Cross-order transfer of *Wolbachia* from *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae) to *Drosophila simulans* (Diptera: Drosophilidae)

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Bacteria of the genus *Wolbachia* are widespread in arthropods and can induce different effects on the host such as cytoplasmic incompatibility (CI), thelytoky (T) or feminization (F). In some *Wolbachia*-infected hosts, no effect (N) has been found. Successful transfer of *Wolbachia* by microinjection from one host to an uninfected one has been established with CI, F, N-*Wolbachia* but not with T-*Wolbachia*. In this paper a transfer experiment of T-*Wolbachia* from the parasitoid *Muscidifurax uniraptor* to *Drosophila simulans* is described. The infection could be detected in the new host for several generations by polymerase chain reaction (PCR). However, no specific effects on the host were detected, and the bacteria were not stably maintained.

Keywords: cytoplasmic incompatibility, *Drosophila*, microinjection, *Muscidifurax*, thelytoky, *Wolbachia*.

Introduction

Intracellular bacteria of the genus *Wolbachia* (Rickettsiales) are found in many different insect species. Although infection with these bacteria was known from mosquitoes at the beginning of this century (Hertig, 1936), it was only with the availability of polymerase chain reaction (PCR) technology at the end of the 1980s that the widespread occurrence of these bacteria was revealed. Screening of insect species from different orders from Panama revealed an infection rate of 16% (Werren *et al.*, 1995a).

Wolbachia are cytoplasmically inherited and can have a number of different effects on their hosts: (i) cytoplasmic incompatibility (CI), which is a crossing incompatibility in which sperm from *Wolbachia*-infected males cannot successfully fertilize eggs from uninfected females, whereas all other crosses are fertile (Yen & Barr, 1971; Trpis *et al.*, 1981; Noda, 1984; Wade & Stevens, 1985; Hoffmann *et al.*, 1986; Breeuwer & Werren, 1990); (ii) thelytoky (T), which is found in Hymenoptera — unfertilized eggs from *Wolbachia*-infected females develop into females, whereas they would normally develop into males

(Stouthamer *et al.*, 1990, 1993; Zchori-Fein *et al.*, 1995); (iii) feminization (F), which has only been found in isopods (Crustacea) in which the infection causes genetic males to reproduce as females (Juchault *et al.*, 1994); (iv) no apparent effect (N) on the reproduction of infected individuals at all, and this has been found in several *Drosophila* species (Giordano *et al.*, 1995; Hoffmann *et al.*, 1996).

The phylogeny of *Wolbachia* has been studied extensively based on several *Wolbachia* genes: 16S rDNA (O'Neill *et al.*, 1992; Rousset *et al.*, 1992; Stouthamer *et al.*, 1993) and the cell cycle gene *ftsZ* (Holden *et al.*, 1993; Werren *et al.*, 1995b). These studies show that two main groups of sequences can be distinguished and these have been named group A and group B (Werren *et al.*, 1995b). However, it appears that *Wolbachia* inducing similar effects on hosts are not monophyletic and are present in both groups. Very closely related *Wolbachia* can have quite different effects on their host. This absence of an association between monophyly and the different effects on the host has led to several hypotheses. One hypothesis is that a non-specific *Wolbachia* strain induces a distinct host response, e.g. a *Wolbachia* strain inducing CI in one host would induce thelytoky in a hymenopter host, but so far this has

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not been found. Alternative hypotheses for the lack of congruence between effect on host and phylogeny are: (i) *Wolbachia* genes coding for a certain effect (T, CI or F) are located on a plasmid that can jump relatively easily from one *Wolbachia* strain to another; (ii) *Wolbachia* genes inducing T, CI or F can cross over between different strains in double-infected hosts; (iii) *Wolbachia* genes causing T, CI or F are highly congruent and can readily evolve from each other.

