Wolbachia variant that induces two distinct reproductive phenotypes in different hosts

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Wolbachia is an intracellular endosymbiont that induces a variety of reproductive alterations in diverse arthropods. The almond moth, *Cadra cautella*, is double infected with two *Wolbachia* variants, *wCauA* and *wCauB*, and expresses complete cytoplasmic incompatibility (CI). The individual contribution of *wCauA* and *wCauB* to the expression of CI are unclear, however, because the two variants have not been separated in this host. The effect of *wCauA* is of particular interest because it induces male killing when transferred into the Mediterranean flour moth, *Ephestia kuehniella*. In the present study, we generated *C. cautella* infected with only *wCauA* by treating double-infected insects

with tetracycline. Single-infected *C. cautella* exhibited strong CI, demonstrating that *wCauA* induces two distinct reproductive phenotypes in different hosts: CI in *C. cautella* and male killing in *E. kuehniella*. CI was also observed in the cross of double-infected males and single-infected females. Comparison of the single- and double-infected insects by real-time quantitative polymerase chain reaction suggested that the *wCauA* density is not affected much by the presence or absence of *wCauB*.

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Introduction

Wolbachia is a maternally inherited intracellular symbiont that is widespread in arthropods (Bouchon *et al*, 1998; Jeyaparakash and Hoy, 2000) and filarial nematodes (Bandi *et al*, 1998). In its arthropod hosts, *Wolbachia* induces a variety of reproductive alterations such as cytoplasmic incompatibility (CI), thelytokous parthenogenesis, male killing, and feminization (reviewed in Werren, 1997; Bourtzis and Braig, 1999; Stouthamer *et al*, 1999).

There are two types of CI: unidirectional and bidirectional. Unidirectional CI typically occurs when infected males mate with uninfected females, whereas the crosses of other combinations are compatible. This phenomenon is generally explained by 'modification' of the sperm and 'rescue' in the infected eggs (Werren, 1997). Unidirectional CI decreases the reproductive success of uninfected females, and promotes the spread of infection (Turelli and Hoffmann, 1991). Bidirectional incompatibility often occurs when males and females are infected with different variants of *Wolbachia*, and is explained by variations in the 'modification' and 'rescue' functions of the different *Wolbachia* variants (O'Neill and Karr, 1990; Rousset and De Storéur, 1994).

The variety of reproductive alterations induced by *Wolbachia* raises the question of how different phenotypes arise. Some experimental transfers of *Wolbachia* between different host species revealed that *Wolbachia*

induces similar phenotypes in the recipient host as in the donor host, but host differences might affect the penetrance of the phenotype (eg, Boyle *et al*, 1993; Clancy and Hoffmann, 1997; Poinot *et al*, 1998). Transfers of feminizing *Wolbachia* among isopods resulted in four possible outcomes: no reproductive effect, expression of feminization, death of recipients, and failure of transinfection (Bouchon *et al*, 1998). Thus, the reproductive phenotypes appear to result from a complex interaction of host and bacterial components.

The almond moth, *Cadra cautella*, is double infected with *wCauA* and *wCauB*, which belong to the A- and B-*Wolbachia* groups designated by Werren *et al* (1995), respectively, and expresses complete CI (Sasaki and Ishikawa, 1999). When the *Wolbachia* were transferred from *C. cautella* into the Mediterranean flour moth, *Ephestia kuehniella*, in which *wCauA* and *wCauB* were segregated, *wCauA* induced male killing in the new host (Sasaki *et al*, 2002). This suggests that *wCauA* induces different phenotypes in the two host insects. The effect of *wCauA* on its natural host *C. cautella*, however, is not clear because the host is double infected, and single-infected insects have not been obtained.

In this study, we generated a single-infected *C. cautella* strain carrying only *wCauA* by treating the double-infected insects with tetracycline. The primary aim was to examine the phenotype expressed in the single-infected insects. Crossing experiments demonstrated that *wCauA* alone induces strong CI. We also investigated two aspects of double infection using single- and double-infected *C. cautella* strains. First, we examined the crossability of the two strains, and observed that double-infected males were incompatible with single-infected females. Second, we used real-time polymerase chain

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reaction (PCR) to measure the density of *Wolbachia* in ovaries, in which no significant difference in the density of *wCauA* was found between the two lines. These results are discussed in conjunction with how double infection is maintained.

Materials and methods

Insects

C. cautella, which was double infected, was originally collected in Tsuchiura, Japan. The uninfected strain was generated by tetracycline treatment (Sasaki and Ishikawa, 1999). The insects were reared on a diet consisting of wheat bran, dried yeast, and glycerol (20:1:2) at 25°C under a 16 h light:8 h dark photoperiod.

