Wolbachia Transfer from *Drosophila melanogaster* into *D. simulans*: Host Effect and Cytoplasmic Incompatibility Relationships

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Manuscript received September 23, 1997
Accepted for publication June 8, 1998

ABSTRACT

Wolbachia are maternally transmitted endocellular bacteria causing a reproductive incompatibility called cytoplasmic incompatibility (CI) in several arthropod species, including Drosophila. CI results in embryonic mortality in incompatible crosses. The only bacterial strain known to infect *Drosophila melanogaster* (*w*Dm) was transferred from a *D. melanogaster* isofemale line into uninfected *D. simulans* isofemale lines by embryo microinjections. Males from the resulting transinfected lines induce >98% embryonic mortality when crossed with uninfected *D. simulans* females. In contrast, males from the donor *D. melanogaster* line induce only 18–32% CI on average when crossed with uninfected *D. melanogaster* females. Transinfected *D. simulans* lines do not differ from the *D. melanogaster* donor line in the Wolbachia load found in the embryo or in the total bacterial load of young males. However, >80% of cysts are infected by Wolbachia in the testes of young transinfected males, whereas only 8% of cysts are infected in young males from the *D. melanogaster* donor isofemale line. This difference might be caused by physiological differences between hosts, but it might also involve tissue-specific control of Wolbachia density by *D. melanogaster*. The *w*Dm-transinfected *D. simulans* lines are unidirectionally incompatible with strains infected by the non-CI expressor Wolbachia strains *w*Ki, *w*Mau, or *w*Au, and they are bidirectionally incompatible with strains infected by the CI-expressor Wolbachia strains *w*Ha or *w*No. However, *w*Dm-infected males do not induce CI toward females infected by the CI-expressor strain *w*Ri, which is found in *D. simulans* continental populations, while *w*Ri-infected males induce partial CI toward *w*Dm-infected females. This peculiar asymmetrical pattern could reflect an ongoing divergence between the CI mechanisms of *w*Ri and *w*Dm. It would also confirm other results indicating that the factor responsible for CI induction in males is distinct from the factor responsible for CI rescue in females.

WOLBACHIA are maternally transmitted endocellular bacteria infecting numerous species of arthropods (Werren and O'Neill 1997). Their presence can lead to reproductive alterations (Werren 1997; Bourtzis and O'Neill 1998), such as feminization (Rigaud 1997), thelytokous parthenogenesis (Stouthamer 1997), or cytoplasmic incompatibility (CI; Hoffmann and Turelli 1997). CI is a reproductive incompatibility causing embryo mortality. It appears when a male infected by one (or more) Wolbachia strain(s) is crossed with a female that is either devoid of Wolbachia or does not harbor the strain(s) found in the male. Wolbachia strains can be classified on the basis of their CI type or by using described Wolbachia gene sequences, *i.e.*, 16S *rDNA* (Breeuwer *et al.* 1992; O'Neill *et al.* 1992; Rousset *et al.* 1992; Stouthamer *et al.* 1993), the bacterial cell cycle genes *ftsZ* (Holden *et al.* 1993; Werren *et al.* 1995) and *dnaA* (Bourtzis *et al.* 1994), and *wsp*, a very variable surface protein gene (Braig *et al.* 1998; Zhou *et al.* 1998).

Three CI-expressor Wolbachia strains have been described in *Drosophila simulans*: *w*Ri (Hoffmann *et al.* 1986), *w*Ha (O'Neill and Karr 1990), and *w*No (Merçot *et al.* 1995). Strains infected by *w*Ri, *w*Ha, or *w*No are all bidirectionally incompatible; *i.e.*, CI will occur (typically with 70–100% embryonic mortality) in both directions of crosses. Three more strains that do not cause detectable CI have been found in *D. simulans*. One (*w*Ma) is restricted to Madagascar (Rousset and Solignac 1995), another (*w*Au) has been found in Australia and America (Turelli and Hoffmann 1995; Hoffmann *et al.* 1996), and the most recently discovered, *w*Ki (Merçot and Poinsot 1998b), is known from a single population from Mount Kilimanjaro (Tanzania).

In contrast with this diversity, only one Wolbachia CI

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type has been described in the sibling species *D. melanogaster*. In this species, the situation is as follows: (i) CI is generally weak, *i.e.*, 20–30% embryonic mortality (Hoffmann 1988; Bourtzis *et al.* 1994, 1996; Hoffmann *et al.* 1994), although it can vary between strains of hosts, from being undetectable to >70% embryonic mortality (Solignac *et al.* 1994), and (ii) all infected strains are mutually compatible, regardless of their capacity to induce detectable levels of CI (Solignac *et al.* 1994). The Wolbachia infecting five different *D. melanogaster* strains were tested using the highly variable sequence of the Wolbachia surface protein *wsp*. Four had identical sequences, and one differed from the other by 2 out of 565 bp (Zhou *et al.* 1998). As a comparison, the same authors find that *wsp* sequences of *D. simulans* Wolbachia strains can vary by as much as 162 bp.

The apparent difference of behavior of Wolbachia in these two sibling species of Drosophila prompts the following question: Does the level of CI expression vary because of differences between Wolbachia strains, or is it host dependent? Boyle *et al.* (1993) transferred the Wolbachia strain *w*Ri from *D. simulans* into an uninfected *D. melanogaster* strain, with a low CI as a result (15–35% in the absence of selection). However, when back transferred into a *D. simulans* background, *w*Ri expressed >75% embryonic mortality in incompatible crosses. In this case, the host seemed to play a key role in the level of CI. Moreover, these authors noted that the higher CI induced by *D. simulans* males was associated with higher levels of bacteria in the eggs of infected females from the same stock, compared to the Wolbachia load in *D. melanogaster* eggs. This brings forth a second question: Could the difference of CI levels be explained simply by different bacterial loads; *i.e.*, could it be caused by only a dosage effect, as suggested by Breeuwer and Werren (1993) in the microhymenopteran *Nasonia vitripennis*.?

We have generated transinfected lines by injecting in uninfected *D. simulans* embryos the Wolbachia strain found in *D. melanogaster* (in the present work this Wolbachia is referred to as *w*Dm). Our objective was to answer two questions: (i) Would *w*Dm behave differently in a new host as far as CI and bacterial load are concerned? (ii) Would *w*Dm determine a completely new CI type in *D. simulans*, as compared to the other known infections in this species? Once transferred into *D. simulans*, the strain *w*Dm induced a very high CI, while CI expression was low in the donor *D. melanogaster* line. A dot blot assay on DNA extracts from whole flies showed that males of transinfected *D. simulans* lines did not harbor significantly more Wolbachia than males from the *D. melanogaster* donor line. However, the observation of testes using a DAPI coloration revealed that the percentage of infected cysts was 10 times higher in *D. simulans* transinfected males than in males of the *D. melanogaster* donor line.

