Wolbachia Transfer from Rhagoletis cerasi to Drosophila simulans: Investigating the Outcomes of Host-Symbiont Coevolution

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Wolbachia is an endosymbiont of diverse arthropod lineages that can induce various alterations of host reproduction for its own benefit. Cytoplasmic incompatibility (CI) is the most common phenomenon, which results in embryonic lethality when males that bear Wolbachia are mated with females that do not. In the cherry fruit fly, Rhagoletis cerasi, Wolbachia seems to be responsible for previously reported patterns of incompatibility between populations. Here we report on the artificial transfer of two Wolbachia variants (wCer1 and wCer2) from R. cerasi into Drosophila simulans, which was performed with two major goals in mind: first, to isolate wCer1 from wCer2 in order to individually test their respective abilities to induce CI in the new host; and, second, to test the theoretical prediction that recent Wolbachia-host associations should be characterized by high levels of CI, fitness costs to the new host, and inefficient transmission from mothers to offspring. wCer1 was unable to develop in the new host, resulting in its rapid loss after successful injection, while wCer2 was established in the new host. Transmission rates of wCer2 were low, and the infection showed negative fitness effects, consistent with our prediction, but CI levels were unexpectedly lower in the new host. Based on these parameter estimates, neither wCer1 nor wCer2 could be naturally maintained in D. simulans. The experiment thus suggests that natural Wolbachia transfer between species might be restricted by many factors, should the ecological barriers be bypassed.

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Using a microneedle (Femtotips; Eppendorf), cytoplasm was taken from every hour. Recipient eggs were dechorionated manually prior to injection into the body of infection following tetracycline treatment (28). Ago, originally infected by two D. simulans strains were checked by PCR for the presence of Wolbachia. STC strain was performed by cytoplasmic injection (33).

Materials and methods

R. cerasi and D. simulans lines. Larvae of R. cerasi were collected from a wCer1- and -2-infected population on honeysuckle (Lonicera xylosteum) in Vienna, Austria, in 1999. After pupation, puparia were stored under the optimal conditions (37). Emerging enna, Austria, in 1999. After pupation, puparia were stored under the optimal conditions (37). Emerging females were founded into a new host (11). The results presented are partially in agreement with these predictions. Indeed, a fitness cost to the host and low transmission efficiency are observed, as expected, but the level of CI is clearly reduced.

Wolbachia infection and line establishment. The transfer of wCer1 and wCer2 into the D. simulans STC strain was performed by cytoplasmic injection (33). Using a microneedle (Femtotips; Eppendorf), cytoplasm was taken from R. cerasi eggs and injected into the posterior part of recipient eggs. Donor eggs were obtained by dissection directly from ovaries, providing fresh and weakly differentiating embryos. Fresh receiver eggs were collected from the egg-laying plates every hour. Recipient eggs were dechorionated manually prior to injection.

D. simulans females developing from injected eggs represent the generation 0 (G0). Each G0 female was crossed with one G0 male and was left for laying before its infection status was determined by PCR. The infection status of the offspring was determined by PCR on a mass extraction of three G1 females. In lines in which infection was detected in G1, 10 G1 sisters were mated to their brothers and left to lay separately before their infection status was determined.

During the experiment, all lines were maintained at 25°C in larval densities in vials with axenic medium (14). Rates of transmission from mothers to offspring were low in transfected lines, imposing stringent conditions for maintenance of infection. Thus, at every generation, and for every transfected line, six females were left to lay independently before their infection status was determined. The next generation was then started by using offspring from infected females only.

Cl tests. Individual crosses were done with 3-day-old virgin males and 4- to 5-day-old virgin females. Each cross was initiated by placing one male and one female in a vial with axenic medium. Copulation was monitored, allowing the discarding of pairs in which it lasted less than 15 min, to ensure that sperm was actually transferred. The male was then removed, and the female was supplied with an egg-laying plate for 48 h. Upon removal of the female, the eggs were placed at 25°C for 24 h before the egg hatch was measured by counting all eggs. Laying plates with less than 20 eggs were discarded. All individuals from infected strains were checked by PCR for the presence of Wolbachia.

