**Wolbachia** and Bacteriophage WO-B Density of **Wolbachia** A-Infected *Aedes albopictus* Mosquito

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**ABSTRACT.** *Wolbachia* are maternally inherited symbiotic bacteria capable of inducing an extensive range of reproductive abnormalities in their hosts, including cytoplasmic incompatibility (CI). Its density (concentration) is likely to influence the penetrance of CI in incompatible crosses. The variations of *Wolbachia* density could also be linked with phage WO density. We determined the relative density (relative concentration) of prophage WO orf7 and *Wolbachia* (phage-to-bacteria ratio) during early developmental and adult stages of singly infected *Aedes albopictus* mosquito (*Wolbachia* A-infected) by using real-time quantitative PCR. Phage WO and *Wolbachia* did not develop at the same rate. Relative *Wolbachia* density (bacteria-to-host ratio) was high later in development (adult stages) whilst relative prophage WO density (phage-to-bacteria ratio) was low in the adult stages. Furthermore, 12-d-old adults of singly infected female mosquito had the highest *Wolbachia* density. In contrast, the larval stage 4 (L4) contained the highest prophage WO-B orf7 density. The association of hosts–*Wolbachia*–phage among diverse species is different. Thus, if phage and *Wolbachia* are involved in CI mechanism, the information of this association should be acquired for each specific type of organism for future use of population replacement or gene drive system.

**Abbreviations**

<table>
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<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>CI</td>
<td>cytoplasmic incompatibility</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>RH</td>
<td>relative humidity</td>
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<td>RTQ-PCR</td>
<td>real-time quantitative PCR</td>
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<td>STE</td>
<td>sodium chloride–Tris–EDTA</td>
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*Aedes albopictus* is one of the important vectors of dengue fever in various parts of the world (Kambhampati and Rai 1991; Kambhampati et al. 1991; Knudsen 1995). In nature, most of them harbor two types of bacteria *Wolbachia*, wAlbA and wAlbB. *Wolbachia* are a group of intracellular inherited bacteria that infect a wide range of arthropods. They are associated with a variety of reproductive alterations in their hosts, the best known being cytoplasmic incompatibility (CI; Kittayapong et al. 2000). Incompatibility can occur between the sperm of an infected male and the egg of an uninfected female or between the sperm of an individual infected with one strain and the egg of an individual infected with a different strain. Thus matings between infected males and uninfected females are sterile but the reciprocal matings are fertile. Hence uninfected females are at risk of failing to transmit their uninfected cytoplasm, if they cross mate, but infected females are at no such risk. Therefore natural selection favors the infected state (Curtis and Sinkins 1998).

CI promotes the spread of *Wolbachia* through populations and, as a result, has been proposed as a gene-driving system for the distribution of disease-blocking transgenes through populations of mosquito vectors. At present, effective gene drive systems for spreading genes that can block the transmission of insect-borne pathogens are much needed (Sinkins and Gould 2006) for population replacement and in reducing disease transmission. In *A. albopictus* these dynamics are extremely favorable, with very high maternal transmission fidelity and levels of incompatibility recorded. Correspondence between measurements taken in the laboratory and field is much better than in the *Drosophila simulans* model system (Sinkins 2004).

The exact mechanisms by which *Wolbachia* induce CI are still unknown. Several factors have been found to modulate CI strength (i.e. egg hatchability), such as bacterial and host genotypes or bacterial density, and these factors may interact in complex ways (Weeks et al. 2002). *Wolbachia* density is likely to influence the rate of maternal transmission and could also affect the penetrance of CI in incompatible crosses (Sinkins 2004).

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The lack of congruence between the phylogeny of *Wolbachia* and incompatibility types has led some to propose that genes responsible for *Wolbachia* incompatibility are conveyed by extrachromosomal particles, such as plasmid or phages (Guillemaud et al. 1997; Stouthamer et al. 1999). Masui et al. (2000) identified a bacteriophage-like genetic element of *Wolbachia*, which was tentatively named bacteriophage WO. It has been suggested that this element could have a significant effect on genome organization and host reproduction, and might increase the rate of evolution (Brownlie and O’Neill 2005). The variations of *Wolbachia* density could also be associated with phage WO density as observed in the *Nasonia* wasp (Bordenstein et al. 2006). Thus, phages might replicate independently from *Wolbachia* and play a significant role in the expression of CI (Duron et al. 2006).

