Wolbachia Bacteria Effects after Experimental Interspecific Transfers in Terrestrial Isopods

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INTRODUCTION

Wolbachia α-proteobacteria are a striking and intriguing case of intracellular parasites. Wolbachia are probably the most widespread bacteria in invertebrates, infecting 20–80 % of all insects (e.g., Werren and Windsor, 2000; Jeyaprakash and Hoy, 2000), some mites (e.g., J ohanowicz and Hoy, 1995), numerous terrestrial isopods (Bouchon et al., 1998), and filarial worms (Bandi et al., 1998). Wolbachia are maternally transmitted, through the cytoplasm of the eggs. Instead of increasing their hosts' reproduction or survival, as expected from classical theory (Fine, 1975; Yamamura 1993), they evolved toward “reproductive parasitism,” i.e., they alter hosts' reproduction to facilitate their spread in host populations (O'Neill et al., 1997). Some Wolbachia strains bias the sex ratio of their host toward females, i.e., the transmitting sex (reviewed in Bandi et al., 2001). In terrestrial isopods particularly, they are responsible for the feminization of genetic males (Martin et al., 1973; Bouchon et al., 1998). Other Wolbachia strains induce parthenogenesis and male-killing in insects (Bandi et al., 2001). Another Wolbachia-induced alteration of reproduction is the cytoplasmic incompatibility (CI) expressed in numerous (if not all) host taxa infected by this symbiont (O'Neill et al., 1997).

The repartition of Wolbachia strains across hosts and the diversity of their effects cannot be explained by strict vertical transmission only. Infectious (i.e., horizontal) transmission between different host species or taxa is required to explain the overall incongruence between host and symbiont phylogenies and the widespread Wolbachia infection among arthropods (Rousset et al., 1992; Rigaud and Rousset, 1996; Vavre et al., 1999).

Several studies using experimental infections revealed that Wolbachia can often infect a foreign indi-
vidual host. However, a stable implantation of the infection in lineages after vertical transmission through generations is not always achieved. Failures often occur when Wolbachia are transinfected in a host phylogenetically distant from their native host (Rigaud and Juchault, 1995; Van Meer and Stouthamer, 1999; Heath et al., 1999; but see Pintureau et al., 2000), whereas successes are generally found between closely related species (Boyle et al., 1993; Clancy and Hoffmann, 1997; but see Braig et al., 1994). Furthermore, Wolbachia implantation in a foreign host lineage often leads to loss of strength of their phenotypic expression. Differences in these cases were found to be due to different Wolbachia density or distribution in their new host’s tissues (Breeuwer and Werren, 1993; Clancy and Hoffmann, 1997; Poinso et al., 1998; Pintureau et al., 2000). All these data suggest that only a few attempts of Wolbachia horizontal transfers in the wild have led to permanent establishment in a new host species.

Most of these data concerned CI or parthenogenesis-inducing Wolbachia in insects and only a few feminizing Wolbachia in crustaceans. The aim of this study was to follow the behavior of isopod feminizing Wolbachia transferred into several foreign host species, to answer the following questions: Can a feminizing Wolbachia be established in foreign host lineages after a horizontal transfer? Do closely related Wolbachia induce different effects when transferred into the same foreign hosts? And if so are the differences explained by differences in bacterial load?

MATERIAL AND METHODS

Wolbachia and Their Hosts

Wolbachia came from infected host lineages maintained in the laboratory for several years. Two Wolbachia strains were used; one named wAv from Armadillidium vulgare, collected in 1990 at Cells sur Belles, France, and one strain named wAn from A. nasatum, collected in 1994 at Mignaloux, France. These strains are phylogenetically closely related; their 16S rDNA sequences are identical (Bouchon et al., 1998), whereas sequences of the variable wsp gene show a divergence of 0.48% (Cordaux et al., 2001). Both strains induce feminization in their original host in the wild and in the lab (i.e., genetic males infected with Wolbachia generally develop into females; when feminization is incomplete, they can develop an intersex phenotype). Infected females thus produce highly female-biased progenies: 95% of daughters are generally obtained on average (e.g., Juchault and Legrand, 1979; Rigaud and Juchault, 1993).