When confronted with the natural Wolbachia strains found in *D. simulans*, *w*Dm exhibited strong unidirectional CI against non-CI-expressor strains *w*Au and *w*Ki (as well as against the nonexpressor *w*Mau strain found in *D. mauritiana*). CI was strong and bidirectional between *w*Dm and the CI-expressor strains *w*Ha and *w*No. In contrast, we present evidence that *w*Dm is completely compatible with the CI-expressor strain *w*Ri in one direction of cross, with the reciprocal cross exhibiting partial CI. Such an asymmetry (which is not caused by the genome of the host) had not been reported previously between two Wolbachia strains capable of inducing CI.

MATERIALS AND METHODS

Strains: *D. melanogaster* Wien 5 is an isofemale line that was established in 1994 from a naturally infected population (Vienna, Austria). It is used as the source of *w*Dm Wolbachia. MelO is a naturally uninfected strain from Nasr'allah, Tunisia. It is used in crosses as a standard uninfected control. *D. simulans* SimO is a naturally uninfected strain from the same location as MelO, *i.e.*, Nasr'allah, Tunisia (Montchamp-Moreau *et al.* 1991). It is used in crosses as a standard uninfected control. R1A, NHa, and R3A derive from a naturally infected population (Nouméa, New Caledonia). In the wild, Nouméa individuals are either infected simultaneously by *w*Ha and *w*No, or they are mono-infected by *w*Ha. The laboratory strain R1A only harbors bi-infected individuals. NHa is a Nouméa stock that was found in the laboratory to be infected by *w*Ha only, and R3A was obtained by substituting the Nouméa genome by that of SimO for 11 generations. It was then found to be infected by *w*No only (Merçot *et al.* 1995). NHaTC is an uninfected stock derived from NHa by an antibiotic treatment (tetracycline) that cured its Wolbachia infection (Poinsot and Merçot 1997). It is used as the uninfected recipient *D. simulans* strain in the transinfection experiments. ME lines are the transinfected lines obtained after injection of *w*Dm into NHaTC embryos. DSR is a Californian strain infected by *w*Ri (Hoffmann *et al.* 1986). Coffs Harbour is an Australian strain founded using only infected flies from a 1993 collection. It is carrying the variant *w*Au (Hoffmann *et al.* 1996). Kc9 is an isofemale line from the Kilimanjaro 1996 strain (Mount Kilimanjaro, Tanzania) and is infected by the variant *w*Ki (Merçot and Poinsot 1998b). DSW(Mau) is an isofemale line obtained by transinfecting *w*Mau from *D. mauritiana* into the *D. simulans* Californian uninfected strain Watsonville (Giordano *et al.* 1995). The *w*Mau strain is the equivalent in *D. mauritiana* of the *w*Ma Wolbachia strain found in a Madagascar population of *D. simulans* (Rousset and Solignac 1995).

Rearing conditions: To ensure the most favorable conditions for the infection, all strains used were maintained at 25° on axenic medium (David 1962) as low-density mass cultures (unless otherwise stated) by crossing 20 virgin females aged 3–5 days with 25 virgin males aged 3 days. Low-density rearing is preferable because larval crowding can have a negative effect on the expression of CI (Sinkins *et al.* 1995). Crosses with young males ensure a maximum selection against possible uninfected eggs because CI is highest in young males (Hoffmann *et al.* 1986; Montchamp-Moreau *et al.* 1991); Wolbachia is gradually eliminated from the testes as the males become older (Bressac and Rousset 1993).

Microinjections: Microinjections were carried out as described in Santamaria (1987). Using a microcapillary needle (Boehringer Femtotips), cytoplasm was drawn from infected Wien 5 embryos and then injected into slightly dehydrated,

uninfected NHaTC embryos. Isofemale lines were established after crossing the emerging females with NHaTC males.

PCR conditions: Total DNA was extracted from the ovaries of individual females, following the method of Kocher *et al.* (1989), or from whole individuals, following the method of O'Neill *et al.* (1992). The Wolbachia 16S ribosomal subunit DNA sequence was amplified using specific primers 99F and 994R as described in O'Neill *et al.* (1992). Alternatively, Wolbachia-specific primers for the *dnaA* bacterial gene were used as described in Bourtzis *et al.* (1994).

Restriction fragment length polymorphism: To further ensure that the infection found in ME lines resulted from the microinjection and not from a resurgence of the *w*Ha Wolbachia infecting the NHa strain before tetracycline treatment, PCR amplification products of ME lines were subjected to *Mwo*I digestion at 37° during 3 hr. The 16S *rDNA* sequence of *w*Dm presents a *Mwo*I restriction site, whereas the sequence of *w*Ha does not, which allows discrimination of the two strains by RFLP.

Wolbachia load in individual males: Total DNA was extracted by the STE method (O'Neill *et al.* 1992) from individual males used in incompatible crosses. One microliter (out of 50) of the supernatant was used as a template for PCR analysis with Wolbachia-specific primers for the *dnaA* gene. The rest was dot blotted on a zeta probe blotting membrane and hybridized with a 480-bp PCR-derived *dnaA* fragment; the *dnaA* gene is known to be a single-copy gene in Wolbachia (Bourtzis *et al.* 1994). The image analysis of the autoradiograms and the calculation of the Wolbachia densities, expressed as bacterial equivalents per male, have been described previously (Bourtzis *et al.* 1996). Statistical analysis of bacterial densities was carried out using the Tukey test (Sokal and Rohlf 1995) after square root transformation because group variances were proportional to the means.

Wolbachia load in sperm cysts: Relative Wolbachia densities were estimated in very young males (a few hours old) by DAPI staining of sperm cysts, as described in Bressac and Rousset (1993).

Wolbachia load in embryos: Total DNA was extracted from groups of 50 embryos (0–2 hr old), and a dot blot assay was carried out following the procedure described above.

CI measurements (mass crosses): Tests were performed at 25°. Fifteen 4- or 5-day-old virgin females were allowed to mate for 8 hr with 25 virgin 3-day-old males in a bottle with standard axenic medium. Flies were transferred for oviposition on fresh axenic medium. After 24 hr, the adults were discarded and the eggs were kept at 25° for at least another 24 hr before the hatch rate was estimated, generally on 200 eggs per line.

CI measurements (individual tests): In the crosses using F_1 individuals or flies infected by *w*Au, *w*Ki, or *w*Mau, 3-day-old males were crossed with females aged 3–5 days. All matings were monitored, and inseminated females were placed individually for 48 hr on small petri dishes with fresh medium. Upon removal, the dishes were kept at 25° for at least 24 hr before the hatch rate was estimated on the total egg count. In the experiment using males for which global bacterial density was also assessed, the same protocol was used, except that virgin males were aged 1–2 days and females were aged 1–3 days. In addition, males were frozen immediately after mating for future DNA extraction.

