**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**

CI cytoplasmic incompatibility  
EDTA ethylenediaminetetraacetic acid  
PCR polymerase chain reaction  
RH relative humidity  
RTQ-PCR real-time quantitative PCR  
STE sodium chloride–Tris–EDTA

*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 WO orf7 primers: WOorf7F (5’-GAA ATG CTT GTT CAG GTA ATA GC-3’) and WOorf7R (5’-ATA AAT TCT CCT 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 102–109 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 A260.

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 $\alpha = 0.05$).
RESULTS

Phage WO-B orf7 density in egg and L1 stages were very low compared with the other stages (Table I), nevertheless, the prophage copy numbers in these two stages were detected. However, when compared with Wolbachia copy number (phage-to-bacteria ratio), the relative densities were still very low comparing to the others. The relative density of prophage WO-B orf7 was high in L2 and significantly higher ($p = 0.00$) than L3 and pupal stages ($p = 0.00$). The highest density level was found in L4 with significant differences ($p = 0.000$ in all categories; compared with egg, L1, L2, L3 and pupa).

Table I. Relative prophage WO-B orf7 (WO-B) and Wolbachia (WO) density$^a$ during egg to pupal stages and during the adult stages of $A$. albopictus KOH

<table>
<thead>
<tr>
<th>Egg to pupal stages$^b$</th>
<th>egg</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>pupa</th>
</tr>
</thead>
<tbody>
<tr>
<td>WO-B</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>56.2 ± 5.0</td>
<td>12.8 ± 1.1</td>
<td>103 ± 9.8</td>
<td>29.0 ± 2.4</td>
</tr>
<tr>
<td>WO</td>
<td>0.046 ± 0.007</td>
<td>0 ± 0</td>
<td>0.002 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

| Adult stages, d |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Egg to pupal stages$^b$ |                |                |                |                |                |
| WO-B ($\times 10^{-4}$) | 6.6 ± 1.3 | 22.9 ± 3.4 | 4.9 ± 2.2 | 12.8 ± 1.5 | 10.3 ± 4.5 | 1.5 ± 0.6 | 22.0 ± 4.7 | 20.4 ± 7.0 | 38.8 ± 6.3 |
| WO               | 3.4 ± 0.4 | 2.8 ± 0.2 | 5.4 ± 0.5 | 11.0 ± 1.1 | 2.3 ± 0.1 | 6.0 ± 0.6 | 9.2 ± 0.1 | 6.3 ± 0.6 | 6.3 ± 0.7 | 8.5 ± 1.0 |

$^a$Means of relative phage WO-B orf7 densities (phage-to-bacteria ratio) and Wolbachia densities (bacteria-to-host ratio) ±SD.

$^b$L1–L4 – larval stage 1 to stage 4.

Relative Wolbachia densities in egg, L1, L2, L3, L4 and pupal stages were very low compared with the adult stages (see Table I). All larval and pupal stages did not show any relative Wolbachia density (but with some amount of Wolbachia copy numbers which were very rare compared with host cell copy numbers, defensin gene). Egg had the highest Wolbachia density than the other stages but with no significant difference ($p = 1.00$). Relative Wolbachia density of all the adult stages was remarkably higher than that of all earlier stages (egg to pupa). Twelve-d-old adult mosquito had the highest Wolbachia density with significant differences compared with adult stages 3–30 d ($p = 0.000$ in all categories).

All prophage WO-B orf7 relative densities were very low in adults (phage-to-Wolbachia ratio in the range of 0.0001–0.004); nevertheless, the prophage density in all adult stages was lower than in the egg (phage-to-Wolbachia ratio, relative density of 0.012). All adult stages of the mosquito here had relative prophage density significantly lower than in the earlier stages except for L1.

Among all adult stages 30-d-old adults had the highest prophage density and 21-d-old adults contained the lowest prophage WO orf7 density. Earlier stages in singly Wolbachia-infected A. albopictus contained higher prophage density than in the later development and, as the result showed, opposite correlation with Wolbachia density.

When we compare prophage WO-B orf7 and Wolbachia density in early development, the prophage WO-B density was by several orders higher than the relative Wolbachia density. Wolbachia and prophage WO-B orf7 showed nearly inverse relationship in L2, L3, L4 and pupal stages but not directly proportional.

DISCUSSION

This work partly supported the results of Bordenstein et al. (2006) who showed that, in Nasonia wasp, the phage density is negatively associated with Wolbachia density. The identification of factors that can modulate the CI rate remains a pivotal step in the understanding of the basic mechanisms responsible for incompatibility. The study by Duron et al. (2006) in Culex pipiens differs from those obtained in other insect species, suggesting that hypotheses drawn from the Drosophila model cannot be generalized directly. It would appear wise to conduct experimental studies on a wide range of hosts–Wolbachia–phage association before constructing a general model of the host–reproductive parasite interactions (Duron et al. 2006). Re-
revealing the microbial genetic factors that modulate CI will significantly enhance our understanding of how Wolbachia endosymbionts override normal host reproductive strategies (Bordenstein et al. 2006).

From the results of Wolbachia and phage WO-B orf7 density assays, prophage and Wolbachia did not develop at the same rate. The high level of Wolbachia density was found in the adult stages while prophage WO-B density was very low. There were fluctuations of phage and Wolbachia densities in each specific age of female mosquito. While Wolbachia densities were positively associated with incompatibility levels in many systems (Poinsot et al. 1998; Perrot-Minnot and Werren 1999; Noda et al. 2001; Veneti et al. 2003), phage replication could lead to a reduction in CI (Bordenstein et al. 2006) which corresponds to the results obtained in singly infected A. albopictus mosquito.

The association of hosts–Wolbachia–phage among diverse species requires further investigation for each one of them before considering the CI mechanism of each organism and/or for population replacement in the field. Our study is the first that shows the relative Wolbachia density in correlation to prophage WO-Orf7 density in female singly infected A. albopictus mosquito, improving the recent knowledge and supporting the possible involvement of phage WO-B in the CI mechanism. Prophage WO-B might be related to the CI mechanism due to the reduction of density in the adults. Prophage and Wolbachia relative density in female A. albopictus provide additional information which can contribute to elucidating the CI mechanism and to improving the data for the use of Wolbachia as a gene drive system.

Crossing experiments with laboratory colonies showed that aged superinfected males could express strong CI when mated with young uninfected or wAlbA infected females; these results provide additional evidence that the CI properties of Wolbachia infecting A. albopictus are well suited for application strategies that seek utilization of Wolbachia in host-population modification (Kittayapong et al. 2002). The CI expression ought to be further investigated in singly infected A. albopictus mosquito to explain the role of phage WO orf7 and wAlbA in the CI mechanism.

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