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Transinfection of *Wolbachia* in the Mediterranean Flour Moth, *Ephestia kuehniella*, by Embryonic Microinjection

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Wolbachia are maternally transmitted intracellular bacteria found in many arthropod species. They cause a reproductive incompatibility called cytoplasmic incompatibility (CI) in several hosts, including the Mediterranean flour moth, *Ephestia kuehniella*. Two strains of *E. kuehniella*, one from Yokohama city and the other from Tsuchiura city, express different levels of CI: the Yokohama strain expresses CI at a higher level than the Tsuchiura strain. In order to determine whether the difference of CI levels depends on *Wolbachia* or the host, we performed transinfection experiments in *E. kuehniella* by means of embryonic microinjection, and successfully transferred *Wolbachia* carried by the Yokohama strain into the Tsuchiura strain, from which the original *Wolbachia* had been removed by tetracycline treatment. The resulting transinfected strain expressed CI at a level near that of the Yokohama strain, suggesting that, in these strains of *E. kuehniella*, the level of CI is determined by *Wolbachia* rather than by the host.

Keywords: cytoplasmic incompatibility, embryonic microinjection, *Ephestia kuehniella*, *Wolbachia*.

Introduction

Wolbachia are maternally transmitted bacteria found in many insects and other arthropods. *Wolbachia* infections often lead to reproductive alterations of the hosts, including thelytokous parthenogenesis, feminization of genetic males into functional females, male-killing and cytoplasmic incompatibility (CI) (reviewed in Stouthamer *et al*., 1999). CI is a reproductive incompatibility that results in the death of the embryo. In the simplest case, CI is expressed when *Wolbachia*-infected males mate with uninfected females.

The strength of CI varies among host species (Bourtzis & O'Neill, 1998). Almost complete incompatibility has been observed in flour beetles of the genus *Tribolium* (Wade & Steven, 1985), the almond moth *Ephestia cautella* (Brower, 1976; Kellen *et al*., 1981; Sasaki & Ishikawa, 1999) and the small brown planthopper *Laodelphax striatellus* (Noda, 1984). Weak expressions of CI have been reported for *Drosophila melanogaster* (Hoffmann *et al*., 1994), *D. sechellia* (Giordano *et al*., 1995) and *D. ananassae* (Bourtzis *et al*., 1996). The strength of CI also varies even within a species depending on the *Wolbachia* variant (Merçot *et al*., 1995). Some *Wolbachia* that infect *D. simulans* induce relatively strong CI (Hoffmann *et al*., 1986; O'Neill & Karr, 1990), while others do not induce detectable CI (Rousset & Solignac, 1995; Hoffmann *et al*., 1996; Bourtzis *et al*., 1998; Merçot & Poinsot, 1998).

Does the level of CI vary due to the difference in *Wolbachia* or hosts? To answer this question, investigators have transferred *Wolbachia* between different hosts. In some experiments, *Wolbachia* transferred into new hosts expressed CI as they did in their natural hosts, suggesting that the strength of CI largely depends on *Wolbachia* (Rousset & De Stordeur, 1994; Giordano *et al*., 1995; Sinkins *et al*., 1995; Clancy & Hoffmann, 1997). On the other hand, other experiments supported the view that the host plays an important role in the determination of the CI level. When a *Wolbachia* variant known to induce strong CI was transferred from *D. simulans* into *D. melanogaster*, the transferred *Wolbachia* did induce CI in *D. melanogaster* but at a lower level than in *D. simulans* (Boyle *et al*., 1993). Poinsot *et al*. (1998) performed the converse transinfection, namely from *D. melanogaster* to *D. simulans*, and reported that the resulting transinfected lines expressed almost complete CI, even though the donor *D. melanogaster* showed only weak CI.

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We previously examined *Wolbachia* infection in two strains of the Mediterranean flour moth *Ephestia kuehniella*, one from Yokohama city and the other from Tsuchiura city. Both strains were infected with *Wolbachia* that belonged to the A group designated by Werren *et al*. (1995). Phylogenetic analysis based on the *wsp* gene, the gene coding for a *Wolbachia* surface protein (Braig *et al*., 1998), suggested that the two *Wolbachia* variants are closely related: there was only one synonymous nucleotide substitution between the two sequences. Crossing experiments showed that the Yokohama-strain males expressed significantly higher levels of CI in both intrastrain and interstrain crosses with uninfected females than did the Tsuchiura-strain males (Sasaki & Ishikawa, 1999). Does the difference in the CI levels expressed by the two strains depends on *Wolbachia* or the host? In the present study, in an attempt to answer this question, we performed transinfection experiments in *E. kuehniella* by means of embryonic microinjection, and successfully transferred *Wolbachia* from the Yokohama strain to the Tsuchiura strain. The level of CI expressed by the transinfected strain was comparable to that of the donor strain.