Level of cytoplasmic incompatibility: When reciprocal crosses with an uninfected strain were available, we used CI_{corr}, a corrected index of CI. The aim was to minimize the background noise caused by the natural mortality of the cross (which is unrelated to CI). This background noise is estimated by the compatible cross mortality (CCM), *i.e.*, the mortality observed in the cross between standard uninfected males and infected females of the strain under test. CI_{corr} is then defined as the percentage of eggs that do not hatch among those that would have survived in the absence of CI. Then $CI_{corr}(\%) = [(CI_{obs} - CCM)/(100 - CCM)] \times 100$, where CI_{obs} is the percentage of unhatched eggs observed in the incompatible cross. The CI_{corr} of a given male was set at 0 whenever $CI_{obs} <$ CCM. All statistical analyses were carried out after arcsine transformation. By definition, the CI_{corr} index does not apply in crosses where both directions are incompatible. In such cases, CI in a given direction of cross was simply estimated by the percentage of unhatched eggs.

RESULTS

Establishment of transinfected lines: We injected 215 eggs of the uninfected *D. simulans* strain NHaTC with cytoplasm of eggs from the infected *D. melanogaster* Wien 5 isofemale line. Out of the 33 fertile females recovered, 17 gave a positive PCR signal with 16S *rDNA* Wolbachia-specific primers. Amplification products obtained from transinfected ME lines were of the *w*Dm type and not of the *w*Ha type, according to their RFLP pattern after *Mwo*I digestion. A similar pattern was found in the *Mwo*I-digested amplification product from Wien 5 females, whereas the *MwoI*-digested amplification product from a NHa control yielded the expected *w*Ha RFLP pattern (data not shown). The infection found in ME lines is, therefore, a consequence of *w*Dm transfer and not of imperfect elimination of *w*Ha by tetracycline treatment of the NHa strain. As a first step, among the isofemale lines founded by PCR-positive G0 females, 5 were chosen at random for CI testing and were maintained in bottles under the same conditions as Wien 5 and all infected strains during the experiment, *i.e.*, low-density bottles by crossing 20 virgin females to 25 young virgin males for 24 hr. The other 13 lines were initially maintained by mass transfer in vials. In G3, virgin males from the ME lines kept in bottles were tested for CI. At the same time, all 17 ME lines were tested again by PCR. It was then found that 2 of the 5 lines kept in bottles (ME 4 and ME 17) had lost the infection and that 9 out of the 13 lines maintained in vials by mass transfers had also lost the infection. All infected lines kept in vials were then transferred to bottles under the same conditions described above. No further loss of infection was found in the subsequent generations.

CI induced by *w*Dm as a function of the host: In G3, the five lines maintained in bottles were tested for the expression of cytoplasmic incompatibility. The results (Figure 1) show that all three infected lines expressed moderate to very high levels of CI, while the two uninfected ones did not. CI was also measured in G6. At this time, the infection was clearly established and the expression of CI was very high in all lines, with embryonic mortality >98% (Figure 1). These values are in sharp contrast with the moderate amount of CI expressed by the Wien 5 donor line in mass crosses with the uninfected strain MelO (23.5% CI on average, based on the observation of 1000 eggs). However, it has been

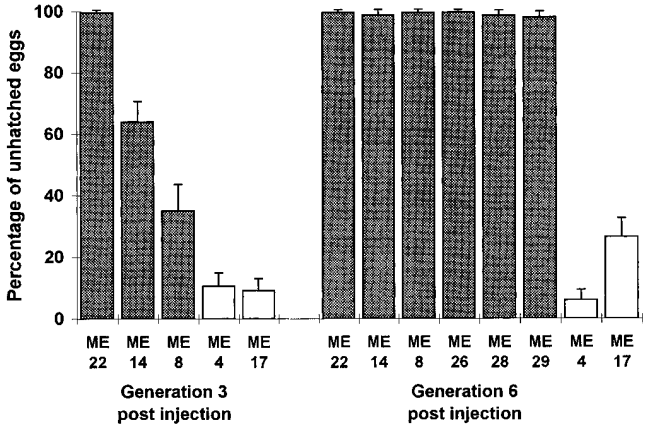

Figure 1.—Cytoplasmic incompatibility expressed by males of *D. simulans* isofemale lines transinfected with *w*Dm. Shaded bars, infected lines; white bars, lines that were founded by a PCR-positive transinfected G0 female but found to be uninfected in G3. CI is estimated by the percentage of unhatched eggs in mass crosses with uninfected SimO females (117–200 eggs per cross). Error bars are 95% confidence intervals.

shown that naturally infected strains of *D. melanogaster* can be polymorphic for the infection, with a proportion of the individuals uninfected (Hoffmann *et al.* 1994; Solignac *et al.* 1994). Our mass crosses did not allow us to determine whether this effect could explain the difference of CI expression. A comparison was then made between ME lines and the Wien 5 line, using individual crosses for which all males were tested by PCR to exclude the uninfected ones from the analysis. The results (CI being expressed as CI_{corr}) are presented in Table 1. The ANOVA shows that the line effect is highly significant ($F^{7}_{170} = 102.8; P < 0.001$), and a Tukey test indicates that this results from the CI_{corr} of Wien 5 being significantly lower than that of ME lines; the ME lines are not significantly different among themselves.

The Wien 5 line was further tested two generations later and was again found to induce a moderate amount of CI_{corr} (N = 30 crosses, 2612 eggs, $CI_{corr} = 17.8 \pm 2.9\%$). This figure is even significantly lower than the one presented in Table 1 ($t = 2.21$; 50 d.f.; $P < 0.05$).

TABLE 1

Means of corrected cytoplasmic incompatibility (CI_{corr}) and bacterial equivalents per male

Line	*N* crosses	*N* eggs	% CI_{corr} ± SE	Bacteria/male ±SE ($\times 10^6$)
ME 8	20	956	90.1 ± 2.1	46.41 ± 5.36
ME 14	24	1073	94.5 ± 0.6	47.09 ± 3.06
ME 16	22	947	92.9 ± 0.9	35.08 ± 4.72
ME 22	25	1045	93.3 ± 0.8	29.12 ± 2.63
ME 26	24	1006	90.8 ± 1.5	44.84 ± 2.55
ME 28	20	914	94.0 ± 0.7	33.12 ± 2.37
ME 29	21	929	94.5 ± 0.7	28.28 ± 1.63
Wien 5	22	1367	31.8 ± 4.2	48.20 ± 6.48

Such a phenomenon is in agreement with previous observations showing that CI can vary significantly in only a few generations in *D. melanogaster* (Solignac *et al.* 1994).