One method to determine whether (i) a specific *Wolbachia* strain causes a distinct effect on the host or (ii) different hosts experience different effects with the same *Wolbachia* strain is to create new combinations of host and symbiont. Such transfer experiments have been carried out successfully with CI-*Wolbachia* among several *Drosophila* spp. both intraspecifically (Rousset & De Stordeur, 1994) and interspecifically (Boyle *et al.*, 1993; Clancy & Hoffmann, 1997) and interspecifically in *Tribolium confusum* (Chang & Wade, 1994). In addition, interfamilial transfer of CI-*Wolbachia* has been realized. *Wolbachia* from the mosquito *Aedes albopictus* were transferred to the fruitfly *D. simulans*, and this revealed that the transinfected *Drosophila* strain became bidirectionally incompatible with all other naturally infected strains (Braig *et al.*, 1994). F-*Wolbachia* causing feminization have also been transferred inter- and intraspecifically in several species of isopods (Juchault *et al.*, 1994) and induced similar effects in their hosts. Finally, N-*Wolbachia* causing no obvious effect on the host were transferred from *D. mauritiana* to *D. simulans* in which they also failed to cause any obvious effect (Giordano *et al.*, 1995). From these studies, it became clear that similar effects were found in the new host-*Wolbachia* combination as in the original one.

No such transfer experiments have been performed with T-*Wolbachia*. Here, we report on experiments we have conducted to determine whether T-*Wolbachia* found in the hymenopteran

Muscidifurax uniraptor would cause a specific effect in the dipteran host *Drosophila simulans*. It was determined whether injection of T-*Wolbachia* induced mortality in *D. simulans* eggs and if the infection would become established in the new host. Secondly, when the infection succeeded, effects on the host were studied by: (i) measuring egg mortality of infected eggs; and (ii) performing crosses to investigate whether T-*Wolbachia* were able to induce CI in its new host. CI-*Wolbachia* were also injected in uninfected *D. simulans* to verify the microinjection protocol.

Materials and methods

Insect strains

The insect and *Wolbachia* strains used in the injection experiment are shown in Table 1.

Flies were maintained in bottles containing *Drosophila* medium [20 g of agar, 135 g of sugar, 38 g of yeast, 0.12 g of nipagine (10 g L⁻¹ nipagine in 70% ethanol) made up in 1 L of water] and incubated at 20°C. The fly pupa parasitoid *M. uniraptor* originated from Puerto Rico (Legner, 1985) and was reared on *Musca domestica* pupae and incubated at 25°C.

Microinjection

Microinjection was performed according to the method of Santamaria (1986) and Boyle *et al.* (1993) with some modifications. Embryos were collected over a 30-min period, transferred to double-sided sticky tape on a slide, dechorionated manually and transferred to a new slide. The embryos were partially desiccated by exposure to the air for up to 4 min (depending on the relative humidity) and subsequently covered with heavy mineral oil (Sigma, no. 400–5). Using a microcapillary needle (Boehringer femtotips), ooplasm containing *Wolbachia* (wUni or wRi) was drawn from infected eggs and then injected into the posterior poles of unin-

Table 1 Insect species and *Wolbachia* strains used in the microinjection experiment

Species		<i>Wolbachia</i> strain abbreviation	Effect in host	Abbreviation for new transinfected species
<i>Drosophila simulans</i> Riverside	D	wRi	CI	—
<i>Muscidifurax uniraptor</i>	D	wUni	T	—
<i>Drosophila simulans</i> Watsonville	A	Injected with wRi	CI	DSW(CI)
<i>Drosophila simulans</i> Watsonville	A	Injected with wUni	?	DSW(T)

Ooplasm was isolated from the donor species (D) and injected into the acceptor (A).

infected *D. simulans* Watsonville embryos before pole cell formation. Injected embryos were incubated overnight at 25°C (18 h). On the following day, hatching larvae were isolated at 2-h intervals and transferred to vials with *Drosophila* medium. After pupation and eclosion, isofemale lines were started. Adult virgin females were crossed with uninfected males, because some males from the injected eggs failed to mate or were sterile. A sample of the injected eggs was used to measure the mortality of eggs, larvae and pupae.