Tetracycline treatment

One approach to obtain *wCauA*-single-infected *C. cautella* is to transfer *wCauA* back into uninfected *C. cautella* from the transinfected *E. kuehniella* bearing only this *Wolbachia* variant, but artificial transfer of *Wolbachia* into *C. cautella* has not been successful. Therefore, we attempted to use antibiotic treatment to remove *wCauB* from double-infected *C. cautella*.

The duration of *C. cautella* larval development at 25°C is approximately 3 weeks. To obtain single-infected lines, the double-infected insects were reared on a diet containing 0.04% tetracycline for the first week during larval development. Approximately 200 eggs were placed on 10 g of the diet with tetracycline so that the larvae were fed the diet as soon as they hatched. One week after hatching, 200 g of the diet without tetracycline was added to the rearing case and mixed gently with the tetracycline diet on which the larvae were growing. The insects treated with tetracycline were referred to as generation 0 (G0).

G1 eggs were collected from individual G0 females, and diagnosed for infection by PCR (see below). G1 and subsequent generations were reared without antibiotics. Selection for single-infected lines was continued for four generations.

Diagnostic PCR for infection

The template DNA was prepared from eggs or adults according to O'Neill *et al* (1992). Each egg was homogenized in 15 µl STE (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.4 mg/ml proteinase K, and incubated for 90 min at 55°C and then for 15 min at 95°C. When DNA was extracted from adults, each individual was first homogenized in 100 µl STE without proteinase K, and 10 µl of the homogenate was added to 90 µl STE containing proteinase K.

To test the infection status, two primer sets for the *ftsZ* bacterial cell-cycle gene were used (Werren *et al*, 1995). The A group-specific *ftsZ* primers were *ftsZAdf* (5'-CTC AAG CAC TAG AAA AGT CG-3') and *ftsZAdr* (5'-TTA GCT CCT TCG CTT ACC TG-3'), and the B group-specific primers were *ftsZBf* (5'-CCG ATG CTC AAG CGT TAG AG-3') and *ftsZBr* (5'-CCA CTT AAC TCT TTC GTT TG-3'). PCR was performed in a 20-µl reaction mixture using Takara EX Taq. The 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.

Incompatibility test

Crosses were performed using single pairs of virgin individuals. Since the male larva is easily distinguished by a dark patch (the testes) on its back, we separated the females and males at the late larval stage to obtain virgin moths. A female and a male, less than 2 days after emergence, were put in a plastic cup (3 cm in diameter, 5 cm in height) and left there for 3 days. Successful mating was judged from the number of eggs deposited, as unmated females produce few eggs. From the mating cups in which more than 50 eggs were found, 50–100 eggs were collected and placed onto 1% agarose in a plastic dish (35 mm in diameter). After incubation for 7–8 days at 25°C, the hatching rate was scored for each single-pair cross.

Real-time quantitative PCR

DNA was extracted from ovaries of individual females by phenol extraction followed by ethanol precipitation, as previously described (Ikeda *et al*, 2003a). Quantitative PCR was performed in a LightCycler (Roche Diagnostics) using variant-specific primers and probes for the *groEL* gene according to Ikeda *et al* (2003b). The *groEL* of *wCauA* was amplified using the primers *groEL415AF* (5'-CAA TGT CTC GTA CAA TTT CTC-3') and *groEL641AR* (5'-GAT TAT CAA GCT CCA CGA-3'). The PCR product was detected with the probes *groELA1* (5'-AAA GAG TTA GAA GTT GAG CTG ACT ACT GG-3'-FITC) and *groELA2* (LC-Red 640-5'-ATG CAA TTT GAT CGC GGT TAT CTC TCT CCG-3'). The *groEL* of *wCauB* was amplified with the primers *groEL415BF* (5'-CAA TGT CTC GCA CAA TTT CTT-3') and *groEL641BR* (5'-GAT CAT CAA GCT CCA CGC-3'), and the product was detected with the probes *groELB1* (5'-GAA GTT GAA CTT ACA ACT GGT ATG CAG TT-3'-FITC) and *groELB2* (LC-Red 640-5'-GAC CGT GGT TAT CTC TCT CCA TAC-3').

The 10-µl reaction mixture consisted of 1 µl LightCycler Master Mixture (Roche Diagnostics), 30 nmol MgCl₂, 5 pmol each primer, 2 pmol probe 1, 4 pmol probe 2, and 1 µl template DNA. The PCR cycling conditions were 30 s at 95°C followed by 45 cycles of 1 s at 95°C, 10 s at 52°C, and 30 s at 72°C.

