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Properties of *Drosophila simulans* strains experimentally infected by different clones of the bacterium *Wolbachia*

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Maternally inherited bacteria of the genus *Wolbachia* are responsible for reproductive incompatibilities between strains of *Drosophila simulans*. Such incompatibilities are known in several types of crosses, including both directions of crossing between two types of infected strains, 'R' and 'S'. To determine whether the bidirectional incompatibility between R and S flies is due only to differences between their bacteria, flies from an uninfected strain have been experimentally infected with bacteria associated with each type. The incompatibility properties of experimental strains are close to those of original strains harbouring the same bacteria and therefore independent of nuclear background. Backcross experiments, however, show that the infection level of a strain depends on the nature of paternal ancestors. This is not explained by nuclear effects but is possibly the result of an interaction between the infection levels of both parents, in which the infection level of S strains is an equilibrium between a tendency for females to produce weakly infected offspring and selection of more infected eggs by sperm from infected males.

Keywords: cytoplasmic incompatibility, cytoplasmic injection, *D. simulans*, nuclear–cytoplasmic interactions, *Wolbachia*.

Introduction

In a variety of insect species, maternally inherited bacteria of the genus *Wolbachia* are responsible for sterile crosses known as cytoplasmic incompatibilities (Yen & Barr, 1973; Kellen *et al.*, 1981; Hsiao & Hsiao, 1985; Binnington & Hoffmann, 1989; Breeuwer *et al.*, 1992; O'Neill *et al.*, 1992; Rousset *et al.*, 1992a, b). These sterilities result from the interruption of embryonic development after irregular syngamy (Ryan & Saul, 1968; Jost, 1971; Breeuwer & Werren, 1990; O'Neill & Karr, 1990).

The bacteria are present in ovaries and testes and only inherited through the egg cytoplasm. Uninfected males are compatible with all females, infected or not. Incompatibilities are observed in crosses between uninfected females and infected males and occasionally between infected individuals. This last situation, which is known in the moth *Ephestia cautella* (see Brower in Kellen *et al.*, 1981), the hymenopteran *Nasonia* (Conner & Saul, 1986), the mosquitoes *Culex pipiens* (Barr, 1982; Subbarao, 1982) and *Aedes albopictus* (Kambhanpati *et al.*, 1993) and the fruit fly *Drosophila simulans*, is commonly assumed to result from the presence of different bacteria (Barr, 1982; O'Neill & Karr, 1990; Rousset *et al.*, 1991).

In *D. simulans*, several types of strains have been distinguished on the basis of their crossing relationships (O'Neill & Karr, 1990; Montchamp-Moreau *et al.*, 1991; Nigro, 1991). Infected strains of the incompatibility type 'R' are incompatible in both directions when crossed with strains of type 'S'. The molecular identification of the bacteria through partial 16S rDNA sequencing has revealed the existence of different bacterial strains associated with the different incompatibility types of *D. simulans* (Rousset *et al.*, 1992b). Apart from these sterilities, little is known of the relationships between *Wolbachia* and *Drosophila*. *Wolbachia* can be visualized in cysts (immature sperm) by DAPI-staining (Bressac & Rousset, 1993). The cytoplasm of sperm cysts is eliminated in a waste bag at the posterior extremity of sperm. In infected cysts, the waste bag shows aligned spots of DAPI-stained material corresponding to *Wolbachia* that are eliminated during sperm maturation. Preliminary observations have revealed that the frequency of infected cysts is variable between different infected strains and there

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may be a relationship between this infection level and the level of incompatibility (Bressac & Rousset, 1993).

Although there may be a strict association between the bacterial types and incompatibility types, a demonstration of the purely bacterial determination of the incompatibilities between R and S individuals is lacking. R and S flies also differ in their mitochondrial and nuclear genomes. Three mitochondrial types have been distinguished in *D. simulans* (Baba-Aïssa *et al.*, 1988) and there is a strict association between two of them and the incompatibility types R and S, R being associated with mitochondrial type *si*II and S with *si*I (Montchamp-Moreau *et al.*, 1991). A genetic differentiation may also exist at the nuclear level between *si*I and *si*II flies (Hyytia *et al.*, 1985; Choudhary & Singh, 1987).

Experimentally infected strains, therefore, have been established through the injection of cytoplasm from infected eggs into uninfected eggs. The comparison of flies with the same bacterial type and different nuclear genomes can point to the involvement of nuclear factors in the behaviour of the strains. Conversely, the comparison of strains with the same nuclear genome and different bacteria is clear evidence for the role of the different bacteria in the determination of incompatibility. The incompatibility properties of some strains obtained through injection experiments have already been studied by Nigro (1991) but as the recipient strain was itself infected and there was no information on the nature of bacteria in the experimental lines, those results were difficult to interpret.