Rearing of isofemale lines

In the first generation after injection, all females were tested for *Wolbachia* infection. From the offspring of each of these isofemale lines, five new isofemale lines were started. After 1 week, these five females were tested for *Wolbachia* infection. Offspring from infected mothers were used to start new isofemale lines. In the 3rd–7th generations, new DSW(T) lines were established from females that had the highest proportion of infected offspring. Adult transinfected females were tested for *Wolbachia* infection by PCR amplification of the *ftsZ* gene (Holden *et al.*, 1993).

Egg mortality, fecundity and incompatibility testing

To investigate whether *wUni* infection in DSW(T) caused egg mortality, the number of hatched eggs of infected DSW(T) females was counted. Females were taken from an infected DSW(T) line and the uninfected DSW line (control). They were placed on agar dishes covered with fresh yeast. After 48 h, these females were placed on a new agar dish with yeast. When the agar dishes had been incubated at 20°C for 48 h, the numbers of eggs and larvae were counted. At the end of the experiment, females were prepared for PCR to check for *Wolbachia* infection. Data from uninfected females from the DSW(T) line, according to PCR, were removed before analysis of the results. Experiments were performed in duplicate. The numbers of eggs from infected and non-infected mothers were also compared.

Egg mortality was used to indicate reproductive incompatibility. To test whether *wUni* induced CI in DSW(T), matings between transinfected males and uninfected females were set up. For each cross, 20 1- to 2-day-old DSW virgin females were each allowed to mate with one 5-day-old DSW(T) male in the second generation or one 1-day-old male in the fourth generation. As a control, 10 virgin DSW females (from the same batch as above) were

crossed with uninfected DSW males. To confirm the unmated status of these females, a control vial of 10 virgin females was taken. All DSW females were checked for *Wolbachia* infection to confirm their uninfected status. After 24 h, males were removed and tested for *Wolbachia* infection. Females were allowed to lay eggs for 2 days on agar covered with fresh yeast. After removing the females, the dishes were incubated for 48 h, and the numbers of eggs and larvae were counted. Experiments were performed in duplicate, and the mortality rates were compared with a Kruskal–Wallis test.

DNA extraction and PCR

Drosophila flies were homogenized with a clean pestle in 100 µL of STE (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) (O'Neill *et al.*, 1992) and incubated with 2 µL of Proteinase K (20 mg mL⁻¹) for 1 h at 37°C, followed by 5 min at 95°C. Samples were spun for 2 min in a centrifuge, and 1 µL of the supernatant was used as template in subsequent PCR reactions. The *Wolbachia ftsZ* gene was amplified using specific *ftsZ* primers (Holden *et al.*, 1993): 5'-GGACCGGATCCGTATGCCGATTGCAGAG-CTTG-3' and 5'-GGACCGAATTCGCCATGAG-TATTCATTGGCT-3'. For PCR, a temperature profile of 94°C for 2 min (1 cycle); 94°C for 1 min; 55°C for 1 min; 72°C for 1 min (40 cycles) was used.

Confirmation of the identity of the Wolbachia strain in the infected Drosophila host

FtsZ sequences of *Wolbachia* from *D. simulans* Riverside *wRi* and *M. uniraptor wUni* were retrieved from GenBank (accession numbers U28178 and U28186) and analysed for differences (Werren *et al.*, 1995b). The *ftsZ* primers (Holden *et al.*, 1993) amplify the region 494–1221 bp of the *ftsZ* gene, which includes a part of the 3' flanking sequence. The 792 bp sequences (including the primers) differ in four nucleotides, and *PvuII* cuts at position 1120 of the *wRi ftsZ* sequence. *PvuII* restriction digestion results in two fragments of 659 bp and 133 bp in the case of the *ftsZ* of *wRi Wolbachia*.