Our initial concern regarding a possible infection polymorphism in Wien 5 was not justified; the fraction of uninfected males found in Wien 5 (2 out of 54) was even lower than in the ME lines tested (17 out of 175), but not significantly so ($\chi^2 = 1.96$; 1 d.f.; NS).

***w*Dm bacteria load in males as a function of the host:** For a given Wolbachia strain, the level of CI has been correlated to the proportion of infected cysts in the testes in Drosophila (Bressac and Rousset 1993; Solignac *et al.* 1994). A similar correlation has also been found with the Wolbachia load in eggs laid by infected females in Drosophila (Boyle *et al.* 1993) and in the parasitoid wasp *N. vitripennis* (Breeuwer and Werren 1993). To establish whether the large difference of CI between ME lines and Wien 5 could result from a difference of Wolbachia load, we assayed bacteria density using different means. First, dot blots were carried out on total DNA extracts from the 178 infected males used in the individual incompatible crosses presented in Table 1. The results indicate that the overall Wolbachia load (expressed as the number of Wolbachia equivalents per individual) in Wien 5 males at the time of the CI tests was not lower than that of ME males (Table 1). Indeed, Wien 5 obtained the highest load score in this first experiment. The line factor is significant ($F^{7}_{170} = 66.15; P < 0.001$), but a Tukey test reveals that the only significant differences are among ME lines: ME 14 *vs.* ME 29, ME 14 *vs.* ME 22, and ME 22 *vs.* ME 26.

A further dot blot assay was carried out two generations later using 30 Wien 5 males assayed for CI expression that exhibited very low CI_{corr} (see the section above). The bacterial load of Wien 5 flies during this second assay was significantly lower than that found previously ($28.72 \pm 3.83 \times 10^6$ bacterial equivalents per male *vs.* $48.2 \pm 6.48 \times 10^6$: $t = 2.59$; 50 d.f.; $P < 0.05$). However, it was similar to that of some ME lines that exhibited a very high CI (see ME 22 and ME 29 in Table 1).

To ensure that the comparable Wolbachia loads we found in *D. melanogaster* and *D. simulans* males did not hide tissue-specific variations, we also assessed the specific load in the testes using a DAPI-staining technique. This time, the results reveal a striking difference: young Wien 5 males exhibit 10 times less infected cysts than ME males of the same age (Table 2). Because CI is caused by the presence of Wolbachia in male reproductive cells, this 10-fold difference alone would seem sufficient to explain the very low CI phenotype exhibited by Wien 5 males in contrast with the high CI exhibited by transinfected ME males. Such patterns of cyst infection are in agreement with previous results regarding the variants *w*Ha and *w*Ri in *D. simulans* (Bressac and Rousset 1993) and with *w*Dm in *D. melanogaster* (Solignac *et al.* 1994).

TABLE 2

Percentage of infected cysts in young males and Wolbachia load in embryos

Line or strain	*N* cysts[a]	Infected cysts ±SE (%)	Bacterial equivalents in 50 embryos[b] ± SE
Wien 5 (*w*Dm)[c]	394	8.2 ± 0.9	$4.34 \pm 0.83 \times 10^6$
ME 8 (*w*Dm)	355	81.0 ± 1.8	$8.36 \pm 0.96 \times 10^6$
ME 16 (*w*Dm)	351	82.9 ± 1.4	$6.67 \pm 1.14 \times 10^6$
ME 29 (*w*Dm)	353	82.0 ± 2.0	$5.64 \pm 0.75 \times 10^6$
DSR (*w*Ri)	363	81.7 ± 2.6	$7.46 \pm 1.06 \times 10^6$

[a] DAPI observation of 18–33 cysts per male, 15 males per line or strain.
[b] Dot blots carried out on total DNA extracts, $N = 10$ groups of 50 embryos aged 0–2 hr per line.
[c] The infection status of each line or strain is indicated between brackets.

We also assessed the Wolbachia load in early embryos (0–2 hr old) by dot blot. The results show that the bacterial loads of Wien 5 or ME embryos do not differ significantly (Table 2). Indeed, although the line effect is significant ($F^4_{45} = 2.67$; $P < 0.05$), a Tukey test reveals two largely overlapping groups with the only significant difference found between Wien 5 and ME 8. This suggests that the lower load in the testes of Wien 5 males does not result from Wien 5 eggs initially harboring fewer Wolbachia than ME eggs, but would depend on events taking place during later developmental stages. The average number of Wolbachia per mature egg can be estimated from our results at more than 100,000 Wolbachia equivalents per egg (based on 2500 eggs, *i.e.*, 50 groups of 50 eggs). This estimate is lower than but comparable to the 500,000 figure estimated elsewhere by DAPI staining on eggs from the DSR strain (T. Karr in Bourtzis *et al.* 1996).

CI relationships between *w*Dm and the natural CI-expressor Wolbachia strains of *D. simulans*: Infected ME lines were crossed in both directions with the *D. simulans* strains R1A, NHa, R3A, and DSR. This allowed us to establish the CI relationships between *w*Dm and the three CI-expressor Wolbachia strains (*w*Ha, *w*No, and *w*Ri) found in natural populations of *D. simulans.* ME lines were bidirectionally incompatible with *D. simulans* strains infected by *w*Ha or *w*No or carrying the bi-infection *w*Ha + *w*No (Table 3). A strikingly different pattern appeared when we crossed ME lines with the DSR strain carrying the continental Wolbachia strain *w*Ri (Table 4):

1. Males from the seven ME lines infected by *w*Dm induced only a very limited mortality when crossed with *w*Ri-infected females (average of 18.3% unhatched eggs). Although this value is low, it might represent some CI because it is significantly higher than what is observed in crosses involving males from the two uninfected ME lines (average of 7.5%; infected *vs.* uninfected: $t = 2.49$; 7 d.f.; $P < 0.05$). On the other hand, this mortality is comparable to that observed within the DSR strain (which ranges from 13 to 28.5%) or within infected ME lines (Table 4). These latter intraline mortalities might be caused in part by inbreeding: although CI could also play a role, intraline mortality is not significantly higher in the seven infected ME lines compared to the two uninfected ME lines (17.0 *vs.* 11.25%: $t = 1.09$; 7 d.f.; NS).
2. In contrast, DSR males clearly induced significant CI against ME females infected by *w*Dm (Table 4; average of 59.8% mortality *vs.* average of 18.3% in the reciprocal cross: $t = 12.64$; 9 d.f.; $P < 0.001$). Yet, this level of CI (59.8%) is significantly reduced compared to the CI induced by DSR males against uninfected ME females (average of 95.5%: $t = 15.72$; 7 d.f.; $P < 0.001$).