Materials and methods

Insects

Two laboratory strains of the Mediterranean flour moth *Ephestia kuehniella* were reared on a diet consisting of wheat bran, dried yeast and glycerol (20:1:2 w/w) at 25°C with a 16-h light: 8-h dark photoperiod. They had been collected originally in Yokohama and Tsuchiura, cities located in the central part of the main island of Japan, and have since been maintained in laboratories for several years. *Wolbachia*-uninfected strains were established by rearing the insects on a diet containing tetracycline at a final concentration of 0.04% (w/w) for two generations (Sasaki & Ishikawa, 1999). All strains were maintained under uncrowded conditions at a density of about one larva per 0.5 g of the diet.

Microinjection

Injections were performed at room temperature using a microscope (Nikon SMZ-U) equipped with a 3D micromanipulator (Narishige MMN-1) and a microinjector (Narishige IM-6). A 1×90 mm Pyrex glass capillary was pulled in a Narishige needle puller (PN-3), and the tip was cut manually with a razor to make an angled point with a diameter of about 8 μm. The microneedle was connected to the microinjector by a Teflon tube filled with water.

Freshly laid eggs (< 1-h old) of the uninfected Tsuchiura strain were attached to a piece of double-sided tape on a slide. The eggs were aligned near the edge of the tape with a small paint brush. Ovaries of the donor strain (Yokohama strain) collected by dissection from the adult females were also placed on a piece of double-sided tape on a slide. The ooplasm taken out of the donor ovary was injected into one end of each recipient egg at random because the posterior and anterior ends of the ellipsoidal egg of *E. kuehniella* can not be clearly distinguished. This protocol allowed us to inject approx. 50 eggs per hour. The injected eggs on the slide were placed in a plastic dish (9-cm diameter) containing a piece of moist filter paper, and kept at 25°C. Five days after injection, the sticky surface of the tape was covered with flour, and the slide was transferred onto the diet in a plastic container. The eggs hatched on the sixth or seventh day after injection. Because the larvae moved into the diet, counting the cast-off shells left on the tape checked the rate of egg hatching.

Adults emerged about one month after eggs hatched. Females were then transferred individually into 30 mL plastic cups and mated with males. The males used were either those developed from the injected eggs or those collected from the stock culture of the uninfected Tsuchiura strain. Eggs laid by each female were diagnosed for infection status by PCR.

Diagnostic PCR for detection of Wolbachia

The presence or absence of *Wolbachia* was tested by diagnostic PCR assays using *Wolbachia*-specific primers for the *ftsZ* bacterial cell-cycle gene. The template DNA for PCR was prepared from eggs or larvae by the crude STE boiling method (O'Neill *et al*., 1992). Ten to 20 eggs were homogenized in 50 μL of STE [100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] containing 0.4 mg mL^{-1} proteinase K, and incubated for 90 min at 55°C followed by 15 min at 95°C. After brief centrifugation, 1 μL of the supernatant was used as the template DNA for PCR. In order to prepare DNA from a single egg, the volume of the extraction solution was reduced to 15 μL. A larva was first homogenized in 100 μL of STE without proteinase K, and 10 μL of the homogenate was added to 90 μL of STE containing proteinase K.

PCR was performed in a 20 μL reaction mixture using Takara EX *Taq*. The primers were ftsZAdf (5′-CTC AAG CAC TAG AAA AGT CG-3′) and ftsZAdr (5′-TTA GCT CCT TCG CTT ACC TG-3′), which amplify the *ftsZ* gene of A-group *Wolbachia* (Werren *et al*., 1995). PCR cycling conditions were 94°C for 3 min followed by 35 amplification cycles of 94°C for

30 s, 55°C for 30 s and 72°C for 1 min, and finally 72°C for 5 min.

Crossing experiments

The adults of *E. kuehniella* do not feed, and survive for about one week. During this period, the female mates once or twice. Crossing experiments were performed using single pairs of virgin individuals. As the male larvae are easily distinguished by dark patches (the testes) on the back, we separated the females and males during the late larval stages. A female and a male, younger than 3 days after emergence, were set in a 30-mL plastic cup and left there for 3 days. Most females deposited more than 100 eggs onto the wall of the cup. Cups in which fewer than 50 eggs had been laid were discarded. From each pair, 50–100 eggs were collected and placed onto 1% agarose in a plastic dish (35-mm diameter). After incubation for 6–7 days at 25°C, the hatching rate was scored for each single pair cross. The data were analysed by Mann–Whitney *U*-tests.