Wolbachia were transferred into uninfected strains of the following species: A. vulgare (from Nice, France), A. nasatum (from Mignaloux) (both Armadillididae family), Oniscus asellus (Oniscidae family, from Quincié, France), and Porcellio scaber (Porcellionidae, from Celles sur Belle, France). In these uninfected lineages, there was no bias in sex ratio and sex is genetically determined by sex chromosomes. In A. vulgare and O. asellus WZ individuals are female and ZZ individuals are male. In A. nasatum, XX individuals are female, XY are male. The heterogamety system is unknown in P. scaber. The woodlice that were used have been maintained by outbreeding in the lab for at least 5 years; they have a generation time of approximately 1 year. The different host families used were phylogenetically well differentiated (Michel-Salzat and Bouchon, 2000).

Inoculations

The transfer was made by the injection of a homogenate of infected tissues into the general cavity of adult females, as described in Rigaud and Juchault (1995). Per combination of host species and Wolbachia strain, between 10 and 20 females were injected. Between 12 and 20 females were kept as negative controls. No control consisting of injection of uninfected extract was made, since a previous experiment concluded that the presence of Wolbachia in an homogenate was required to affect female reproduction (Juchault and Mocquard, 1989). Injected females were kept for 250 days before crosses were performed, a delay allowing Wolbachia to infect host tissues and express a feminizing effect (if any) (Rigaud and Juchault, 1995; Bouchon et al., 1998). Injected and control females were crossed with uninfected males. Their offspring were reared in separate boxes and were sorted by sex around 3 months later, after sexual differentiation. During all the experiment, animals were fed ad libidum with carrot slices and dead leaves. The same procedure was followed to maintain strains for further generations, 1 generation being obtained per year.

PCR Assays

The success of the transfer was controlled by PCR screening of the females injected (after they released the young) and in young 6 months after their birth, except those kept for crossing, which were tested later. Total DNA was extracted and PCR amplifications were performed following Bouchon et al. (1998). The Wolbachia-specific primer set for the 16S rDNA was used (99F–994R, O’Neill et al., 1992). In samples testing negative for Wolbachia, a mitochondrial primer set was used in a separate reaction to ensure that the host’s cytoplasmic DNA was accessible as described in Bouchon et al. (1998).

Fluorescence in Situ Hybridization

Wolbachia density was compared using fluorescence in situ hybridization (FISH) adapted from the tech-
The homogenate was then subjected to 10 s of ultrasound, allowing destruction of the cell wall, but preserving nuclei (see Fig. 2). The solution was then fixed for 5 min by adding 200 μl 4% paraformaldehyde and 100 μl was spread on a polylysine-coated slide and air dried. The FISH technique was then performed, as described in Heddi et al. (1999). The Wolbachia-specific probe W2 was used (Heddi et al., 1999), labelled with rhodamine at the 5' end (Eurogentec). After hybridisation, slides were mounted in 50μl Fluorep medium (BioMérieux) containing DAPI (1μg/ml). Observations were made under Zeiss Axioplan microscope equipped for fluorescence, using a x60 oil objective. For each individual, 10 objective fields were sampled randomly on the slide, and all Wolbachia (stained with Rhodamine) and nuclei (stained with DAPI) were counted. For each slide, there was no significant difference in the number of nuclei and bacteria between the ten fields ($\chi^2$ for homogeneity N.S. in all cases, data not shown). The total number of Wolbachia and nuclei were therefore pooled to estimate the ratio Wolbachia/nuclei for a given individual. This method provides a measure of relative bacteria load, not an absolute number per individual.