Standard DNAs were prepared from PCR products amplified from *wCauA* and *wCauB* using the primers *groEL49F* (5'-GTT GCA AGA AGC CTT TCG TG-3') and *groEL827R* (5'-CCA AAA CCT GGA GCT TTT ACT G-3'). The PCR products were electrophoresed on 1.0% agarose gel, extracted from the gel using QIAquick Gel Extraction Kit (Qiagen), and quantified based on the optic absorbance at 260 nm. Dilutions (10-fold) from 10 pg/µl to 1 fg/µl were used to draw standard curves, in which amplification kinetics showed a linear relationship with the concentration of template DNA. We also confirmed that the amplification efficiency of the standards is not affected by the addition of the DNA from the ovaries of uninfected females extracted in the same way as the sample DNA, so the presence of any PCR inhibitors in the sample is negligible.

Results

Selection of *wCauA*-single-infected lines

G1 eggs were collected from 28 females that were treated with tetracycline during the first week of the larval

period. Infection was examined by testing two eggs from each brood. Of the 56 eggs examined, 20 were *wCauA* positive and *wCauB* negative, two were *wCauA* negative and *wCauB* positive, 29 were double positive, and five were double negative. The broods in which at least one *wCauA*-positive and *wCauB*-negative egg was found were maintained for further selection of *wCauA*-single-infected lines. Selection was performed for three subsequent generations and nine single-infected lines were obtained at G4. When G5 eggs were collected from 20 females, the females were diagnosed after oviposition. We confirmed that all mothers were infected with *wCauA*, but not with *wCauB*, and the G5 eggs were pooled as the *wCauA*-single-infected strain.

We also attempted to obtain *wCauB*-single-infected lines from the G1 broods in which *wCauA*-negative and *wCauB*-positive eggs were found. All 30 eggs examined at the G2 generation, however, were double positive, and we did not obtain *wCauB*-single-infected lines.

Incompatibility

Crosses were performed using the *wCauA*-single-infected strain at generations 9 and 10. Prior to the experiments, infections of the single-infected strain at generation 9 and double-infected strain were examined by testing 46 eggs selected at random for each strain. All eggs from the single-infected strain were positive for *wCauA* and negative for *wCauB*, and all eggs from the double-infected strain were positive for both *Wolbachia* variants, suggesting that the transmission of *Wolbachia* is very stable.

Incompatibility assays are shown in Table 1. Since the data had skewed distributions, differences in mortality rate among treatments were assessed using nonparametric Mann–Whitney *U*-test. Normal approximation was used in comparisons including a sample whose size was greater than 20. Single-infected males are incompatible with uninfected females (*A vs B*, $U = 288$, $P < 0.001$), indicating that *wCauA* alone induces CI in *C. cautella*. The incompatibility was almost complete: of the 16 crosses, no eggs hatched in 14 crosses and only one egg hatched in each of the other two crosses.

Unidirectional incompatibility also occurred between single-infected and double-infected strains (*F vs H*, $Z = 5.38$, $P < 0.001$). In addition, there was a slight but significant increase in mortality in the cross of double-infected individuals, compared with crosses between uninfected males and single- or double-infected females (*I vs D*, $Z = 2.59$, $P < 0.01$; *I vs G*, $Z = 3.24$, $P < 0.01$).

Wolbachia densities in single- and double-infected

C. cautella

Wolbachia density in ovaries was measured by real-time quantitative PCR when the single-infected strain was at G11 (Table 2). The data were normalized with the tissue mass rather than with a host gene as an internal control, because most *Wolbachia* were expected to be in ova while most host's DNAs originated in the cells of ovarian tubes surrounding the ova. There was no significant difference in *wCauA* densities between the single- and double-infected strains (Student's $t = 0.94$, $P > 0.2$).

In the double-infected strain, the density of *wCauA* was higher than that of *wCauB* ($t = 4.66$, $P < 0.001$). This difference in density might, in part, be the reason for the difficulty in obtaining *wCauB*-single-infected lines.

Discussion

We demonstrated that *wCauA* induces CI in *C. cautella* (Table 1). *wCauA* induces male killing in *E. kuehniella* (Sasaki *et al*, 2002). Thus, *wCauA* includes two distinct types of host-dependent reproductive alterations.

There are at least two possible explanations for the induction of two different phenotypes by *wCauA*. One is that *wCauA* has different genes to induce CI and male killing. *C. cautella*, but not *E. kuehniella*, might be resistant to the action of the male-killing gene, or the gene expression is suppressed exclusively in *C. cautella*. The second explanation is that the same gene is responsible for the expression of the two phenotypes and the same bacterial action induces different responses in the hosts. CI is believed to result from sperm modification by *Wolbachia* in the testis, whereas male killing occurs due to the presence of *Wolbachia* in eggs. Therefore, *wCauA* might affect male-specific gene expression mechanisms common to spermatogenesis and male embryonic

Table 2 Infection density of *Wolbachia* in *Cadra cautella* ovaries

Host	<i>Wolbachia</i>	groEL copies ($\times 10^7$)/ovary	groEL copies ($\times 10^7$)/mg ovary
Single infected	<i>wCauA</i>	5.28 ± 1.49	1.03 ± 0.26
Double infected	<i>wCauA</i>	6.34 ± 1.50	1.12 ± 0.23
	<i>wCauB</i>	4.36 ± 0.71	0.78 ± 0.12

Ovaries were collected from females 60–72 h after adult emergence. The *Wolbachia* population was expressed in terms of *groEL* gene copy number. The mean $\pm$ SD ($n = 12$) is given for each *Wolbachia* strain.