Maternal transmission rates. Maternal transmission was first roughly estimated as the proportion of infected female daughters from infected mothers during the line establishment, up to G10. The proportion of infected males was similarly assessed in G0, G5, and G10. If CI occurs, this infection rate is an overestimate of the actual transmission rate: CI will increase the proportion of infected adults, because uninfected eggs tend to die. The actual maternal transmission rate of two lines was thus estimated after crossing infected females with uninfected males in G20.

Measurements of fitness effects. Female fertility and fecundity were taken as parameters for the fitness effects of infections. These were investigated during CI assay experiments and therefore by using the same mating protocol. For fertility assays, uninfected males were crossed to infected and uninfected females, and hatching rates were compared. For fecundity assays, infected and uninfected males were crossed with infected and uninfected females. Fecundity was estimated by counting the eggs laid per female in 48 h.

PCR-RFLP and sequencing. DNA was extracted from flies according to the method described by O’Neill et al. (25). The PCR primers used were general primer 8IF-691R of the Wolbachia surface protein gene wsp (44) as well as wCer1- and wCer2-specific wsp primer pairs (32), fzu1-fzu2 of the cell cycle gene fzu2 (41), and the 16S rRNA-specific primer for Wolbachia (25). PCRs were run in reaction volumes of 12.5 µl for the infection screening or in 50 µl for post-PCR procedures: 1 or 4 µl of template DNA, 1 x reaction buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.2 µM forward and reverse primers, and 0.5 or 2 U of Taq DNA polymerase (Gibco), and sterile water was added to the final volume. PCR was run under conditions described by Zhou et al. (44), wsp, fzu2, and 16S rRNA PCR products from wCer1-infected R. cerasi, wCer2-infected D. simulans, and wAu infected D. simulans were cycle sequenced with Big Dye (Perkin-Elmer). wCer2 and wAu differ in their 8IF-691R wsp sequence by one substitution (32). This mutation site proved to be a wCer2-specific restriction site for Fnu4HI. wCer2-infected lines were PCR-restriction fragment length polymorphism (RFLP) digested with Fnu4HI (New England Biolabs) under the standard conditions recommended by the restriction enzyme provider, in order to exclude any line or strain contamination with wAu.

Statistical analysis. CI and fertility data were analyzed with Wilcoxon’s non-parametric tests. Fecundity data were analyzed by analysis of variance (ANOVA).

Nucleotide sequence accession number. The ftsZ sequences from wCer1, wCer2, and wAu have been deposited in the GenBank nucleotide sequence database under accession no. AY227737 to -39, respectively. The 16S rRNA gene sequences from wCer1, wCer2, and wAu have been deposited under accession no. AY227740 to -42, respectively.

Results

Line establishment. A total of 1,036 embryos of the uninfected D. simulans STC line were injected with cytoplasm of wCer1- and -2-infected R. cerasi. From these, 82 embryos developed into adult females, 51 of which were infected. The different infection types were wCer1 and -2 (n = 31), wCer2 (n = 12), and wCer1 (n = 8). Thus, segregation between wCer1 and wCer2 already occurred after injection into generation 0 (G0). Transmission of wCer1 and/or wCer2 from G0 to G1 was found in 18 females. From these G0 females, about 10 daughters were taken for line establishment. Only 3 out of 187 G1 females were superinfected with wCer1 and -2, 38 were infected with wCer2, and 8 were infected with wCer1. wCer1 was lost from all lines between G1 and G2, despite efforts to detect rare infected G2 females. In G5, six isofemale lines remained infected by wCer2: RC20, RC21, RC33, RC45, RC50, and RC78. The six lines were from six different G0 females infected with wCer1 and -2 cytoplasm. Uninfected lines RC200, RC210, RC330, RC450, RC500, and RC780 were founded with uninfected G1 females, sisters of the infected females used for the establishment of the infected lines.