Results

Mortality rates of injected eggs

Mortality rate differences were found between eggs injected with *Drosophila* cytoplasm containing *wRi* or *Muscidifurax* cytoplasm containing *wUni* (Table 2). Eggs injected with *wUni* showed higher

Table 2 Mortality rates of different life stages of *Drosophila simulans* Watsonville injected with different cytoplasm types and *Wolbachia* strains

Injected	Egg to larva			Larva to pupa		
	Alive	Dead	Total	Alive	Dead	Total
<i>wRi</i>	98	142	240	58	40	98
<i>wUni</i>	140	75	215	66	74	140

Injected	Larva to adult			Pupa to adult		
	Alive	Dead	Total	Alive	Dead	Total
<i>wRi</i>	48	50	98	48	10	58
<i>wUni</i>	52	88	140	52	14	66

Adults				
Injected	Total females	Total females with offspring	Total males	Total males + females
<i>wRi</i>	22	11	26	48
<i>wUni</i>	25	11	27	52

hatching rates than eggs injected with *wRi*. However, this difference is not seen when mortality rates of larvae or pupae are compared. Of the resulting adult females, approximately 50% produced offspring.

Wolbachia infection rates

One positive DSW(CI) line was found from 11 lines injected with *wRi*. From this infected mother, all offspring were infected (five individuals tested per generation) in the third and consecutive generations. The infection remained stable for at least 10 generations. Of 41 isofemale lines injected with *wUni*, 10 lines had infected offspring rates ranging from 20% to 80%. The percentage infected offspring of infected mothers decreased to 60% at maximum in successive generations, and no infected offspring were found in the seventh and consecutive generations (Table 3). PCR with the *ftsZ* primers was performed on *D. simulans* Watsonville (DSW) and Riverside, *M. uniraptor* and the transinfected lines DSW(CI) and DSW(T). No product was amplified from DSW, whereas all other samples resulted in a PCR product of the expected size (792 bp). Restriction analysis on these *ftsZ* products confirmed the identity of the *wUni* strain in *D. simulans* (data not shown).

Effects on host resulting from wUni infection in D. simulans

In the second generation after injection, effects on the host caused by the *wUni* infection in DSW(T) were studied by determining female fecundity and egg mortality rates from infected mothers. No differences were found between infected and DSW mothers (Table 4). Secondly, expression of CI by infected *wUni* males was studied. If *wUni* induces CI in its host, reduced egg hatching rates should be found in the crossing of DSW females $\times$ DSW(T) males. But no reduced egg hatching rates were found in the putative incompatible crossing in comparison with the control crossing (Table 4).

In the fourth generation, similar egg mortality results were obtained from the putative CI and control crossing (Table 4). Egg mortality from DSW(T) mothers was determined in the fourth generation as well but, because of the low transmission rate (26%; Table 3), insufficient positive samples were obtained.

Discussion

It was not known whether the injection of foreign cytoplasm into *D. simulans* eggs would result in higher mortality. Because of the different microin-

jection conditions between the actual experiments, our experimental set-up is not suitable for addressing this issue. However, comparing the mortality in both experiments, it becomes clear that *Drosophila* eggs can survive the introduction of foreign cytoplasm (Table 2). In addition, *w*Uni injection does not result in excessive mortality in the different developmental stages of *Drosophila*.

Wolbachia could be detected by PCR in the DSW(CI) and DSW(T) lines in the second generation after injection. The transmission was 100% in the second generation for DSW(CI) and was stable for at least 10 generations. For DSW(T), however, the highest transmission rate was found in the second generation (80%) and decreased to 0% in the seventh generation.

Table 3 Number of isofemale lines of *Drosophila simulans* tested, number of individuals tested per line and offspring infection rates from 2nd to 10th generations of DSW(T) injected females

Generation after injection	No. of isofemale lines tested	No. of individuals per isofemale line	% infected offspring
2	31	5	0
	3	10	20
	3	5	40
	1*	40	47.5
	1	5	60
	2	5	80
3	1	5	0
	1	5	20
	3	5	60
4	1*	38	26
	3	10	40
5	2	5	0
	1	5	20
	3	5	60
6	2	10	0
	1	10	50
7	2	20	0
8, 9, 10	2	5	0

*Individuals from these isofemale lines were used in egg mortality and CI experiments.

