obtained via treatment of early instars with diluted tetracycline solution. These

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larvae were used as the negative control group (Yen and Barr 1973).

Intrathoracic Inoculation. To evaluate the effect of *Wolbachia* on JEV replicating in *Ar. subalbatus*, a virus suspension (4.5×10^8 plaque-forming units [PFU]/ml; $0.17 \mu\text{l}$ per mosquito) was intrathoracically inoculated into newly emerged adult female *Ar. subalbatus* following the method described previously (Chen et al. 1993). At least three mosquitoes were sampled daily and dissected to collect SGs for virus detection until 20 d postinoculation.

Immunofluorescent Antibody Test (IFA). Virus infection was determined by using IFA for detection of viral antigens. The method followed our previous description (Chen et al. 2000). Briefly, SGs dissected from mosquitoes were moved onto wells of 12-well Teflon-coated slides. The slides were immersed in cold acetone (-20°C) for 10 min and then air-dried. One drop of monoclonal antibodies specific to JEV (Trop-Bio, Queensland, Australia) was added to the wells. The slides were washed with phosphate-buffered saline (PBS) after they have been incubated at 37°C for 30 min. Subsequently, goat anti-mouse IgG antibody conjugated with fluorescein isothiocyanate (Sigma-Aldrich, St. Louis, MO) was added to the wells. The slides were washed in PBS again after another incu-

bation under the same conditions, and the specimens were mounted with a mixture of PBS and glycerol (3:7). The specimens were then examined under an epifluorescent microscope (BX50, Olympus, Tokyo, Japan).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). For detection of viral RNA extracted from mosquito salivary glands, RT-PCR was used for amplification of a gene fragment of the E protein following steps outlined in Chen et al. (2000). Primer pairs included the sense primer (5'-AGTTAACATCAG-GCCACCTGA-3') and the complementary primer (5'-GTTCCATCTCGACCAGCAC-3') (Chung et al. 1996). For identification of *Wolbachia* infection in mosquito SGs, PCR also was applied to amplify a fragment of the *Wolbachia* surface protein (*wsp*) gene as described previously (Tsai et al. 2004). The amplified cDNA fragments were run through electrophoresis on a 2% (wt:vol) agarose gel containing $10 \mu\text{l}$ of ethidium bromide (0.1 mg/ml in RNase-free water) once PCR cycles have been completed.

Virus Titration. Plaque assay on BHK-21 cells, following a previous description (Chiou and Chen 2001), was used to determine the virus titer produced in SGs of inoculated mosquitoes. The virus titer is presented as plaque-forming units per milliliter.

Electron Microscopy. Ultrastructural studies followed the method described in our previous report (Chen et al. 2005). In brief, SGs removed from adult mosquitoes with intrathoracic inoculation were fixed immediately in 2% glutaraldehyde for 2 h and then postfixed in 1% osmium tetroxide for 2 h at room temperature. The specimens were dehydrated through an ascending graded series of ethanol. The tissues were embedded in Spurr's resin and polymerized at 70°C . The tissue blocks were sectioned with an ultramicrotome (Reichert Ultracut S, Leica, Vienna, Austria). Thin sections were stained with uranyl acetate and lead citrate in sequence and observed under an electron microscope (JEOL-JEM-2000, JOEL, Tokyo, Japan) at 100 kV.

Results and Discussion

According to the results of IFA (Fig. 1), 76.67% (23/30) and 73.33% (22/30) of dissected SGs were positive to JEV for *Wolbachia*-infected and -free colonies, respectively (Table 1). The results of RT-PCR showed that JEV infected most examined SGs (Fig. 2); the positive rates were 86.57% (13/15) and 80.00% (12/15) for *Wolbachia*-infected and -free colonies, respectively (Table 1). There was no significant difference between the infection rates of two colonies detected by either analytical method ($\chi^2 = 0.08$ and 0.24 for IFA and RT-PCR, respectively; $P > 0.05$). Moreover, an undifferentiated daily dynamic trend of virus titers was shown in mosquitoes with or without *Wolbachia* infection. We suggest that the two mosquito colonies were not significantly different in production of JEV (Fig. 3).

JEV must infect and subsequently pass through the epithelium of mosquito midguts to reach SGs for fur-

Fig. 1. JEV (T1P1 strain) was detected by immunofluorescent antibody test 7 d postinoculation, showing that viral antigens existed in the medial lobe and proximal-lateral lobes of salivary glands dissected from inoculated *Ar. subalbatus*. (a) Positive. (b) Negative. Original magnification, $200\times$.