Transmission rates. The infection rates in offspring from wCer2 mothers were measured during the line establishment from generations 1 to 10, giving the following estimates: 54% in RC20 (n = 30; 95% confidence interval, 36.2 to 71.8%), 61% in RC21 (n = 102; 95% confidence interval, 51.5 to 70.5%), 65% in RC33 (n = 50; 95% confidence interval, 51.8 to 78.2%), 80% in RC45 (n = 129; 95% confidence interval, 73.1 to 86.9%), 52% in RC50 (n = 43; 95% confidence interval, 37 to 66.9%), 86% in RC78 (n = 33; 95% confidence interval, 74.2 to 97.8%). These infection rates are overesti-
mates of the maternal transmission rate, because the infection status of fathers was not checked. The proportion of infected individuals could be greater in crossings between infected females and infected males than between infected females and uninfected males, because CI selects for higher infection rates in the offspring. The actual maternal transmission rates in RC21 and RC45 were estimated in G20 by crossing infected females with infected males. The transmission rates were 77% for RC21 (n = 60; 95% confidence interval, 66.3 to 87.8%) and 55% for RC45 (n = 71; 95% confidence interval, 43.4 to 66.6%).

**TABLE 1.** Crossing experiments to test whether wCer2 does induce cytoplasmic incompatibility in *D. simulans*

| Generation<sup>a</sup> | Fly<sup>b</sup> | No. of crosses | No. of eggs counted | Mean % embryonic mortality (SE) | Wilcoxon's test result<sup>c</sup> | p<sup>d</sup> |
|------------------------|----------------|---------------|---------------------|-------------------------------|-----------------------------------|--------|
| G<sub>0</sub>          | RC21 (wCer2)   | 15            | 1,613               | 29.2 (4.5)                    | 2.607                             | <0.01  |
| G<sub>0</sub>          | RC21Ø (Ø)      | 10            | 1,093               | 11.7 (5.9)                    | 2.378                             | <0.02  |
| G<sub>0</sub>          | RC21 (wCer2)   | 4             | 491                 | 40.5 (9.2)                    | 2.938                             | <0.01  |
| G<sub>0</sub>          | RC21Ø (Ø)      | 8             | 791                 | 14.9 (3.7)                    | 2.887                             | <0.01  |
| G<sub>10</sub>         | RC21 (wCer2)   | 9             | 1,014               | 33.1 (4.6)                    | 2.355                             | <0.02  |
| G<sub>10</sub>         | RC21Ø (Ø)      | 8             | 849                 | 13.5 (3.6)                    |                                    |        |
| G<sub>0</sub>          | RC33 (wCer2)   | 5             | 584                 | 14.7 (4.5)                    | 0.183                             | <0.86  |
| G<sub>0</sub>          | RC33Ø (Ø)      | 6             | 593                 | 13.9 (4.3)                    | 2.983                             | <0.01  |
| G<sub>10</sub>         | RC33 (wCer2)   | 14            | 1,465               | 13.6 (2.6)                    | 2.548                             | <0.02  |
| G<sub>10</sub>         | RC33Ø (Ø)      | 8             | 722                 | 4.8 (1.3)                     | 2.699                             | <0.01  |
| G<sub>0</sub>          | RC45 (wCer2)   | 19            | 1,896               | 36.5 (4.2)                    | 1.540                             | <0.13  |
| G<sub>0</sub>          | RC45Ø (Ø)      | 8             | 717                 | 24.5 (9.3)                    | 2.964                             | <0.01  |
| G<sub>0</sub>          | RC45 (wCer2)   | 9             | 1,077               | 64.6 (9.5)                    | 3.464                             | <0.001 |
| G<sub>0</sub>          | RC45Ø (Ø)      | 8             | 920                 | 11.9 (8.3)                    |                                    |        |
| G<sub>10</sub>         | RC45 (wCer2)   | 9             | 1,061               | 44.8 (9.9)                    | 2.699                             | <0.01  |
| G<sub>10</sub>         | RC45Ø (Ø)      | 8             | 819                 | 5.8 (1.3)                     | 2.699                             | <0.01  |
| G<sub>0</sub>          | RC50 (wCer2)   | 9             | 784                 | 43.2 (8.2)                    | 2.983                             | <0.01  |
| G<sub>0</sub>          | RC50Ø (Ø)      | 7             | 608                 | 14.1 (2.8)                    | 2.449                             | <0.02  |
| G<sub>10</sub>         | RC50 (wCer2)   | 4             | 289                 | 33.8 (8.9)                    |                                    |        |
| G<sub>10</sub>         | RC50Ø (Ø)      | 5             | 528                 | 6.8 (1.3)                     |                                    |        |