## RESULTS

All injected females that were tested were positive for Wolbachia infection, confirming the efficiency of the injection technique (Table 1). A decrease in fertility was observed in transinfected females (Table 2), except in A. nasatum infected with wAn, the only case in which the hosts were infected with a Wolbachia strain that came not only from the same species but also from the same population. In A. vulgare, the wAv bacteria and the transinfected host were between two sets of host populations distant by 800 km. The decrease of fertility was very strong in O. asellus, whatever the

### TABLE 1

<table>
<thead>
<tr>
<th>Recipient species</th>
<th>Wolbachia type</th>
<th>Mothers$^a$</th>
<th></th>
<th>F1 daughters$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Examined</td>
<td>Infected</td>
<td>Examined</td>
</tr>
<tr>
<td>P. scaber</td>
<td>wAv</td>
<td>4</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>wAn</td>
<td>4</td>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td>O. asellus</td>
<td>wAv</td>
<td>5</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>wAn</td>
<td>5</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>A. vulgare</td>
<td>wAv</td>
<td>5</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>wAn</td>
<td>5</td>
<td>5</td>
<td>32</td>
</tr>
<tr>
<td>A. nasatum</td>
<td>wAv</td>
<td>5</td>
<td>5</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>wAn</td>
<td>5</td>
<td>5</td>
<td>20</td>
</tr>
</tbody>
</table>

$^a$ Females sampled at random among injected females.

$^b$ Offspring sampled at random in four broods of mothers controlled positive for Wolbachia infection.
symbiont they received, and more moderate in P. scaber and A. vulgare.

The Wolbachia vertical transmission to offspring differed greatly between species. Transmission was weak in daughters from P. scaber and O. asellus, while transmission was strong in A. vulgare and A. nasatum, independent of the bacterial strain they received (Table 1). In line with the poor transmission to offspring, no significant bias in offspring sex ratio was observed in P. scaber and O. asellus (Table 2). Strong excesses of females were observed in broods of A. nasatum females, whatever the symbiont they received (Table 2). A similar excess of females was observed in A. vulgare females infected with their native Wolbachia, while wAn induced moderate sex ratio biases, intermediate between those of control and wAv-infected females (Table 2).

To follow the infection over time, F2 generations were obtained from F1 females in P. scaber infected with wAn and A. vulgare and A. nasatum infected with wAn and wAv symbionts, respectively (only broods from females that were tested positive for Wolbachia infection were kept for further investigation). In P. scaber, one infected F1 daughter produced 21 males and 25 females; all of 10 females that were tested were PCR negative for Wolbachia infection. No subsequent generation was continued.

For A. vulgare infected with wAn and A. nasatum infected with wAv the evolution of the proportion of males and the proportion of intersexes (i.e., individuals infected by Wolbachia but imperfectly feminized, see Rigaud and Juchault, 1998) are given in Fig. 1. During the 3 generations following the transfer, there was no significant change in the proportion of males, neither in A. vulgare (Kruskal-Wallis test: \( \chi^2 = 2.03; P > 0.35 \)) nor in A. nasatum (K-W test: \( \chi^2 = 0.97; P > 0.60 \)). The proportion of intersexes was very low and stable in A. nasatum infected with wAv (K-W test: \( \chi^2 = 0.01, P > 0.99 \)), a situation commonly found in naturally infected lineages (Juchault and Legrand, 1979; Rigaud and Juchault, 1998). However, the proportion of intersexes changed with generations in A. vulgare (K-W test: \( \chi^2 = 16.95, P < 0.001 \)), reaching unusually high values (near 9%) in the third generation following transfer.

Intersexes reflect a lower efficiency of feminization of Wolbachia (Rigaud and Juchault, 1998). The dissection of 218 intersexes revealed that 82.6% were sterile. They possess both male and female external characteristics and have nonfunctional gonads that have a structure between male and female. The remaining intersexes (17.4%) had female gonads and a tiny male appendix masculina. To test if the intersex phenotype could be related to a lower bacterial load, the FISH technique was used to compare Wolbachia amount in cells of A. vulgare females and intersexes. The Wolbachia-specific probe previously used in weevils and Triatoma (Heddi et al., 1999; Pintureau et al., 2000) allows a clear detection of isopod Wolbachia (Fig. 2). A comparison of the bacterial load was impossible between female and intersex gonads because of their dissimilarity, which could lead to differences in bacterial number due to differences in cytoplasm amount (there is more cytoplasm present in oocytes than in undifferentiated cells). We therefore compared the bacterial load in a somatic organ: the nerve chord and associated fat tissues. The counting of host nuclei was used as a reference to standardize the Wolbachia num-