We then tried to determine whether the partial and possibly unidirectional CI pattern between *w*Ri and *w*Dm could be explained by the number of Wolbachia in the testes of *w*Ri-infected DSR males being too high for CI to be rescued by the number of Wolbachia present in eggs laid by *w*Dm-infected ME females. The analysis of the results, shown in Table 2 (see above), is clearly in opposition with this simple quantitative hypothesis because (i) we did not find any significant difference in the percentage of infected cysts in young males between infected ME lines and the DSR strain, and (ii) the Wolbachia loads of the eggs laid by *w*Dm- or *w*Ri-infected females are not significantly different.

We also tried to rule out the possibility that apparent partial incompatibility in the direction of cross *w*Ri-infected male × *w*Dm-infected female was in fact caused by poor fertility in DSR males or, in general, by the genetic background of the host. The first possibility was suggested by the quite high intrastrain mortality in our DSR strain (up to 28.5% in some mass crosses), which led us to suspect that by inbreeding and drift, this laboratory strain might have fixed male sterility alleles. F_1 individuals were then obtained from both directions of cross between DSR and the infected line ME 29, using old nonvirgin males to minimize selection caused by CI. These individuals are genetically similar but are infected

TABLE 3

CI relationships[a] between *w*Dm and the CI-expressors *w*Ha and *w*No

Cross male × female	ME lines under test (ME_{UT})			
	ME 8	ME 14	ME 22	ME 4[b]
ME_{UT} (*w*Dm)[c] × NHa (*w*Ha)	70.5	100.0	99.0	13.0
NHa (*w*Ha) × ME_{UT} (*w*Dm)	98.5	97.0	96.5	95.5
ME_{UT} (*w*Dm) × R3A (*w*No)	95.5	100.0	100.0	24.0
R3A (*w*No) × ME_{UT} (*w*Dm)	97.0	87.6	82.5	79.5
ME_{UT} (*w*Dm) × R1A (*w*Ha + *w*No)	84.0	99.5	100.0	7.0
R1A (*w*Ha + *w*No) × ME_{UT} (*w*Dm)	91.0	97.5	96.0	93.0

[a] CI is estimated by the percentage of unhatched eggs in mass crosses of 25 males × 15 females, calculated from 200 eggs per cross.
[b] ME 4 is an uninfected ME line.
[c] The infection status of each line or strain is indicated between brackets.

by *w*Ri or *w*Dm, depending on whether their mother was a DSR or a ME female. The individual CI tests carried out using these F_1 individuals are shown in Table 5. Reciprocal crosses confirmed the existence of CI between 3-day-old males infected by *w*Ri (= F_{1wRi} males) and females infected by *w*Dm (= F_{1wDm} females). The mean percentage of embryonic mortality induced by F_{1wRi} males crossed with F_{1wDm} females is 30.5%. This value is significantly higher than that obtained in the reciprocal cross (8.1%: $t = 4.62$; 21 d.f.; $P < 0.001$) and in the cross between F_{1wDm} individuals (8.6%: $t = 4.40$; 24 d.f.; $P < 0.001$). The CI induced by *w*Ri in a F_1 background was significantly lower than that in a DSR background (Table 4; average = 59.8%: $t = 5.31$; 18 d.f.; $P < 0.001$). This might result from poor fertility in DSR males. Alternatively, the F_1 background might have depressed CI induction by *w*Ri. However, this second possibility is very unlikely, considering that young F_{1wRi} males induce a near total CI (99.8% mortality) against uninfected females (Table 5).

The results in Table 5 also allow us to conclude that *w*Dm-infected males are not able to induce CI against *w*Ri-infected females. The egg mortalities found in the crosses F_{1wDm} male × F_{1wRi} female and F_{1wDm} × F_{1wDm} are not significantly different, regardless whether males are 3 days old (8.1 *vs.* 8.7%: $t = 0.01$; 28 d.f.; NS) or 7 days old (6.5 *vs.* 5.4%: $t = 0.37$; 24 d.f.; NS). Thus, although *w*Ri and *w*Dm are both able to induce very high levels of CI, they seem to be partially compatible in one direction of cross and fully compatible in the reciprocal direction, a pattern not described before.

The possibility remained that the partial compatibility between F_{1wRi} males and F_{1wDm} females was caused only by a quantitative difference, not in bacterial load directly, but in a factor produced by Wolbachia to induce (or rescue from) CI. The results shown in Table 5 suggest that this is not the case. First, it must be noted that 7-day-old (hereafter noted as "old") F_{1wRi} males induce significantly more egg mortality than old F_{1wDm} males when crossed with F_{1wDm} females (12.3 *vs.* 5.4%: $t = 3.07$; 26 d.f.; $P < 0.01$). This is not caused by a lower fertility in aging F_{1wRi} males because old F_{1wRi} males do not induce significantly more egg mortality than 3-day-old (hereafter noted "young") F_{1wRi} males when mated with F_{1wRi} females (3.4 *vs.* 4.4%: $t = 0.60$; 26 d.f.; NS). According to the hypothesis that *w*Ri and *w*Dm differ only by a quantitative factor, the weak but significant CI expressed by old F_{1wRi} males toward F_{1wDm} females would imply that the CI capability of old F_{1wRi} males is at least equivalent to that of young F_{1wDm} males. This hypothesis would then predict that the CI induced by old F_{1wRi} males should at least be equivalent to that of young F_{1wDm} males toward uninfected females. This is clearly not the case: when crossed with uninfected females, old F_{1wRi} males induce

TABLE 4

CI relationships[a] between the CI expressors *w*Dm (ME lines) and *w*Ri (DSR strain)

Cross male × female	ME 8	ME 14	ME 16	ME 22	ME 26	ME 28	ME 29	ME 4[b]	ME 17[b]
ME (*w*Dm) × ME (*w*Dm)	15.5	18.5	24.5	12.0	26.0	17.0	6.5	14.5	8.0
ME (*w*Dm) × DSR (*w*Ri)	24.0	19.5	20.5	16.0	13.0	26.5	8.5	9.5	5.5
DSR (*w*Ri) × ME (*w*Dm)	60.0	60.5	65.5	54.5	62.5	60.5	55.0	95.0	96.0

[a] CI is estimated by the percentage of unhatched eggs in mass crosses of 25 males × 15 females, calculated from 200 eggs per cross.
[b] ME 4 and ME 17 are uninfected ME lines.