Table 1 Mortality rates in crosses between *Cadra cautella* strains

Cross	Female	Male	No. of pairs	Mortality (%)	
				Median	Interquartile range
A	Tet	Tet	18	14.8	[9.9–30.3]
B	Tet	<i>wCauA</i>	16	100.0	[100.0–100.0]
C	Tet	<i>wCauA</i> + <i>wCauB</i>	19	100.0	[100.0–100.0]
D	<i>wCauA</i>	Tet	15	11.1	[8.1–19.2]
E	<i>wCauA</i>	<i>wCauA</i>	22	21.2	[8.4–24.8]
F	<i>wCauA</i>	<i>wCauA</i> + <i>wCauB</i>	26	94.0	[87.7–94.3]
G	<i>wCauA</i> + <i>wCauB</i>	Tet	14	10.0	[8.3–13.0]
H	<i>wCauA</i> + <i>wCauB</i>	<i>wCauA</i>	16	21.3	[7.5–28.1]
I	<i>wCauA</i> + <i>wCauB</i>	<i>wCauA</i> + <i>wCauB</i>	23	23.0	[13.9–24.6]

development. Different phenotypes could be expressed if the male-specific gene expression mechanisms are similar but not the same in the two host insects.

We assume that the second possibility is more likely, because if *wCauA* has different genes to induce CI and male killing as in the first explanation, it is difficult to explain how the gene for male killing is maintained in *wCauA* that does not express this phenotype in its natural host. The presence of a common mechanism for CI and male killing has also been suggested in *Drosophila bifasciata*, in which a change of phenotype from male killing to CI was observed (Hurst *et al*, 2000). The authors reported that the females infected with male-killing *Wolbachia* laid eggs with decreased bacterial density when they were exposed to elevated temperatures, and the male offspring produced following the high temperature treatment exhibited weak CI.

Two other significant observations in the present study are that unidirectional incompatibility occurs between single- and double-infected strains, and that the density of *wCauA* does not differ significantly between the two strains. These observations provide insight into the mechanism of how double infection is maintained.

There are double infections of *Wolbachia* in many species (eg, Werren *et al*, 1995; Jeyaprakash and Hoy, 2000; Kittayapong *et al*, 2003). It has been shown that double-infected males are incompatible with single-infected females in some insects such as *D. simulans* (Merçot *et al*, 1995; Rousset and Solignac, 1995), *Aedes albopictus* (Sinkins *et al*, 1995), and *Nasonia vitripennis* (Perrot-Minnot *et al*, 1996). This incompatibility has been explained by the specific interaction between sperm modification and rescue as follows: each *Wolbachia* variant modifies the sperm in its particular way and the sperm modified by two different *Wolbachia* variants cannot be rescued in single-infected eggs. This incompatibility is considered to be the mechanism by which double infection spreads and is maintained in a host population. Therefore, it is expected that incompatibility between double- and single-infected individuals is common in double-infected hosts. Our observation in *C. cautella* provides additional evidence to support this view.

There was a slight increase in mortality in the cross of double-infected males and females. Such mortality within multiple-infected insects is also observed in *D. simulans*, and is explained by an incomplete rescue effect that possibly occurs due to an unequal transmission of some of the *Wolbachia* variants to the embryos (Rousset *et al*, 1999).

Besides incompatibility, the maternal transmission rate of *Wolbachia* is a critical factor that determines the spread and maintenance of infection, and the transmission is more or less affected by the density of *Wolbachia*. To understand the transmission of double infection, it is important to clarify how the density of the two different *Wolbachia* variants is regulated. There might be an interaction such as competition between the two *Wolbachia* variants. Alternatively, proliferation of the two *Wolbachia* variants might be independent of each other. Our data from quantitative PCR suggest that there is no strong competitive interaction between *wCauA* and *wCauB* in the double infection. This is in agreement with previous studies of two transinfected insects, *D. simulans* (Rousset *et al*, 1999) and *E. kuehniella* (Ikeda *et al*,

2003a,b), and naturally infected *Leptopilina heterotoma* (Mouton *et al*, 2003). Independent regulation does not decrease the density of each *Wolbachia* variant, and therefore appears to be suitable for stable transmission of double infection.

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