### Table 2

<table>
<thead>
<tr>
<th>Recipient species</th>
<th>Infection</th>
<th>N</th>
<th>Young/brood (mean ± SEM)</th>
<th>Percentage of males (mean ± SEM)</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. scaber</td>
<td>None</td>
<td>12</td>
<td>52.01 ± 3.84&lt;sup&gt;*&lt;/sup&gt;</td>
<td>46.72 ± 1.85</td>
<td>**</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>wAv</td>
<td>8</td>
<td>33.54 ± 3.97&lt;sup&gt;*&lt;/sup&gt;</td>
<td>46.20 ± 2.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>wAn</td>
<td>14</td>
<td>35.64 ± 4.19&lt;sup&gt;*&lt;/sup&gt;</td>
<td>46.71 ± 1.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O. asellus</td>
<td>None</td>
<td>11</td>
<td>36.83 ± 2.17&lt;sup&gt;*&lt;/sup&gt;</td>
<td>52.63 ± 1.29</td>
<td>***</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>wAv</td>
<td>14</td>
<td>5.44 ± 1.16&lt;sup&gt;*&lt;/sup&gt;</td>
<td>43.51 ± 3.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>wAn</td>
<td>11</td>
<td>6.81 ± 2.87&lt;sup&gt;*&lt;/sup&gt;</td>
<td>42.79 ± 5.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. vulgare</td>
<td>None</td>
<td>17</td>
<td>111.26 ± 6.95&lt;sup&gt;*&lt;/sup&gt;</td>
<td>50.47 ± 0.80&lt;sup&gt;*&lt;/sup&gt;</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td></td>
<td>wAv</td>
<td>14</td>
<td>73.82 ± 7.84&lt;sup&gt;*&lt;/sup&gt;</td>
<td>5.41 ± 3.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>wAn</td>
<td>18</td>
<td>73.22 ± 6.03&lt;sup&gt;*&lt;/sup&gt;</td>
<td>39.25 ± 2.73&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. nasatum</td>
<td>None</td>
<td>14</td>
<td>38.95 ± 3.48&lt;sup&gt;*&lt;/sup&gt;</td>
<td>47.43 ± 1.41&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>wAv</td>
<td>7</td>
<td>28.86 ± 2.69&lt;sup&gt;*&lt;/sup&gt;</td>
<td>7.36 ± 3.88&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>wAn</td>
<td>10</td>
<td>38.43 ± 5.62&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.03 ± 2.50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: For each recipient species, values followed by different letters were significantly different (P < 0.05) after Tukey-Kramer posthoc test.

* One-way ANOVA test comparing the three groups for each recipient species.

** Kruskal-Wallis nonparametric test comparing the three groups for each recipient species; n.s. P > 0.05.

*** P < 0.01.

**** P < 0.001.
ber estimate (Fig. 2). The Wolbachia number was significantly higher in female tissues than in intersex tissues (Fig. 3, Wilcoxon rank sum test: Z = 3.34; P < 0.001).

DISCUSSION

Wolbachia could survive in their new hosts, but were vertically transmitted only in species closely related to the donors. A general resistance to Wolbachia cannot be involved since P. scaber and O. asellus are known to harbor Wolbachia in the wild (Bouchon et al., 1998). But Wolbachia naturally hosted by these species are phylogenetically divergent from wAv and wAn (Bouchon et al., 1998; Cordaux et al., 2001). Therefore a physiological incompatibility could be present between these Wolbachia and their new hosts. This hypothesis is strengthened by the fact that, in most cases, Wolbachia have a deleterious effect on their new hosts' fertility after transinfection, evidencing a physiological cost of their presence. Such an effect was also found in A. vulgare females that were infected with Wolbachia from another A. vulgare population. This could be due to a local adaptation of the Wolbachia. To test this the same experiment should be repeated with hosts and bacteria from each of the two populations. From our experiment it is not clear whether the decrease in offspring number is due to a direct or an indirect cost, i.e., if the decrease in fertility was due to a lower growth rate in mothers (fertility is correlated to size in isopods; Sutton et al., 1984) or if it was due to a more direct effect, such as disrupting vitellogenesis or inducing a higher mortality in young. A decrease in growth rate has already been observed after intraspecific (but interpopulation) Wolbachia transfer in A. vulgare (Juchault and Mocquard, 1989). But the very strong decrease in young number observed in O. asellus nevertheless cannot be due to an effect on mother size only (the difference would suppose a difference in size on the same order as the decrease in fertility, which was not detected by eye) and is more likely to be due to a direct pathogenic effect of the symbiont. Results obtained within the Armadillidium genus showed that there was no major incompatibility between Wolbachia and recipient species: symbionts were transmitted to almost all offspring (as in naturally infected lineages) and kept their feminizing effect. However, a dissymmetry in the capacity of Wolbachia to disturb host sexuality was observed. While wAv have a similar feminizing effect on A. nasatum as wAn, the reverse is not true. The wAn symbiont induced a moderate sex ratio bias and a strong proportion of intersexes in A. vulgare, which revealed a weaker capacity to induce feminization in its new host. This dissymmetry highlights the fact that these closely related symbionts adapted to their hosts in a different manner. The wAn symbiont could have adapted more closely to its host than wAv. We can therefore suggest that wAv has kept a wider potential for infecting new hosts with success than wAn.