TABLE 5

Egg mortality induced by young and aged F_1 males

Cross male × female	Males aged 3 days: *N* crosses	*N* eggs	% Egg mortality ±SE	Males aged 7 days: *N* crosses	*N* eggs	% Egg mortality ±SE
F_1(*w*Ri)[a] × SimO	10	330	99.8 ± 0.2	10	331	70.4 ± 5.3
SimO × F_1(*w*Ri)	13	884	1.1 ± 0.3	12	465	1.9 ± 0.8
F_1(*w*Dm)[b] × SimO	11	434	100.0	10	334	61.3 ± 6.3
SimO × F_1(*w*Dm)	16	747	4.2 ± 0.9	11	452	1.7 ± 0.6
F_1(*w*Ri) × F_1(*w*Dm)	17	754	30.5 ± 4.9	14	829	12.3 ± 2.0
F_1(*w*Dm) × F_1(*w*Ri)	14	919	8.1 ± 1.1	12	423	6.5 ± 2.4
F_1(*w*Ri) × F_1(*w*Ri)	18	1105	3.4 ± 1.0	10	312	4.4 ± 1.0
F_1(*w*Dm) × F_1(*w*Dm)	16	736	8.7 ± 1.5	14	557	5.4 ± 2.3
SimO × SimO	7	156	6.3 ± 3.2	10	266	13.1 ± 4.4

[a] F_1(*w*Ri) = individuals infected by *w*Ri, resulting from a cross between ME 29 males and DSR females.
[b] F_1(*w*Dm) = individuals infected by *w*Dm, resulting from a cross between DSR males and ME 29 females.

70.4% egg mortality and young F_{1wDm} females induce 100% egg mortality ($t = 6.96$; 9 d.f.; $P < 0.001$). Accordingly, it would seem that *w*Ri and *w*Dm are qualitatively different as far as their CI mechanisms are concerned.

CI relationships between *w*Dm and non-CI-expressor strains of Wolbachia: Individual tests were set up between infected ME lines and the *D. simulans* stocks Kc9, DSW(Mau), and Coffs Harbour. This allowed us to establish the CI relationships between *w*Dm and, respectively, Wolbachia strains *w*Ki, *w*Mau, and *w*Au. In all cases, males from ME lines infected by *w*Dm were found to be completely incompatible with females infected by *w*Ki, *w*Mau, or *w*Au (Table 6). The latter finding contradicts the unpublished results reported elsewhere (see discussion).

DISCUSSION

Through transinfection experiments, we established *D. simulans* isofemale lines infected by *w*Dm, the Wolbachia strain found in *D. melanogaster*. The transinfected lines allowed us to answer two questions, which we will consider in turn: (i) Will *w*Dm behave in the new host as in *D. melanogaster*, *i.e.*, being able to develop, be transmitted to the offspring by females, and induce only moderate amounts of CI through males? (ii) What will be the CI relationship between *w*Dm and the CI-expressor strains naturally found in *D. simulans*?

Behavior of *w*Dm in a new host: The infection was initially detected by PCR in 17 out of 33 surviving G0 females. Seven transinfected ME lines were still infected in G3 and have remained infected ever since. Because no further loss of the infection was found in the next generations, spanning almost 2 yr in the laboratory, we suppose that the apparent massive infection loss (59%) in the first three generations postinjection could be attributed to an initial low density of the symbiont. An alternative hypothesis is that some G0 females were scored as PCR positive while Wolbachia had not reached their germline (although PCR was carried out on ovaries, contamination by Wolbachia from somatic tissues might have occurred during dissection). Therefore,

TABLE 6

CI relationships between *w*Dm and the non-CI-expressor Wolbachia stains *w*Ki, *w*Mau, and *w*Au

Cross male × female	*N* crosses	*N* eggs	% Egg mortality ±SE
ME 29 (*w*Dm)[a] × Kc9 (*w*Ki)	12	840	99.6 ± 0.3
Kc9 (*w*Ki) × ME 29 (*w*Dm)	11	785	16.0 ± 2.4
ME 29 (*w*Dm) × DSW(Mau) (*w*Mau)	10	815	99.8 ± 0.2
DSW(Mau) (*w*Mau) × ME 29 (*w*Dm)	11	847	18.1 ± 3.7
ME 14 (*w*Dm) × Coffs Harbour (*w*Au)	7	607	100.0
ME 16 (*w*Dm) × Coffs Harbour (*w*Au)	15	669	99.9 ± 0.1
ME 29 (*w*Dm) × Coffs Harbour (*w*Au)	7	299	100.0
ME 26 (*w*Dm) × Coffs Harbour (*w*Au)	31	2162	98.5 ± 1.5
Coffs Harbour (*w*Au) × ME 26 (*w*Dm)	19	960	23.0 ± 3.4

[a] The infection status of each line is indicated between brackets.

after the early installation phase, it appears that the infection is efficiently maintained in the lines through maternal transmission.

The donor *D. melanogaster* line Wien 5 shows a moderate level of CI (18–32%), which is typical of many *D. melanogaster* strains (Hoffmann 1988; Boyle *et al.* 1993; Bourtzis *et al.* 1994, 1996; Hoffmann *et al.* 1994; Solignac *et al.* 1994). In contrast, six generations postinfection, males from transinfected *D. simulans* ME lines induced >90% CI when crossed with uninfected females. Such high levels of CI are common in natural *D. simulans* infections (Hoffmann *et al.* 1986; O'Neill and Karr 1990; Montchamp-Moreau *et al.* 1991; Merçot *et al.* 1995; Rousset and Solignac 1995), especially with the Wolbachia strain *w*Ri.

Such a host-specific difference in the expression of CI has already been described in Drosophila. Boyle *et al.* (1993) successfully injected the *w*Ri strain into an uninfected *D. melanogaster* strain. The CI induced in transinfected lines was low (maximum of 35% egg mortality), *i.e.*, typical of naturally infected strains of *D. melanogaster*, yet the Wolbachia caused a high level of CI when it was injected back into an uninfected *D. simulans* strain (75% egg mortality). These authors noted a correlation between the level of CI induced by males and Wolbachia density in the eggs of the lines under test. They were also able to increase the CI in one of their *D. melanogaster* transinfected lines through an artificial selection experiment, with a correlative increase in the density of Wolbachia in the eggs of the selected line. Our results show a different situation. As it could be expected, a dot blot assay on total DNA extracts revealed that males from ME transinfected lines harbored high densities of Wolbachia, comparable to the highest loads reported in *D. simulans* using this technique (*i.e.*, for the *w*Ri strain, see Bourtzis *et al.* 1996). Yet, surprisingly, the same dot blot assay on total DNA extracts revealed that Wien 5 males, which induced low levels of CI, harbored a bacteria load comparable to that found in ME males. Moreover, Wien 5 embryos were found to harbor Wolbachia loads similar to those of ME embryos. These results suggest that the overall growth of *w*Dm is not inhibited in *D. melanogaster* compared to its growth in transinfected *D. simulans* lines. However, we found that the percentage of infected cysts in the testes of young ME males was 10 times higher than that in Wien 5 males. This large difference would seem sufficient to explain the phenotypic gap between the high CI expressed by ME males and the moderate CI exhibited by Wien 5 males. The tissue-specific difference of Wolbachia load found in Wien 5 compared to ME lines (similar density in the eggs and the soma but very lower density in the testes) is of particular interest. Indeed, it is exactly the pattern expected if *D. melanogaster* had developed some control over the symbiont. This is because the relationship between the host and the Wolbachia depends strongly on the sex of the host. Because males can be sterilized by the Wolbachia present in their testes, any mutation in the genome of the host leading to a reduction of Wolbachia load in the male germline will be favored by natural selection. On the other hand, there will be no such strong selection to eliminate Wolbachia from the soma (unless they represent an important physiological cost) because these somatic bacteria appear to have no bearing on the sterilization of male reproductive cells. A buffering effect caused by Wolbachia present in somatic cells might explain why we could not detect any significant difference in Wolbachia load between Wien 5 and ME lines when using total DNA extract from whole males. In females, on the contrary, any mutation that would eliminate Wolbachia from the germline will be selected against because uninfected eggs laid by such females would die when fertilized by sperm from an infected male. Therefore, a specific decrease of Wolbachia load in the ovaries is not to be expected.