Materials and methods

Original strains

We have used the following strains.

Watsonville (Wa). An uninfected strain from California (Hoffmann *et al.*, 1986), of mitochondrial type *si*II.

Riverside (Ri). An uninfected strain from California, of incompatibility type R (Hoffmann *et al.*, 1986) and mitochondrial type *si*II. Its bacterial type as identified by Rousset *et al.* (1992b) and O'Neill *et al.* (1992) will be called *wR*.

Hawaii 4 (Ha). An infected isofemale strain of mitochondrial type *si*I and incompatibility type S (Montchamp-Moreau *et al.*, 1991). It is incompatible in both directions of crossing with R flies (Montchamp-Moreau *et al.*, 1991; Table 1). Two types of bacteria have been found in S flies. The ones found in this strain (Rousset *et al.*, 1992b) will be called *wHa*. As the incompatibility properties of S flies are heterogeneous (Montchamp-Moreau *et al.*, 1991) and this heterogeneity may be related to the presence of two different bacterial types (Rousset *et al.*, 1992b), it is better to characterize the crossing properties of the Hawaii strain as the 'S_H' incompatibility type.

Table 1 Incompatibility tests (eclosion rates)

♀ \ ♂	RiWa2	RiWa8	HaWa3	HaWa4	Ri	Ha
RiWa2	94	0	2	68	2	13
RiWa8	67	87	12	n.d.	83	24
HaWa3	88	5	61	66	1	75
HaWa4	91	1	21	75	0	41
Wa	97	1	13	7	n.d.	n.d.
Ri	78	94	15	47	95	26
Ha	82	4	83	85	0	68

For each cross, the eclosion rate has been estimated from 100–300 eggs. To avoid complications resulting from a possible evolution of strains in the course of generations, all crosses have been performed within one generation, for all strains (F_7 for RiWa strains and F_5 for HaWa strains). n.d.: not simultaneously done (Ri and Ha males are incompatible with Wa females although Ha males are not completely so (Hoffmann *et al.*, 1986; Montchamp-Moreau *et al.*, 1991; and unpublished data)).

Experimentally-infected strains

Wolbachia were introduced into uninfected eggs by cytoplasmic injections according to the method of Santamaria (1987). About 40 pl of cytoplasm taken from the anterior pole of a donor egg (Ri or Ha) (where, at least in another host species, *Culex pipiens*, the bacteria are abundant (Yen, 1975)) were injected in the posterior pole of the recipient egg (Wa), where the germ line differentiates.

Each of the two infected strains Ri and Ha have been used as donor for cytoplasmic injection in the uninfected strain Wa. In each case, viable females grown from injected eggs were isolated and crossed with Wa males, the offspring of these crosses being the F_1 of each line. For each donor strain, several independent isofemale strains have thus been established (RiWa strains from injection of Ri cytoplasm and HaWa from Ha cytoplasm). Some of them, RiWa2, RiWa8, HaWa3 and HaWa4, where infection could be detected in the first five generations, have been chosen for further experiments.

Backcross lines

For backcross experiments, six females and six males were taken from parent strains at each generation. Experimental lines have been backcrossed to their donor strain.

F♂ lines were obtained from the backcross of females of the donor strain to males of the experimental strain. Thus, the F♂3 and F♂4 strains are the results of backcrossing the descendants of Ha females each generation to HaWa3 and HaWa4 males, respectively. F6♂3 individuals are the offspring of six generations of backcrosses to HaWa3 males.

F♀ lines were similarly obtained as the descendants of females of the experimental strain backcrossed to males of the donor strain. F6♀3 individuals are the descendants of HaWa3 females after six generations of backcrosses to Ha males.

Incompatibility properties

Strains were reared at 20°C. For each cross, six 1–3-day-old virgin females were allowed to mate with six 1-day-old virgin males. Females were allowed to lay eggs 1 day later on blackened agar, sweetened with sucrose and covered with fresh yeast. The first 100 eggs were disposed on clean agar and eclosions were counted.

Infection level of males

DAPI (4′,6-diamidine-2-phenylindole-dihydrochloride) forms fluorescent complexes with double-stranded DNA and therefore visualizes bacterial DNA in the cytoplasm of infected sperm cysts. The infection level, defined as the frequency of sperm cysts that are infected in the testes of a 2-day-old individual, was determined for both testes after dissection, ethanol fixation and DAPI staining as described in Bressac & Rousset (1993).