We determined prophage WO orf7 along with *Wolbachia* densities in singly infected female *A. albopictus* mosquito to improve the knowledge in the correlation of phage WO orf7 and *Wolbachia* replication during mosquito development and in specific adult age.

**MATERIALS AND METHOD**

*Mosquito specimens*. The female mosquito *A. albopictus* KOH (wAlbA infected from Koh Samui, Thailand; Kambhampati et al. 1993) was used. All mosquitoes were naturally *Wolbachia* A-infected. Female mosquitoes were mated and blood-fed. The mosquito colony was maintained in the insectary at the Center for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University (Thailand) at 75 % RH and 25–27 °C.

**DNA extraction and PCR detection.** All DNA from mosquito samples was extracted using the crude boiling method of O’Neill et al. (1992). Five mosquitoes and larvae were used in each specific stage, 100 for egg stage. All mosquito samples (egg, larva, pupa and adult – 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 d) were ground and homogenized in 100 µL of STE buffer (in mmol/L: NaCl 100, Tris-HCl 10, EDTA 1; pH 8.0), heated for 10 min at 95 °C and centrifuged.

**PCR amplification was done on a Hybaid OmniGene thermal cycler using 20-µL volumes of reaction mixture to check for the presence of phage WO-B using WOorf7 primers: WOorf7F (5’-GAA ATG CTT GTT CAG GTA ATA GC-3’) and WOorf7R (5’-ATA AAT TCT CTT ATT TTT TCT GGC A-3’) (Masui et al. 2000).** The PCR thermal profile used was: 1 cycle (3 min 95 °C) followed by 35 cycles (30 s 95 °C, 30 s 52 °C, and 1 min at 72 °C), and 1 cycle of 5 min at 72 °C. Each PCR reaction mixture contained 13 µL double-distilled H2O, 2 µL 10× buffer (Promega), 2 µL 25 mmol/L MgCl2, 0.5 µL dNTPs (10 mmol/L each), 0.5 µL of 20 µmol/L primers, 1 U of Taq DNA polymerase (Promega) and 2 µL of template DNA. Defensin primers (Ruang-Areerate and Kittayapong 2006) (encoding an insect immunity of the mosquito) were used as a quality control for DNA extraction.

**RTQ-PCR.** The relative densities of prophage WO in mosquitoes were quantified by a real-time PCR-based method in an ABI PRISM® 7000 Sequence Detection System (*Applied Biosystems*). The amplification reaction was monitored using a SYBR green. Each run consisted of a series of DNA standards prepared from plasmid DNA, containing phage WO orf7 from *Wolbachia*-infected *A. albopictus* mosquitoes (with $10^3$–$10^6$ copies of standard DNA as template). Two and three replicated reactions were done for each standard and sample, respectively. PCR products were cloned into pGEM-T vectors (*Promega*) according to the manufacturer’s recommendations. Quality and concentration of all purified standard DNA were determined spectrophotometrically at 260 nm; molar concentrations and the orf7 gene copy number of all DNA were calculated after the determination of $A_{260}$.

The signal curves of standards and samples measured in the same run were used for quantification and done automatically using software. The reaction mixture (25 µL) which consisted of 12.5 µL of 2× SYBR® Green PCR Master Mix (*Applied Biosystems*), 500 nmol/L of each primer, 2 µL of template DNA sample (or standard DNA) was used in each well. The real-time PCR cycling included 1 cycle (3 min 95 °C) followed by 45 cycles (30 s 95 °C, 30 s 52 °C) and, finally, 1 cycle (30 s, 72 °C).

**Primers used for *Wolbachia* copy quantitation were GF (5’-GGT TTT GCT GGT CAA GTA A-3’) and AR (5’-GCA TCT TGG GTA ACT ACT TTT-3’) (Ruang-Areerate and Kittayapong 2006).** The real-time PCR cycling consisted of 1 cycle (15 min at 95 °C), followed by 45 cycles (1 min 94 °C, 1 min 50 °C) and 1 cycle (1 min at 60 °C). Defensin primers were used to quantify mosquito gene copy numbers (Ruang-Areerate and Kittayapong 2006).

All statistical analysis was done using SPSS (one way ANOVA; at $\alpha = 0.05$).