<sup>a</sup> Generation following injection.<br><sup>b</sup> The infecting *Wolbachia* variant is shown in parentheses. Ø, uninfected.<br><sup>c</sup> The Wilcoxon's tests were performed by comparing each cross involving infected males with the corresponding control cross, in which the male is not infected.<br><sup>d</sup> P, associated a probability.

As shown in Table 1, wCer2 was found to induce CI in 8 out of 10 experiments, although at a low level.

The ability of wCer2 to rescue its own CI expression was tested by crossing infected males with infected and uninfected females. Rescue is observed if embryonic mortality is significantly lower when females are infected. This was investigated by using two infected lines (RC21 and RC45) and their uninfected counterparts (RC21Ø and RC45Ø). As shown in Table 2, significant rescue was found in both experiments.

To test if this rescue was complete, infected females were crossed with infected and uninfected males. Rescue can be considered as complete if embryonic mortality is not significantly higher when males are infected. This was investigated by using two infected lines (RC21 and RC45) and their uninfected counterparts (RC21Ø and RC45Ø). As shown in Table 3, rescue was not found complete in the experiment involving the RC45 and RC45Ø lines, while P was found just above the 5% level.

**TABLE 2.** Test of whether wCer2 is able to rescue its own modification in *D. simulans*<sup>a</sup>

| Fly<sup>b</sup> | No. of crosses | No. of eggs counted | Mean % embryonic mortality (SE) | Wilcoxon's test result<sup>c</sup> | p<sup>d</sup> |
|----------------|---------------|---------------------|-------------------------------|-----------------------------------|--------|
| RC21 (wCer2)   | 13            | 1,502               | 35.4 (3.9)                    | 2.393                             | <0.02  |
| RC21 (wCer2)   | 12            | 1,249               | 22.8 (3.0)                    |                                    |        |
| RC45 (wCer2)   | 18            | 2,138               | 54.7 (6.9)                    | 2.938                             | <0.01  |
| RC45 (wCer2)   | 20            | 1,744               | 28.2 (3.3)                    |                                    |        |

<sup>a</sup> Data were pooled from two experiments (performed in G<sub>0</sub> and G<sub>10</sub>), after testing for homogeneity.<br><sup>b</sup> The infecting *Wolbachia* variant is given in parentheses. Ø, uninfected.<br><sup>c</sup> The Wilcoxon's tests were performed by comparing each pair of crosses.<br><sup>d</sup> P, associated a probability.
threshold in the experiment involving RC21 and RC21Ø. Thus, the data suggest that wCer2 does not fully rescue its own CI. As discussed below, imperfect transmission is thought to be the likely explanation.

**Fitness effects.** The effect of wCer2 on female fertility can be tested by crossing infected males with infected and uninfected females. A positive or negative effect on fertility is detected if hatching rates differ in the two crosses. This was investigated by using two infected lines (RC21 and RC45) and their uninfected counterparts (RC21Ø and RC45Ø). As shown in Table 4, wCer2 was not found to affect female fertility.