Control of the presence of bacteria

The presence of bacteria in the different strains has been controlled for by PCR amplification of a fragment of the 16S ribosomal DNA. DNA was extracted from ovaries or testes of individual flies by the method of Gloor & Engels (1992). The primers were 5′TTGTAGCTTGCTATGGTATAACT3′ on positions 76–99 of *E. coli* 16S rDNA and a primer from O'Neill *et al.* (1992), on positions 1014–994 of the rDNA. These primers can amplify the different *Wolbachia* present in *D. simulans*. The amplification programme was 5 mn at 93°C and 38 cycles of 1 mn at 93°C, 1 mn at 48°C, 1 mn at 72°C. The presence of bacteria was also detected by DAPI-staining of sperm cysts of at least four males for each line.

Determination of molecular types

Bacterial types were distinguished by sequencing diagnostic regions of 16S ribosomal DNA by the methods described in Rousset *et al.* (1992a). The mitochondrial type of flies was checked by *Hpa*II digestion as in Solignac *et al.* (1986).

Results

Injections

From 95 Wa eggs receiving Ri cytoplasm, eight isofemale lines (RiWa1 to RiWa8) were obtained. *Wolbachia* were detected, at least transiently, by PCR or DAPI-staining in four of them. From 85 Wa eggs receiving Ha cytoplasm, four HaWa isofemale lines were obtained, two of them being infected.

The strain RiWa2 lost its bacteria between F_5 and F_7. They could no longer be detected by PCR amplification or DAPI-staining. The nature of bacteria in the other strains has been determined by sequencing and as expected was that of the donor strain. Although cytoplasmic injections can lead to replacement of the mitochondrial genome of the recipient strain by that of the donor strain (de Stordeur *et al.*, 1989), this has not occurred in the HaWa3 and HaWa4 lines and it could not be detected in RiWa flies, as Ri and Wa flies share identical mitochondrial *Hpa*II profiles.

The original and experimental strains have been crossed to determine their incompatibility type (Table 1).

If we first consider the relationships between experimental and original strains, RiWa2 behaves as an uninfected strain, in agreement with the fact that it was no longer infected at the time of these tests. RiWa8 behaves as Ri and HaWa3 as Ha. HaWa4 behaves as a mixture of Ha and uninfected individuals, the females being incompatible with Ri males and partially incompatible with Ha males and the males being incompatible with Ri females.

Considering the crosses between experimental strains, RiWa2 males behave as uninfected (they are compatible with all females) and RiWa2 females are incompatible with males from other strains, although only partially with HaWa4 ones. RiWa8 is similar to Ri. HaWa3 males are strongly incompatible with RiWa8 and also with HaWa4 females. HaWa3 females are similar to Ha. HaWa4 females express only weak incompatibilities except with RiWa8 and HaWa3 males.

Cytoplasmic injections allow the transfer of *Wolbachia* and the modification of the incompatibility type of a strain. In the present experiments, the RiWa8

strain has acquired *wR* bacteria and properties very similar to those of Ri. The HaWa3 and HaWa4 strains have acquired *wHa* bacteria and HaWa3 behaves as Ha. HaWa4 behaves as a mixture of Ha and uninfected individuals. It is possible that weakly infected individuals behave more or less as uninfected ones, so the behaviour of HaWa4 is not evidence for the maintenance of completely uninfected individuals in this strain.

Infection level of backcross lines

To test whether differences between Ha and HaWa4 are due to differences in the nuclear genomes of these strains, it is possible to replace the 'Watsonville' genome of HaWa4 by the 'Hawaii' genome, through backcrosses to Ha males. Several such experiments have been undertaken (see the backcross lines section in Materials and methods). The infection level of males, defined as the number of infected waste bags over the number of maturing cysts in the testes of a male, was determined in males of the original and experimental strains by DAPI-staining as described in Materials and methods. All observations are detailed in Fig. 1. The significance of differences between strains has been evaluated by Mann–Whitney tests.