Table 1. Infection rates of *Wolbachia*-infected and -free *A. subalbatus* colonies with intrathoracically inoculated with 0.17 μ l (original virus titer: 4.5×10^8 PFU/ml) of JE virus (T1P1 strain) suspension and measured 7 d postinoculation

Infection status	% infection rate (no. positive/no. tested)	
	IFA ^a	RT-PCR ^b
<i>Wolbachia</i> -infected	76.67 (23/30)	86.57 (13/15)
<i>Wolbachia</i> -free	73.33 (22/30)	80.00 (12/15)

^a IFA: Immunofluorescent antibody test ($\chi^2 = 0.08$; $P > 0.05$).
^b $\chi^2 = 0.24$, $P > 0.05$.

ther replication before transmission can occur (Doi et al. 1967, Doi 1970, Leake and Johnson 1987). Although the medial lobe of SGs can be infected, JEV infection frequently occurred in proximal-lateral lobes (Fig. 1). In ultrastructure, JE virions were clustered in the cytoplasm and more commonly in apical cavities of SG cells in the proximal region of lateral lobes (Fig. 4). The apical cavity in this region is usually irregular in morphology and contains a lightly filamentous material (Yen and Barr 1973). *Wolbachia* also occurred in SG cells of this region, but generally it was restricted in the cytoplasm (Fig. 5). It has been known that the proximal-lateral lobes are the primary area for synthesis of α -glucosidase, whereas the distal-lateral lobes are the primary synthesis sites for apyrase; these enzymes function in sugar feeding and blood feeding, respectively (Marinotti et al. 1996). Whether the distribution of *Wolbachia*, JEV, or both is related to specific enzyme synthesis is not clear. At least, colocalization of *Wolbachia* with clustered JE virions revealed that these two microorganisms live in same environment but in different ecological niches (Fig. 6).

Fig. 2. Infection of JEV (T1P1 strain) was detected by RT-PCR in salivary glands dissected from *Ar. subalbatus* by intrathoracically inoculated with virus suspension and measured 10 d postinoculation. M, 100-bp DNA ladders; Lane 1, positive control from which RNA templates were extracted from infected C6/36 cells. Lanes 2–6, SGs from virus-inoculated mosquitoes.

Fig. 3. Changing trend of daily titers of JEV (T1P1 strain) grown in salivary glands of *Wolbachia*-infected and -free colonies of *Ar. subalbatus*.

Actively respiring mitochondria characterized as a marked condensation of the cristae were found in the periphery of apical cavities in SG cells infected by JEV (Fig. 4). Migration of mitochondria has been reported in the cytoplasm toward one side of the nucleus in herpes simplex virus or viral assembly sites in African swine fever virus (ASFV)-infected cells (Rojo et al. 1998, Murata et al. 2000). In ASFV-infected cells, mitochondrial alteration from the resting state to actively respiring state was thought to supply energy effectively for the virus morphogenetic process (Rojo et al. 1998). The dramatic shift in the morphology of mitochondria during the assembly process of virus particles is known to be compatible with an increase in respiratory function and ATP production by their mitochondria (Rojo et al. 1998). Eventually, it could lead to an increased production of reactive oxygen species and subsequent damage to mitochondrial DNA and protein (Rojo et al. 1998). In the current study, damaged SG cells were actually observed in *Ar. subalbatus* heavily infected by JEV (data not shown), although its relationship with colocalizing *Wolbachia* remains to be clarified. A degeneration of SGs also has been observed in adult female *Culex*

Fig. 4. Actively respiring mitochondria characterized as a marked condensation of the cristae (thick arrow) were located in the periphery of the apical cavity (AC) of SG cells infected by T1P1 strain of JEV (thin arrow). Bar = 1 μ m.

Fig. 5. (a) *Wolbachia* was observed in the lateral lobe of salivary glands of *Ar. subalbatus*. Bar = 2 μ m. (b) Magnification of the rectangular area shown in a. Bar = 0.2 μ m.

tarsalis (Coquillett) coinfecting with two noninclusion cytoplasmic viruses (Richardson et al. 1974).

Both *Ae. aegypti* and *Ae. albopictus* are thought to be vectors of dengue fever and several other arboviruses (Metselaar et al. 1980, Rai 1991, Gubler 1998, Johnson et al. 2002). However, in the past 25 yr, the global resurgence of epidemic dengue/dengue hemorrhagic fever was thought to primarily be transmitted by *Ae. aegypti*, not *Ae. albopictus* (Degallier et al. 2003, Gubler 2003). Unlike *Ae. aegypti*, *Ae. albopictus* is generally infected with *Wolbachia* groups A and B (Tsai et al. 2004). It is possible that the endosymbiont harbored by mosquitoes may play a role as a competitor, which might be deleterious to the virus or which would make SG cells refractory to subsequent viral infection or transmission. Such species competition between *rickettsia* infections in cat fleas has been documented (Williams et al. 1992). However, these two *Aedes* mosquitoes possessed insignificantly different susceptibility to dengue virus in field-caught mosquitoes (Chow et al. 1998) and to dengue or yellow fever virus in experimental infections (Whitehead et al. 1971, Johnson et al. 2002). There might not be an evident mutual effect between *Wolbachia* and the

Fig. 6. *Wolbachia* (solid arrows) occurred in cytoplasm of the salivary gland cell closely to the apical cavity (AC) in which T1P1 strain of JE virions were clustered (empty arrows). Virions also were seen in the vacuole (Va) located in the cytoplasm. Bar = 0.5 μ m.

virus, even if both of them eventually co-reside within the SG cell. In turn, *Wolbachia* probably did not function to elevate SG escape barrier. Regardless, whether the endosymbiont would interfere with the transmission of arboviruses remains to be demonstrated, especially regarding its effect on depositing viral particles into secretory products within SG lumina. It has been reported that the striking number of viral particles in SG luminal saliva was consistent with the transmission efficiency of arthropods in nature (Whitfield et al. 1971).

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