Results

Establishment of transinfected strain

The ooplasm of *Wolbachia*-infected females of the Yokohama strain was injected into uninfected eggs of the Tsuchiura strain. Out of 321 injected eggs, 127 eggs hatched and 54 females and 41 males emerged. Insects that developed from the injected eggs were designated as generation 0 (G0). The first 50 females that emerged were mated to males for egg collection, while the four females that emerged later were discarded.

The presence of *Wolbachia* in the G1 eggs was examined by diagnostic PCR. At first, 10–20 eggs collected from each female were tested in bulk. In this first assay, we detected *Wolbachia* in 13 broods, which were named line 1 to line 13, respectively (Table 1). The *Wolbachia*-positive broods were then subjected to the second assay in which four eggs from each brood were tested individually. Nine broods, of which all the eggs examined were infected, were pooled as a transinfected strain. Three broods, lines 3, 8 and 11, comprised both infected and uninfected eggs. These were maintained separately, and their G2 eggs were re-examined by the same assays as performed on G1 eggs (Table 2). Testing eight broods for each line, we obtained nine infected broods that were added to the transinfected strain, which had been obtained by pooling the 9 broods of infected G1 eggs.

CI expressed by the transinfected strain

The transinfected strain was reared and PCR assayed to ensure that the infection was stable at G3 and G8. All of the 16 larvae selected at random for each generation were infected. The adult males of G3 and G8 were tested for the expression of CI by crossing them with the uninfected Tsuchiura-strain females (Fig. 1). CI expressed by the naturally infected males of the Tsuchiura and Yokohama strains were also examined simultaneously with the crossing experiment of G8 males. The

Table 2 *Wolbachia* infection in the G2 eggs of transinfected *E. kuehniella* Tsuchiura

	First assay		Second assay	
Line	No. of broods	Infection	No. of broods	Infection ratio
Line 3	5	–		
	3	+	3	4/4
Line 8	4	–		
	4	+	4	4/4
Line 11	6	–		
	2	+	2	4/4

Table 1 *Wolbachia* infection in *E. kuehniella* Tsuchiura injected with egg cytoplasm of *E. kuehniella* Yokohama

G0 females		First assay of G1 eggs		Second assay of G1 eggs	
No. of females	Egg collection	No. of broods	Infection	No. of broods	Infection ratio
6	unsuccessful				
44	successful	31	–		
		13	+	9	4/4
				1 (Line 8)	3/4
				2 (Lines 3 & 11)	1/4
				1 (Line 9)	0/4

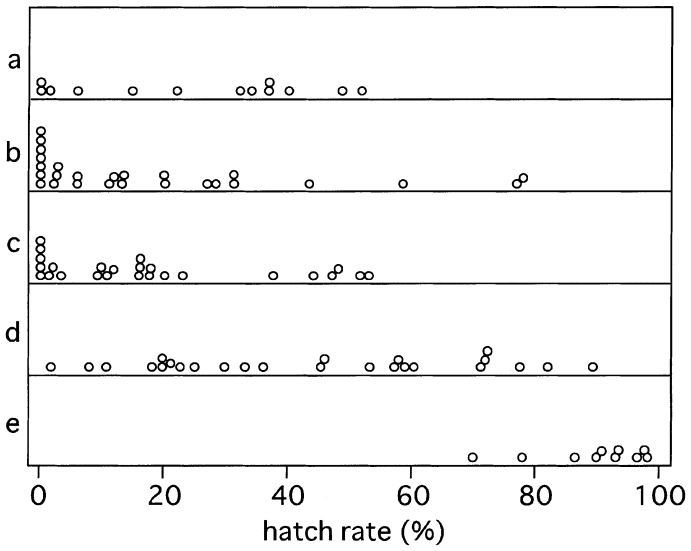

Fig. 1 Cytoplasmic incompatibility expressed by *Ephestia kuehniella*. Uninfected females of the Tsuchiura strain were crossed with transinfected males of generation 3 (a), transinfected males of generation 8 (b), infected Yokohama-strain males (c), infected Tsuchiura-strain males (d), and uninfected Tsuchiura-strain males (e).

levels of CI expressed by the transinfected strain were significantly different from those expressed by the naturally infected Tsuchiura strain (G3 and Tsuchiura: $U = 229$, $P < 0.05$; G8 and Tsuchiura: $U = 509$, $P < 0.001$). On the other hand, no significant difference in CI levels was observed among G3, G8 and the Yokohama strain.