The infection levels in experimental strains HaWa3b (range 1–43.6 per cent), HaWa4b (0.7–18.9 per cent) and RiWa8 (72.2–89.9 per cent) are lower than those of the related donor strains Ha (8.5–57.4 per cent) and Ri (93.1–100 per cent) and these differences are significant (HaWa3b and Ha: $U=91$, $P<0.01$; HaWa4b

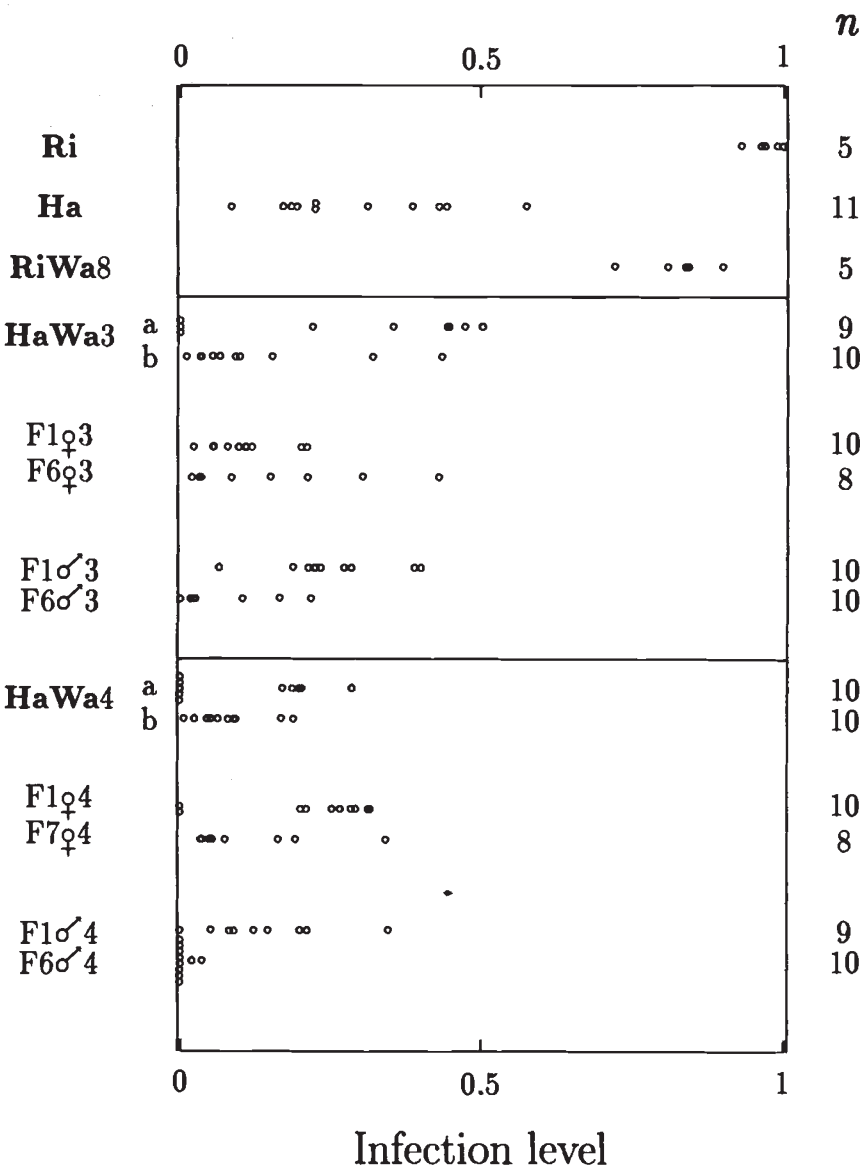

Fig. 1 Infection level of males. HaWa3 and HaWa4 males have been examined simultaneously with F_1 of backcross lines, which was the generation following the incompatibility tests, and simultaneously with F_6. These two series of observations are distinguished as 'a' and 'b', respectively. Numbers on the right (n) stand for the number of males in each sample.

and Ha: $U=104$, $P<0.001$; RiWa8 and Ri: $U=25$, $P<0.005$).

In the HaWa strains, the infection level shows no significant change through time (HaWa3a and HaWa3b, five generations later: $U=57$, $P>0.10$; HaWa4a and HaWa4b: $U=48$, $P>0.10$).

HaWa4 behaved in crosses as a less infected strain than HaWa3, i.e. crosses with HaWa3 males are on the average more incompatible than crosses with HaWa4 ones and the situation is reversed for females (Table 1). Infection levels are indeed slightly lower in HaWa4 (HaWa3a and 4a, $U=66.5$, $P<0.05$ but later HaWa3b and 4b, $U=57$, $P>0.10$).

F♀ strains possess the *wHa* bacterial types and their nuclear genome, originally that of the strain Wa, has been replaced by the Ha genome in the course of generations of backcrosses to Ha males. There is no significant change of infection level in F♀ strains (F1♀3 and F6♀3: $U=44$, $P>0.1$; F1♀4 and F7♀4: $U=56$, $P=0.1$). The infection level remains significantly smaller than in the Ha strain (F6♀3 and Ha, $U=68.5$, $P<0.05$; F7♀4 and Ha, $U=77$, $P<0.005$).

