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Transfer of a parthenogenesis-inducing *Wolbachia* endosymbiont derived from *Trichogramma dendrolimi* into *Trichogramma evanescens*

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ABSTRACT

Wolbachia, which are maternally transmitted endosymbionts, are considered to have moved horizontally between invertebrate hosts multiple times. However, it is not well understood how easily *Wolbachia* are transmitted horizontally between different hosts and how frequently horizontally-transmitted *Wolbachia* become established in their new hosts. We transferred a parthenogenesis-inducing *Wolbachia* endosymbiont derived from the parasitic wasp *Trichogramma dendrolimi* to *Trichogramma evanescens*. Specifically, *Wolbachia* was cultivated in a mosquito cell line and the *Wolbachia*-infected cells were microinjected into uninfected *T. evanescens*. Among 276 pupae inoculated with *Wolbachia*-infected cells, 65 adults emerged (G0). Diagnostic PCR demonstrated that 25 of 37 G0 females (68%) were *Wolbachia*-positive. Among isofemale lines established from G0 females, the proportions of infected lines were 80% (20 of 25) in G1 and 100% (18 of 18) in G2. In an isofemale line, infection was stably maintained for more than 10 generations. These results indicate invasion of *Wolbachia* into the germline of the recipient insect. Quantitative PCR demonstrated that the *Wolbachia* titer in the recipient host was significantly lower than that in the native host. The absence or very low number, if any, of parthenogenetically-reproducing individuals in the recipient host may be caused by the low *Wolbachia* titer. The *Wolbachia* titer in the recipients was lower in G11 than in G5, suggesting a decline in the density. Together with a previous report, our study may imply that *Wolbachia* in *Trichogramma* species are highly adapted to their hosts, which hinders robust expression of the *Wolbachia* phenotype in non-native host species.

1. Introduction

Wolbachia pipientis (also referred to as *Wolbachia*) is a bacterium belonging to the alpha-subdivision of Proteobacteria and is the most ubiquitous endosymbiont among arthropods and filarial nematodes (Zug and Hammerstein, 2012). Various effects on insect reproduction, such as cytoplasmic incompatibility, parthenogenesis induction, male killing, and feminization, have attracted particular attention, but at present, very little is known about their underlying mechanisms (Werren et al., 2008). *Wolbachia* is maternally inherited within the host lineage and does not spread in an infectious manner. However, the topological incongruence observed between the phylogeny of *Wolbachia* and that of its hosts suggests that horizontal transmission of *Wolbachia* has occurred repeatedly in the evolutionary timescale (possibly via parasitism, predation, or cannibalism) (Werren et al., 2008). At present, it is not well understood how often *Wolbachia* are transmitted horizontally between different hosts and how frequently horizontally-transmitted *Wolbachia* become established in their new hosts. To date, experimental transfer of *Wolbachia* endosymbionts between different host species has been repeatedly achieved (Braig et al., 1994; Grenier et al., 1998; Walker et al., 2011). However, the extent to which *Wolbachia* transfer is feasible is not precisely understood, because successful cases are more favorably published.

In the present study, we transinfected a parthenogenesis-inducing *Wolbachia* derived from the parasitic wasp *T. dendrolimi* into the OK94 strain of *Trichogramma evanescens*. Since *T. evanescens* OK94 is highly tolerant of high temperatures (Inoue and Kubo, 2001) and thus a promising candidate for use in greenhouses as a natural enemy against various lepidopteran pests, induction of parthenogenesis would reduce the cost of rearing and contribute to the mass production of this favorable insect.

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2. Materials and methods

2.1. Insects

The *Wolbachia*-infected strain of *T. dendrolimi* and uninfected *T. evanescens* OK94 were reared on UV-irradiated *Ephestia kuehniella* eggs at 25 ± 1 °C and 65 ± 20% relative humidity under a 16-h light/8-h dark photoperiod. *T. evanescens* OK94 is a strain derived from a single female collected in the field, and has been maintained in the laboratory for more than 12 years.

2.2. Cell line

A cell line, NIAS-AeAl-2, derived from *Aedes albopictus* was provided by Dr. Hiroaki Noda in the National Institute of Agrobiological Sciences, Tsukuba, Japan (Noda et al., 2002). The insect cell line was maintained in IPL-41 culture medium (Life Technologies Japan Ltd., Tokyo, Japan) supplemented with 4–5% fetal bovine serum (Cosmo Bio Co. Ltd., Tokyo, Japan) at 25–28 °C using 50-ml plastic culture vessels without CO_2 gas incubation (Noda et al., 2002).

2.3. Inoculation of Wolbachia into the cell line

Wolbachia derived from *T. dendrolimi* (*w*Den) was inoculated into the NIAS-AeAl-2 cell line as previously described (Kubota et al., 2005). After *T. dendrolimi* female adults were surface-sterilized with 70% ethanol for a few minutes, the ovary was dissected in a droplet of IPL-41 medium and then crushed with insect pins in a new droplet of medium containing NIAS-AeAl-2 cells. The droplet containing the ovary fragment homogenate was used as the inoculum and introduced into a 50-ml plastic culture vessel containing the cells. The vessel was kept at 25 ± 1 °C until the cells became confluent, at which point a portion of the cells (approximately 20%) was introduced into a new culture vessel and provided with fresh medium. This handling of the cells was usually performed once every 7–10 days. During the maintenance of the inoculated cell culture, uninfected healthy cells were sometimes added. The inoculated cell culture was observed using an inverted phase contrast microscope (TMD300; Nikon, Tokyo, Japan).