Table 4 Number of eggs with standard error (SE) and mean egg mortality with standard deviation (SD) from different mating combinations between uninfected *Drosophila simulans* (DSW) and *w*Uni-infected DSW(T), two and four generations after injection (crosses b and f represent the putative CI crossing)

Generation tested (after infection)	Cross (female $\times$ male)	Eggs/female $\pm$ SE (number of females tested)	Mean egg mortality $\pm$ SD	Egg mortality comparison
2	a: DSW $\times$ DSW	79 $\pm$ 6 (10)	12.7 $\pm$ 7	a vs. b P = 0.42
	b: DSW $\times$ DSW(T)	86 $\pm$ 8 (12)	10.3 $\pm$ 10	
	c: DSW $\times$ DSW	64 $\pm$ 7 (9)	4.8 $\pm$ 5	c vs. d P = 0.64
	d: DSW(T) $\times$ DSW(T)	78 $\pm$ 9 (9)	3.6 $\pm$ 5	
4	e: DSW $\times$ DSW	59 $\pm$ 5 (10)	8.4 $\pm$ 5	e vs. f P = 0.09
	f: DSW $\times$ DSW(T)	56 $\pm$ 8 (7)	5.3 $\pm$ 5	

Effects on the host were studied for DSW(T) in the second and fourth generations. Because *D. simulans* is not adapted to thelytoky, egg mortality was expected to occur. But no significantly higher mortality was found in eggs from infected mothers in the second generation, which corresponded with the decreasing transmission rates. In addition, no fecundity differences could be found between infected and non-infected females. Because *Wolbachia* can induce CI in *Drosophila* (Hoffman *et al.*, 1986), this phenomenon was tested for with the wUni transinfected *Drosophila* strains. No CI could be detected in the second and fourth generations after injection in the incompatible crosses. In other cytoplasm injection experiments, *Wolbachia* effects in the host could be detected as early as two generations after injection (Boyle *et al.*, 1993). In the injection experiment of Braig *et al.* (1994), *Wolbachia* could only be detected by PCR after six generations. But the set-up of this experiment was different because purified *Wolbachia* were injected, which could have an effect on *Wolbachia* fitness or result in a difference in injected *Wolbachia* dose.

Although the *ftsZ* sequences of wRi and wUni are closely related (0.4% difference), no permanent establishment of *Wolbachia* from *M. uniraptor* infection occurred in *D. simulans*. Because wUni infection could be detected for several generations, *Wolbachia* had to be present in the eggs. However, no effects on the host could be detected, and transmission rates decreased after each generation. Because no correlation was found between the decreased transmission rates and egg mortality, it remains unclear why the infection is not stable in its new host. One explanation might be that, when no CI mechanism is present, this being the driving force for the spread of *Wolbachia* through the population (Turelli, 1994), *Wolbachia* could disappear from the population. However, transfer experiments with N-*Wolbachia* revealed that the new hosts had 100% infected offspring as well, indicating that no CI mechanism was necessary for their persistence (Giordano *et al.*, 1995). A second explanation for the infection not being stable is that wUni is not physiologically adapted to its new host. Consequently, the *Wolbachia* density would not rise sufficiently, explaining the fact that no effects were observed on the host (Boyle *et al.*, 1993; Breeuwer & Werren, 1993). As found in *D. serrata*, a novel host for *Wolbachia*, *Wolbachia* from *D. simulans* Riverside were successfully transferred to this species, but maternal transmission was relatively low (Clancy & Hoffmann, 1997), indicating that host-symbiont interactions are important for success in the estab-

lishment of the infection. An example of *Wolbachia* transmission failure was found in isopods. Injection experiments revealed that *Wolbachia* could be transferred between closely related species of isopods (*Armadillidium nasatum* to *A. vulgare*). However, transmission of *Wolbachia* from *Chaetophiloscia elongata* to *A. vulgare*, which are more distantly related species, failed (Juchault *et al.*, 1994). This might be a similar case in which *Wolbachia* were not adapted to their new host. On the other hand, phylogenetic work on *Wolbachia* (Werren *et al.*, 1995b) provides evidence that, in some cases, this bacterium has successfully bridged large phylogenetic distances in its interspecific movement, and it remains unknown what permits particular inter-order transfers to occur successfully. When practical microinjection problems are overcome, future experiments could reveal whether wUni can be transferred to other hymenopteran species and if similar phenotypes are induced as in the original host.

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