Discussion

In the present study, we transferred *Wolbachia* between infected and uninfected strains of *E. kuehniella* by embryonic microinjection. To our knowledge, this is the first report of a successful transfer of *Wolbachia* in Lepidopteran insects.

To date, protocols of *Wolbachia* transfer have been developed for isopods and several species of insects. In isopods, *Wolbachia* can be transferred via the haemolymph by injection of homogenized tissues (Juchault *et al.*, 1994) and by simple wound-to-wound contact (Rigaud & Juchault, 1995). Grenier *et al.* (1998) reported a method for the transfer of *Wolbachia* in *Trichogramma* wasps, in which purified *Wolbachia* from an infected donor was microinjected into pupae developed *in vitro*. In *Drosophila* (Boyle *et al.*, 1993) and *Tribolium confusum* (Chang & Wade, 1994), *Wolbachia* have been transferred by egg-to-egg microinjection. In a preliminary experiment, we conducted a haemolymph transfer in *E. kuehniella*. However, no infected line was obtained, suggesting that *Wolbachia* rarely or never reaches the germline via the haemolymph in this insect (our unpubl. obs.), and leading us to perform embryonic microinjection.

PCR assays of G1 eggs showed that three broods consisted of a mixture of infected and uninfected eggs. This imperfect transmission occurred probably because the amount of injected ooplasm was insufficient to infect all the germline cells with *Wolbachia*. However, such infection polymorphism within a brood disappeared in G2. Almost complete infections were also observed in both G3 and G8: i.e. all of the randomly selected larvae were shown to be infected by the PCR assay. The level of CI expressed by G3 males was comparable to that expressed by G8 males, suggesting that the transferred *Wolbachia* increased in number in the new host within 4 generations (G0–G3) and remained stable thereafter.

In comparison with naturally infected strains, the transinfected strain expressed CI at a level close to that of the donor strain. Accordingly, it was concluded that the difference in CI levels expressed by the Yokohama and Tsuchiura strains was due to differences in *Wolbachia* rather than in the host insects. Besides the difference in CI levels, the two strains of *E. kuehniella* differ in the colour of the adults, the duration of the larval development, and the sensitivity to a high larval density during rearing (our unpubl. obs.). Although there must thus be some genetical differences between the two strains, the difference in CI levels seems to be independent of the host nuclear background. In the crosses using the naturally infected Tsuchiura-strain males, the strength of CI varied greatly and the hatching rates of two crosses were higher than 80% (Fig. 1). This may lead one to suspect that there were some uninfected individuals in the population of the Tsuchiura strain. To eliminate this possibility, we examined the infection rate by testing 100 eggs randomly selected from the stock culture. All eggs were infected, suggesting that the maternal transmission rate is very high and that uninfected individuals seldom appear.

In this transfer experiment, we obtained 13 transinfected broods out of 321 injected eggs. The relatively

high yield of transinfected lines, in addition to the relatively easy protocol, increases the feasibility of using *E. kuehniella* for transinfection experiments. While we transferred *Wolbachia* intraspecifically in this work, it may be possible to apply the protocol to interspecific transfers of *Wolbachia*.

Among Lepidoptera, three distinct phenotypes of reproductive alterations have been observed. *Wolbachia* induce CI in *E. cautella* (Brower, 1976; Kellen *et al*., 1981) and in *E. kuehniella* (Sasaki & Ishikawa, 1999). *Wolbachia*-mediated male-killing has been reported in the butterfly *Acraea encedon* (Hurst *et al*., 1999). In the Asian corn borer *Ostrinia furnacalis*, a phenomenon of feminization caused by a cytoplasmic agent was observed (Kageyama *et al*., 1998), and this agent was revealed to be *Wolbachia* (Kageyama *et al*., pers. comm.). Using these insects, it may be possible to transfer *Wolbachia* between hosts expressing different reproductive alterations in order to examine the roles played by *Wolbachia* and genetic background of host in determining the phenotype. Although each *Wolbachia* variant may be specialized to its natural host species to some degree, *Wolbachia* may be successfully transferred within the same order of Lepidoptera. In Dipteran insects, successful interfamily transfer has been reported between *Aedes albopictus* and *D. simulans* (Braig *et al*., 1994).

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