2.4. Detection of Wolbachia in the inoculated cell culture

To confirm active proliferation of *Wolbachia* in the cell culture, the cells were sampled every 2–3 days. After centrifugation of the cells at 1100 × *g* for 5 min, the supernatant was removed, and the cell pellet was resuspended in 32 μl of STE buffer (5 N NaCl, 500 nM EDTA pH 8.0, 1 M Tris–HCl pH 8.0) and 2 μl of proteinase K (0.5 mg/ml). This mixture was sequentially incubated at 56 °C for 2 h and 99.9 °C for 3 min. Diagnostic PCR for *Wolbachia* infection was performed using the *Wolbachia*-specific primers *wsp*81F and *wsp*691R that amplify a partial sequence of the *wsp* gene (Braig et al., 1998). Quantification of *Wolbachia* titers was performed by fluorescence real-time quantitative PCR (see below).

2.5. Microinjection of Wolbachia-infected cells into T. evanescens

The *w*Den-infected *A. albopictus* cells after cultivation for more than 3 months was used as a donor for microinjection. The cells constituting a confluent monolayer were gently removed from the 25-ml flask and the volume of 1.5 ml (containing cells and IPL-41 medium) was centrifuged at 1100 × *g* for 5 min. The supernatant was removed and the cell pellet resuspended in 200-ml SPG buffer (218 mM sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, 4.9 mM L-glutamate, pH 7.2) (Xi and Dobson, 2005) was used as a donor for microinjection within 30 min.

An uninfected strain of *T. evanescens* OK94 was used as a recipient for microinjection. At 25 °C, this insect usually pupates at 9–10 days after oviposition. The pupation can be recognized by dark pigmentation of the *E. kuehniella* eggs. *E. kuehniella* eggs in which fully developed wasp pupae (with red eyes) were recognizable were aligned on double-sided tape using a paintbrush. Microinjection needles were made from GD-1 borosilicate glass capillaries (Narishige, Tokyo, Japan) using a PN-3 needle puller (Narishige). Using a disposable razor blade, the tip of a needle was cut to make it approximately 7 μm in diameter. The *Wolbachia*-containing SPG buffer described above was microinjected into the ventral side of *T. evanescens* pupae by penetrating the chorion of *E. kuehniella* eggs using an M152 3D-micromanipulator (Narishige) and an IM-6 microinjector (Narishige) under a TMD300 dissecting microscope (Nikon, Tokyo, Japan).

2.6. Detection of Wolbachia in the recipient wasps

Isofemale lines were established from each of the microinjected *T. evanescens* females. After mass-mating and oviposition, all individuals in each isofemale line were squashed in a 1.5-ml microtube and subjected to the above-mentioned proteinase K treatment. Diagnostic PCR for *Wolbachia* infection performed as described above allowed us to detect the presence or absence of *Wolbachia* in each line.

2.7. Nucleotide sequencing

To confirm that the detected *Wolbachia* in the recipient wasps were *w*Den, the PCR products of the partial sequence of the *Wolbachia wsp* gene were directly sequenced using a BigDye DNA Sequencing Kit ver. 3.0 (PE Applied Biosystems, Tokyo, Japan). The sequencing products were evaluated using an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

2.8. Quantitative PCR

Quantitative PCR was carried out with real-time detection of the 16S rRNA gene of *Wolbachia* in the cell line (*A. albopictus*), the native host (*T. dendrolimi*) and the new host (*T. evanescens*) using an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems). Whole *A. albopictus* cells in the vessels were harvested at intervals of several days, and PCR templates were prepared with the above-mentioned proteinase K treatment. When the bottom of the vessel was covered with a confluent cell monolayer (9 days after inoculation), the cells were diluted fivefold. Six adults were harvested from the native host *T. dendrolimi* (female). Six adults were harvested from the recipient host (*T. evanescens*; female) in each of the G5 and G11 generations. The *Wolbachia* 16S rRNA gene was amplified by PCR using the primers Triwol_16S-8F (5′-GCGCGTAGGCTGGTTAATAAGT-3′) and Triwol_16S-153R (5′-TGGGTGTTCCTCCTAATATTTACGA-3′), and a single-stranded DNA probe RT16S (5′-TCCCGAGGCTTAACCTTGGAATTGCT-3′) as previously described (Kubota et al., 2005).

The *Wolbachia* density data obtained by quantitative PCR were subjected to statistical analyses using R ver. 2.4.0 software (R Development Core Team, 2005). As some of the data sets did not exhibit a normal distribution and/or homogeneous variance, we adopted a generalized linear model (McCullagh and Nelder, 1989) for Gaussian, inverse Gaussian, gamma, or negative binomial distributions, which was selected according to the Akaike information criterion.