Previous observations indicated that Wolbachia infection of the testes was weak in *D. melanogaster* (Solignac *et al.* 1994), whereas it was high in *D. simulans* (Bressac and Rousset 1993). However, a comparison of CI levels could not be made rigorously because the Wolbachia strains studied were not the same in both species. Our transinfection experiment with a single Wolbachia strain confirms that weak infection of the testes is probably the key to explaining the low CI generally expressed by infected *D. melanogaster* males because ME lines demonstrate that *w*Dm is clearly able to induce strong CI when present in the testes in sufficient numbers.

Relationships between *w*Dm and the other Wolbachia strains known in *D. simulans*: Our results reveal a bidirectional incompatibility between *w*Dm and both CI-expressor strains *w*Ha and *w*No. This incompatibility is high (>80% embryonic mortality in both directions of cross), which is similar to the bidirectional incompatibilities already described in *D. simulans* (O'Neill and Karr 1990; Merçot *et al.* 1995; Merçot and Poinsot 1998a). A very high unidirectional CI was also found between *w*Dm-infected males and females infected by the nonexpressor strains *w*Au, *w*Ki, or *w*Mau. This is also in line with previous unidirectional CI relationships described in *D. simulans* between expressor and nonexpressor strains (Giordano *et al.* 1995; Rousset and Solignac 1995; Hoffmann *et al.* 1996; Merçot and Poinsot 1998a,b).

However, our crosses reveal a peculiar CI relationship between the two strong CI expressors *w*Dm and *w*Ri. We found that *w*Dm-infected males from the ME lines were apparently able to induce, at best, very weak CI against *w*Ri-infected females belonging to the DSR strain. In the reverse cross, DSR males were able to induce a clear CI phenotype (albeit at a significantly reduced level of 55–65% egg mortality compared to their usual 95% in crosses against other Wolbachia strains) against ME females. To ensure that this peculiar

CI pattern could not be attributed to host factors, we generated F_1 individuals between ME and DSR. Our results confirm that significant CI is induced only in the cross between F_{1wRi} males and F_{1wDm} females, although its level is ~30% instead of the 55–65% found when using inbred DSR males. The apparent reduction of CI expressed by F_{1wRi} males compared to DSR males cannot be attributed to a depressing influence of the F_1 background on Wolbachia expression because young F_{1wRi} males exhibit complete CI (99.8% egg mortality) when crossed with uninfected females, and we assume it probably reflects the inbreeding of DSR.

In this particular experiment, CI was not detected in the cross F_{1wDm} males × F_{1wRi} females. Again, this is probably not explained by a depressing effect of the F_1 genomic background on CI expression by *w*Dm: F_{1wDm} young males exhibit 100% CI when crossed with uninfected females. We would then assume that the weak mortality induced by ME males might also be caused by inbreeding in these isofemale lines rather than by CI. Therefore, our conclusion is that *w*Dm is fully compatible with *w*Ri in one direction of cross and partially compatible in the other direction.

The unidirectional CI patterns described so far in *D. simulans* between two Wolbachia strains (Giordano *et al.* 1995; Rousset and Solignac 1995; Turelli and Hoffmann 1995; Hoffmann *et al.* 1996; Merçot and Poinsot 1998a,b) involved one strain that was totally unable to induce the CI phenotype (*i.e.*, a nonexpressor Wolbachia strain) and one CI-expressor strain (an expressor strain). The pattern found here between *w*Dm and *w*Ri is unique in that both strains are very strong expressors in *D. simulans*, inducing typically 90% CI or more against uninfected females. This peculiar pattern had been obtained previously in an independent transinfection experiment where the only *w*Dm-transinfected line recovered was unfortunately lost after a few generations (Table 7). It must be noted that both the donor *D. melanogaster* strain (yw^{67C23}) and the acceptor *D. simulans* strain (DSHT) were different from those used in the present work. Yet, early results using the DSR strain were qualitatively similar to those we report with ME lines.

Phylogeny and CI relationships: Do the above CI relationships agree with molecular evidence bearing on Wolbachia phylogeny? Considering the most recent and discriminant Wolbachia phylogeny available, based on the very variable bacterial surface protein gene *wsp* (Zhou *et al.* 1998), we can conclude that the total bidirectional CI between *w*Dm and *w*Ha or *w*No is not surprising. Indeed, the divergence between *w*Dm and these two strains is, respectively, 21.4% (*i.e.*, 121 differences out of 565 bp) with *w*Ha and 24.8% (140 differences out of 565 bp) with *w*No. Similarly, the unidirectional CI induced by *w*Dm toward *w*Mau was to be expected (the *wsp* *w*Mau sequence is identical to that of *w*No). The unidirectional incompatibility of *w*Dm toward *w*Ki cannot be considered at the molecular level because the *wsp* sequence of *w*Ki is not yet available.