The effects of wCer2 on female fecundity were tested by crossing infected and uninfected females with both infected and uninfected males (lines RC21 and RC45 and RC21Ø and RC45Ø, respectively). The results, presented in Table 5, were analyzed by ANOVA (Table 6). In the experiment involving RC21 and RC21Ø, a surprising effect of male infection status was observed. Indeed, females appeared to lay significantly more eggs when mated with infected males. In this experiment, infected females were less fecund than uninfected ones, but this difference was not significant at the 0.05 threshold. In the experiment involving RC45 and RC45Ø, no effect of male infection was found. Again, infected females were less fecund than uninfected ones, and here the difference was significant. Thus, the data suggest that wCer2 reduces fecundity in infected females.

**PCR-RFLP and sequencing.** Sequenced wsp PCR products and PCR-RFLP from single flies of strains RC21 and RC45 confirmed the presence of wCer2 in these lines. Contamination with wAu did not occur. ftsZ PCR products of wCer2-infected D. simulans, wAu-infected D. simulans Coffs Harbor, and of wCer1-infected R. cerasi flies were sequenced. wCer2 and wAu shared the same ftsZ sequences, confirming their close genetic relationship. wCer1 was more distantly related, and sequence divergences in ftsZ (2.23% in 941 bp) and wsp (2.38 to 2.55% to wCer2 and wAu, respectively, in 588 bp) (32) were similar. Interestingly, substitutions were equally spread through ftsZ of wCer1, whereas they were restricted to the 3' region of wsp. Most substitutions in wsp of wCer1 were nonsynonymous. All three strains wCer1, wCer2, and wAu shared the same 16S rRNA sequences.

**DISCUSSION**

**Injection, segregation, and infection loss.** After injection of superinfected R. cerasi into D. simulans, wCer1 and wCer2 segregated in G0. In their original host, segregation of wCer1 and wCer2 was observed at a rate of <1% in field populations, whereby in all cases, wCer1 was the leaking variant (32). High segregation rates during injection most probably result from the low number of bacterial cells that are injected within a single recipient egg and actually survive. Both wCer1 and wCer2 were still detectable by PCR in G1 following injection, suggesting that both variants reached the germ cells of G0 females. However, wCer1 was lost from all lines between G1 and G2, suggesting that it was unable to develop properly in this new host or to actively maintain itself in the germ line. This loss was unfortunate, because it prevented us from determining the phenotypic effects of wCer1, yet it also proved to be an informative result. The incapacity of wCer1 to develop in a new host might reflect a higher genetic divergence from wCer2 and a very tight and specific adaptation to the original host. This interpretation is consistent with the view that wCer1 is a more ancient infection in R. cerasi than is
wCer2, as suggested by infection patterns in natural populations (32). On the contrary, wCer2 was still present in G2. Although the efficiency of maternal transmission is low in D. simulans, imposing a stringent protocol for infection maintenance, we still possess, at the time of writing, the six lines derived from six different G0 females. 

CI levels, fitness effects, and transmission efficiency. We found that wCer2 can induce CI in D. simulans, although embryonic lethality is far from 100%. This confirms that wCer2 is able to induce CI and strengthens the view that it is responsible for the patterns of incompatibility observed between R. cerasi populations (2).