Differences were found in the number of symbionts within cells of adult A. vulgare females and intersexes. This result suggest that a weaker capacity of Wolbachia to infect host tissues can be at the origin of a weaker feminizing effect in their hosts. This dose effect for Wolbachia-induced feminization would be similar to some cases of cytoplasmic incompatibility (Breeuwer and Werren, 1993). However, we must acknowledge that it could be possible that the observation of bacterial density in adults does not reflect the density at the time of sex differentiation (i.e., the moment when the bacterial effect is expressed; in the adults we have tested, the damage has already been done). So more studies are needed to confirm these first observations.

To be established in a new host population after infection, Wolbachia symbionts must select three keys: (i) a “compatibility” key, (ii) a “transmission” key, to infect a novel host's generations, and (iii) a “disruption” key, to turn the host's reproduction at their advantage.
to spread in hosts populations. A previous study (Bouchon et al., 1998) showed that Wolbachia often kept their feminizing effect when transferred into adult male of different species, which could be perceived as a conserved ability to disrupt host sexuality (key iii). Particularly, P. scaber adult males were feminized by wAn. In the present study, wAn transferred in P. scaber females were vertically transmitted in one case but without feminization. This difference may be due to a number of vertically transmitted bacteria too low to induce feminization in embryos. This is supported by the fact that F2 P. scaber daughters were not infected by wAn. Overall, however, our results suggested that feminizing Wolbachia did not keep strong potentialities to propagate by contagion, mainly because they lack the “transmission” key. Also, the reduction of fertility following host changes would not help to successfully infect a new host species. Finally, the moderate feminization expressed by wAn in A. vulgare would probably not allow its persistence in this species. The absence of an important excess of females added to the high proportion of sterile intersexes and the reduction of fertility would not allow this Wolbachia strain to spread deterministically in an uninfected population, because infected females do not produce more daughters than uninfected females (Taylor, 1990; Hatcher and Dunn, 1995). The infection of A. nasatum females with wAv symbionts was more successful. There was a decrease in fertility, but thanks to the high female-biased sex ratio they induce, wAv symbionts would have a good chance to persist and spread in A. nasatum populations.

In conclusion, successful horizontal transmission of Wolbachia between two distantly related terrestrial isopods species would probably require a strong selection on a new Wolbachia variant. This does not mean that such transfers are impossible, as suggested by phylogenetic analyses (Bouchon et al., 1998; Cordaux et al., 2001), but these events will only rarely be successful. Maybe several million years ago Wolbachia had more potentialities (plasticity) to infect several host species than at present, and they may have lost these potentialities following a selection and a coevolution with the peculiar hosts that are isopods. There is a possibility, however, that some Wolbachia lineages are less specialized and can infect other host species more easily. A way to test this hypothesis would be to try to transfer Wolbachia from species where recent phylogenetic work has revealed recent horizontal transfer (Cordaux et al., 2001). Of course it is also possible that chances of horizontal transfers happening have always been small, but in evolutionary history during millions of generations even small chances may have left enough opportunities for horizontal transfers to happen. The present only shows the successes of evolution. Our experiment may have been just one of the many unsuccessful attempts.

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