F♂ strains possess the *wHa* bacterial types and their nuclear genome, originally that of the strain Ha, has presumably been replaced by the Wa genome in the course of generations of backcrosses to HaWa males. The infection level decreases in F♂ strains below their initial level (F1♂3 and F6♂3, $U=95$, $P<0.001$; F1♂4 and F6♂4, $U=85.5$, $P<0.001$) as well as below the infection level of the paternal strains (F6♂3 and HaWa3b, $U=74$, $P<0.05$; F6♂4 and HaWa4b, $U=97$, $P<0.001$).

Discussion

The experimentally-infected strains have acquired incompatibility properties similar to that of the donor strains possessing the same bacteria but a different nuclear genome and, in the case of HaWa flies, a different mitochondrial genome. Therefore, these experiments confirm not only the role of *Wolbachia* in incompatibility, as those of Boyle *et al.* (1993), but also that the differences between the two incompatibility types seem to be determined only by the different bacterial types *wR* and *wHa*. In the hymenopteran genus *Nasonia*, Breeuwer & Werren (1993) have also found that differences between the nuclear genomes of two species had no consequences on the bidirectional incompatibility between them.

Nevertheless, males from experimentally-infected strains appear less infected than those from related donor strains. This can indicate that the nuclear genome of the Wa strain is somewhat unfavourable to the two bacterial types or could be a consequence of the injection procedure. The use of DAPI-staining confirms that some factors can modify the infection level of males also in backcross lines. These experiments show that the nature of the paternal strain has an effect, as the infection level decreases in F♂ strains in the course of generations.

The last phenomenon, however, is not easily explained by nuclear effects. Firstly, the replacement of the Ha genome by the Wa genome in F♂ strains should decrease to infection levels comparable to those of paternal HaWa strains. On the contrary, they decrease more than expected, below the infection levels of the paternal strains. Secondly, the replacement of the Wa genome by the Ha one in F♀ strains should increase the infection level to values comparable to those of the Ha strain. No increase can be detected and the infection level remains significantly smaller than in the Ha strain.

Another explanation can be proposed for these results. The different eggs produced by one female probably harbour a variable number of bacteria and weakly infected eggs could easily be incompatible with sperm from infected males. This hypothesis would have at least two kinds of consequences.

Firstly, the relatively high frequency of non-viable eggs in Ha and HaWa strains (Table 1; see also Montchamp-Moreau *et al.* (1991)) can be a consequence of the continuous production of weakly infected eggs. If the average number of bacteria per egg produced by a female were lower than the number of bacteria in the egg from which it has developed, the maintenance of some infection level in Ha and HaWa strains would be an equilibrium between a tendency to produce weakly infected eggs and 'selection' against weakly infected eggs by sperm from infected males (or infected sperm cysts). The weak infection of eggs would result in the observed low infection level of males of Ha and HaWa strains, which probably explains that Ha males are not completely incompatible with uninfected and Ri females.

Secondly, the infection level in the backcrossed lines will be the result of an interaction between the infection levels of their maternal and paternal ancestors. The simplest prediction is that in backcrosses to weakly infected males, the infection level can decrease rapidly to low values. This is indeed the case for F6♂4, the descendants of weakly infected HaWa4 males, and to a lesser extent for F6♂3, the descendants of the somewhat more infected HaWa3 males. This hypothesis can also explain the results of Montchamp-Moreau *et al.* (1991) who have found that the male offspring of type S females backcrossed with uninfected males behaved as weakly infected males.

The infection level of the different strains may therefore be dependent on the bacterial type (*wR* reaching higher levels than *wHa* in the nuclear context of the strain Wa), as well as a non-genetical interaction

between the infection levels of ancestral strains. In such a situation, no nuclear effect can be demonstrated from our results.

The hypothesis of variable numbers of bacteria in eggs has already been discussed in the context of incompatibility in *Culex pipiens* by Subbarao (1982), who found it inadequate to explain her results in this species. As factors controlling the infection level will have consequences on the evolution of cytoplasmic incompatibility in natural populations, it is necessary to know whether such phenomena are present in other host species. Moreover, these results raise several problems, such as the conditions under which the infection level of a strain can be stable in the course of generations or how an initially weakly infected cytoplasmic lineage can attain high levels of infection when the presence of already highly infected males seems necessary to maintain it at this level.

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