We observed that wCer2 is able to rescue its own CI, but only partially so. This probably results from imperfect maternal transmission (i.e., not all eggs are infected and therefore protected from CI). The transmission rates that would be necessary to explain the imperfect rescue would be 55 to 65% for RC21 and RC45. Similar transmission rate values were observed for both lines at G20. Thus, it seems that wCer2 is not, strictly speaking, self-incompatible. Partial nonrescue is simply due to imperfect maternal transmission.

wCer2 does not affect female fertility, but seems to reduce female fecundity by at least 10%. Negative effects on host fitness have been reported previously in natural as well as artificial Wolbachia-host associations (19, 21). Intriguingly, in one data set (involving lines RC21 and RC21O), females were found to lay more when mated with infected males—a result that we fail to interpret in adaptive terms.

wCer1 was not transmitted after G1, while wCer2 had a lower transmission rate. This can be seen by the infection frequency observed during line maintenance, giving a mean value of 66% for the six transinfected lines. Transmission efficiency per se was estimated at G20 in lines RC21 and RC45, giving a mean value of 65.5%, which is much lower than any maternal transmission rate reported so far for natural Wolbachia-host associations. We observed considerable variability within and between the transinfected lines in their infection rates with wCer2 over a long time, here represented by the data from generations 1 to 10 and from generation 20. This variability was not correlated to generation number or lines. We do not yet have an explanation for this finding.

Testing theory. Theory predicts that Wolbachia-host coevolution should lead to a decline of CI level and fitness costs and to an increase in maternal transmission (30, 35). Inversely, strong CI, strong costs, and low transmission rates are expected in new associations (11). We tested this prediction by creating a new association and measuring the parameters. As expected, fitness costs to the host and low transmission rates were observed, but CI levels were very low. Wolbachia density in male testes has been recognized as a key factor for the expression of CI in Wolbachia associations (8, 12, 40). Whether the lower expression of CI of wCer2 in D. simulans is correlated with a reduced density still needs to be assessed. However, from an evolutionary perspective, there are two possible explanations why CI levels might be low in the novel wCer2 D. simulans association.

First, D. simulans might actively repress the expression of wCer2. This is plausible because wCer2 is very closely related to wAu, a natural Wolbachia variant of D. simulans, which does not appear to induce CI in this host (10, 20, 23, 31). Although wAu might have lost its ability to induce CI, regardless of the host background, a possibility remains that D. simulans actively and specifically represses its expression. This being so, D. simulans might recognize wCer2 as wAu-like Wolbachia and therefore repress it.

Alternatively, the wCer2 infection might be maladapted to the new host and therefore not be able to induce high levels of CI in a new host background. Hence, the prediction that CI should be high in new associations might be incorrect. Levels of CI expressed in different host species have so far only been compared in experiments in which the original and novel host were closely related (5, 11, 27). High levels of CI were observed after the transfer of wRi from D. simulans into Drosophila serrata (11) and after the transfer of wMel-infected D. melanogaster into D. simulans (27). However, these results could reflect the evolutionary closeness of Drosophila species rather than the ability of Wolbachia to express high CI in any background. High CI levels might in fact not always be the sign of a recent Wolbachia-host association. Prout (30) and Turelli (35) demonstrated that within panmictic populations, bacterial variants inducing higher CI levels are not selected for, but Frank (17) showed that if the population is structured, bacte-
The likelihood of horizontal transfers. From phylogenies of Wolbachia and their hosts, as well as direct observation, it is now clear that horizontal transfers between species can occur (18, 22, 25, 38, 41, 44). Wolbachia in arthropods could be seen as a huge metapopulation with infected host species as habitats for various subpopulations (7). Within host species, extinction and colonization might regularly occur through loss or gain of infection, and the current distribution of Wolbachia could represent a global and dynamic equilibrium between these two processes (43).

Following the ideas of Combes (13, 39), it can be generalized that Wolbachia must cross three filters (ecological, physiological, and population) before it is established in a new host species. The ecological filter is defined by the interaction between an existing and a potential new host species. It will only be possible infection frequency at equilibrium for natural means, it would not be able to invade populations of Boller, E. F., K. Russ, V. Vallo, and G. L. Bush. 1976. Incompatible races of European cherry fruit fly, Rhagoletis cerasi (Diptera: Tephritidae), their origin and potential use in biological control. Enтомол. Exp. Appl. 20:237–247.

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