On the other hand, the relationships of *w*Dm toward the expressor strain *w*Ri and toward the nonexpressor strain *w*Au are surprising when considering molecular data. We found, in particular, that *w*Dm-infected males were completely incompatible with females infected by *w*Au (Table 6). This finding contradicts unpublished results reported by Bourtzis *et al.* (1998) using the ME16 line described in our present work. From the molecular data presented in Zhou *et al.* (1998), where *w*Mau and *w*No share an identical *wsp* sequence, Bourtzis *et al.* (1998) had been able to successfully predict the compatibility between these two strains. Using a similar line of argument, these authors had also

TABLE 7

Egg mortality in crosses involving DSHTM, a *D. simulans* line transinfected with *w*Dm

Cross male × female	G3 after transinfection			G4 after transinfection		
	N[a]	Eggs	% CI[b] ± SE	*N*[a]	Eggs	% CI[b] ± SE
DSHTM[c] (*w*Dm)[d] × DSHT (Ø)	18	1646	81.2 ± 4.9	9	567	90.8 ± 2.9
DSHTC (Ø) × DSHTM (*w*Dm)	34	2407	18.3 ± 4.2	21	1077	9.8 ± 1.0
DSHTM (*w*Dm) × DSH (*w*Ha)	21	1737	75.2 ± 5.8	9	585	95.1 ± 2.3
DSH (*w*Ha) × DSHTM (*w*Dm)	31	2089	83.4 ± 4.0	19	1077	96.2 ± 1.0
DSHTM (*w*Dm) × DSR (*w*Ri)	32	2307	22.3 ± 4.1	21	1302	22.6 ± 4.0
DSR (*w*Ri) × DSHTM (*w*Dm)	25	1815	86.1 ± 4.0	19	818	65.7 ± 7.2
DSHTM (*w*Dm) × DSHTM (*w*Dm)	22	1251	10.2 ± 2.7	22	1025	11.7 ± 1.5

[a] Number of individual crosses.
[b] CI is estimated by the mean of the percentage of unhatched eggs.
[c] The DSHTM isofemale line was obtained in two steps. First, the DSH line was cured from its *w*Ha infection through a tetracycline treatment, generating an uninfected strain (DSHT). DSHT embryos were then used as recipients in a transinfection experiment. Embryos from a laboratory strain (yw^{67C23}) infected by *w*Dm were used as a source of infected cytoplasm.
[d] The infection status of each line or strain is indicated between brackets. Ø, uninfected.

assumed that females infected by the non-CI-expressor variant *w*Au would be compatible with males infected by *w*Dm (a difference of only 1 of 565 bp between their *wsp* sequences). Preliminary unpublished results that seemed to support this hypothesis were mentioned (Bourtzis *et al.* 1998). However, the results we present here (and which we have repeated with four ME lines including, of course, ME 16) clearly show that *w*Dm-infected males are totally incompatible with *w*Au-infected females. Our finding that *w*Dm-infected males are at the same time totally compatible with *w*Ri-infected females is therefore surprising because the *wsp* sequences of *w*Dm and *w*Ri differ markedly (55 out of 565 bp, *i.e.*, a 9.7% divergence). Although the discovery, cloning, and sequencing of the very variable *wsp* gene (Braig *et al.* 1998) provided a tool of unprecedented precision as far as Wolbachia phylogeny is concerned, it does not seem possible to infer accurately from these molecular data whether or not two Wolbachia strains will be compatible. The total incompatibility of *w*Dm with *w*Au, despite almost identical *wsp* sequences, could be explained by a "loss-of-function" mutation of *w*Au in the mechanism responsible for CI rescue, which would not have been selected against because *w*Au is a non-expressor strain. However, other nonexpressor strains such as *w*Mau or *w*Ki have clearly kept their CI rescue capability toward CI induced by *w*No intact (Bourtzis *et al.* 1998; Merçot and Poinsot 1998b).

Why is there only unidirectional CI between *w*Dm and *w*Ri? Two kinds of hypotheses can be proposed to explain the partial and unidirectional CI pattern between two such strong CI expressors: (i) *w*Ri and *w*Dm might have identical CI mechanisms but differ in a quantitative way, with *w*Ri being strong enough to induce partial CI against *w*Dm but not *vice versa*, and (ii) *w*Ri and *w*Dm might differ qualitatively, but the CI rescue mechanism of *w*Ri might have a broader spectrum of efficiency than that of *w*Dm (*i.e.*, the CI rescue function of *w*Ri would remain functional even when faced with the divergent CI induction mechanism of *w*Dm). In the reverse cross, the *w*Dm rescue mechanism would be only partly efficient because of a lower versatility, hence partial CI in the cross *w*Ri infected male × *w*Dm infected female.

The simplest quantitative hypothesis would assume a difference in bacterial load. Indeed, the level of CI expression has been correlated to Wolbachia load several times (Boyle *et al.* 1993; Breeuwer and Werren 1993; Bressac and Rousset 1993; Rousset and de Stordeur 1994; Solignac *et al.* 1994; Bourtzis *et al.* 1996). Following the load hypothesis, *w*Dm and *w*Ri would be identical as far as CI mechanisms are concerned, but *w*Ri would reach higher densities than *w*Dm. The partial CI observed would then result from the *w*Dm load in the egg being too low to fully rescue the CI phenotype expressed by males heavily infected by *w*Ri. In the other direction of cross, the *w*Ri load in the egg would always be sufficient to rescue the CI phenotype expressed by males infected by *w*Dm. However, none of our density measurements support this hypothesis. Indeed, the Wolbachia load in ME lines or in DSR is similar both in the eggs and in the testes (Table 2). Moreover, it does not seem that *w*Ri would produce more of the unknown factors that allow the induction and rescue of CI. This is suggested by old F_{1wRi} males being still able to induce detectable CI against F_{1wDm} females, while their CI capability against uninfected females is considerably weaker than that of young F_{1wDm} males (Table 5). Finally, young or old F_{1wRi} males do not induce significantly more CI than F_{1wDm} males of the same age when mated with uninfected females (Table 5).

The asymmetrical CI pattern we find leads us to suggest that the CI mechanisms of *w*Ri and *w*Dm are qualitatively different, but have not diverged enough for full bidirectional incompatibility to appear (the alternative hypothesis being that their CI mechanisms are partially similar by chance, *i.e.*, convergence). We might then be witnessing one of the evolutionary steps leading to two bidirectionally incompatible strains. The asymmetry of the CI relationship we describe is especially interesting with regard to the unknown biochemical mechanisms underlying CI. Indeed, it suggests that the bacterial function responsible for CI induction through the modification of the male reproductive cell can evolve separately from the bacterial function responsible for CI rescue in the infected embryo. Similar conclusions have already been drawn from the discovery of Wolbachia strains, which can fully rescue CI while being totally unable to induce it (Bourtzis *et al.* 1998; Merçot and Poinsot 1998b; Poinsot and Merçot 1998). It would seem that CI is not a one-protein business.

The authors thank Androniki Nirgianaki for excellent technical assistance, as well as Sophia Zabalou and Ioannis Livadaras for helping with early transinfection experiments with the DSHTM strain. The Wien 5 line originates from a sample collected in Vienna by Wolfgang Miller. This work was supported by a grant from the French Ministère de l'Enseignement Supérieur et de la Recherche (MESR) (ACC SV3 no. 9503017), by a John D. and Catherine T. MacArthur Foundation grant and by a grant from the Greek Secretariat for Research and Technology (PENED 15774). D.P. was supported by a Ph.D. grant from the French MESR. K.B. was partially supported by grants from the National Institute of Health (AI34355, AI40620) to Scott O'Neill.

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Communicating editor: A. A. Hoffmann