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INTRA-INDIVIDUAL COEXISTENCE OF A *WOLBACHIA* STRAIN REQUIRED FOR HOST OOGENESIS WITH TWO STRAINS INDUCING CYTOPLASMIC INCOMPATIBILITY IN THE WASP *ASOBARA TABIDA*

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Abstract.—Cytoplasmically inherited symbiotic *Wolbachia* bacteria are known to induce a diversity of phenotypes on their numerous arthropod hosts including cytoplasmic incompatibility, male-killing, thelytokous parthenogenesis, and feminization. In the wasp *Asobara tabida* (Braconidae), in which all individuals harbor three genotypic *Wolbachia* strains (wAtab1, wAtab2 and wAtab3), the presence of *Wolbachia* is required for insect oogenesis. To elucidate the phenotype of each *Wolbachia* strain on host reproduction, especially on oogenesis, we established lines of *A. tabida* harboring different combinations of these three bacterial strains. We found that wAtab3 is essential for wasp oogenesis, whereas the two other strains, wAtab1 and wAtab2, seem incapable to act on this function. Furthermore, interline crosses showed that strains wAtab1 and wAtab2 induce partial (about 78%) cytoplasmic incompatibility of the female mortality type. These results support the idea that bacterial genotype is a major factor determining the phenotype induced by *Wolbachia* on *A. tabida* hosts. We discuss the implications of these findings for current hypotheses regarding the evolutionary mechanisms by which females of *A. tabida* have become dependent on *Wolbachia* for oogenesis.

Key words.—*Asobara tabida*, cytoplasmic incompatibility, multiple infections, mutual dependence, symbiosis, *Wolbachia*.

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Many animal species depend on symbiotic microorganisms for essential functions such as nutrition, locomotion, reproduction, or defense against predators or pathogens (Buchner 1965; Margulis and Fester 1991; Douglas 1994). However, even though the role of the symbiont in these host functions is well characterized in certain cases (Douglas 1998), the nature and the diversity of the evolutionary mechanisms by which the host has become dependent on symbiont to survive or reproduce remains poorly understood. In this regard, the study of the interactions between symbiotic *Wolbachia* bacteria and their numerous invertebrate hosts appears quite promising.

Wolbachia are a monophyletic group of maternally inherited alpha proteobacteria that have been found in numerous insects, mites, spiders, terrestrial crustaceans, and filarial nematodes (Werren and O'Neill 1997; Bandi et al. 1998; Werren and Windsor 2000). Strictly intracellular, they depend on their host habitat to survive and develop. *Wolbachia* are of special interest for studying evolution of symbiosis because of the impressive diversity of association types they have established with their hosts, ranging from parasitism (i.e., infected host individuals have lower fitness than uninfected ones) to mutual dependence (i.e., all host individuals are infected and depend on *Wolbachia* to develop or reproduce). In general, *Wolbachia* are required for development and reproduction in filarial nematodes, but they are facultative for the same functions in the great majority of arthropods (Stouthamer et al. 1999; Bandi et al. 2001). Within arthropods, *Wolbachia* persist in host populations as a result of their ability to manipulate host reproduction to increase their own transmission. These manipulations include cytoplasmic incompatibility, thelytokous parthenogenesis, feminization, and male-killing (Werren 1997; Stouthamer et al. 1999). Interestingly, however, certain studies have shown that *Wolbachia* infection is required for reproduction in several insect species, suggesting that, as in nematodes, host-*Wolbachia* interactions have also evolved in mutual dependence in arthropods (for overviews, see Gottlieb and Zchori-Fein 2001; Dedeine et al. 2003).

In the current paper, we focus on a particular case of mutual dependence involving *Wolbachia* endosymbionts in the wasp *Asobara tabida* (Braconidae). In this case, *Wolbachia* infection is required for female reproduction (Dedeine et al. 2001). Indeed, aposymbiotic females (i.e., females cured from their infection) fail to produce any eggs and consequently cannot reproduce. The possibilities that inhibition of egg production is caused directly by antibiotics or indirectly through the release of endotoxins from decaying bacteria have been ruled out, strongly suggesting that egg production is wholly dependent on presence of *Wolbachia* (Dedeine et al. 2001). Surprisingly, the dependence of *A. tabida* on *Wolbachia* seems to be specific for oogenesis. Aposymbiotic males are fertile and, except for their inability to produce eggs, aposymbiotic females appear to have a normal overall physiological state (normal size, weight, locomotor activity and behavior; Dedeine et al. 2001). Exactly how the dependence of *A. tabida* on *Wolbachia* infection has evolved remains an open question.

It has been shown that all *A. tabida* wasps from a single line simultaneously harbor three different *Wolbachia* strains, all of which belong to the A clade of *Wolbachia* (Vavre et al. 1999a). Intra-individual multiple infections have already

been described in several *Wolbachia*-infected species (for an overview, see Mouton et al. 2003), and typically are characteristic of *Wolbachia* strains that induce cytoplasmic incompatibility (CI). Indeed, among the different phenotypes induced by *Wolbachia*, it appears that, at least theoretically, only CI could actively allow the maintenance of different bacterial strains within host maternal lineages (Frank 1998). CI is a sperm-egg incompatibility that results when a male infected with a certain *Wolbachia* strain(s) mates with either an uninfected female or an infected female harboring a different bacterial strain(s) (reviewed in Bourtzis et al. 2003). These crosses result in mortality in diploids and either increased male production or female mortality in haplodiploids (Breeuwer and Werren 1990; Breeuwer 1997; Vavre et al. 2000, 2001; Bordenstein et al. 2003). Consequently, multiply infected females simultaneously harboring several *Wolbachia* strains have a reproductive advantage over uninfected females or females infected with only a subset of *Wolbachia* strains (Frank 1998). Conversely, in the absence of CI, multiple infections generally are not maintained because incomplete maternal transmission generally leads to a reduction in the within-host diversity of symbionts (Mira and Moran 2002). Therefore, one might predict that one or more *Wolbachia* strains present in the triply infected species *A. tabida* also induce CI.

In the present paper, we address two questions. First, which of the three *Wolbachia* strains present in *A. tabida* is required for oogenesis? Second, if one or more strains is not required for oogenesis, do any of these strains induce CI, as predicted theoretically?

Materials and Methods

Insect Biology

Asobara tabida develops as a solitary larval endoparasitoid of various *Drosophila* species (van Alphen and Janssen 1982; Carton et al. 1986). Female wasps deposit their eggs into first or second instar *Drosophila* larvae, within which parasitic larvae subsequently feed and develop. All experiments were performed using *A. tabida* individuals derived from a single line established in 1998 and originating from Pierrefeu, France. This line was initiated with the offspring of 20 females caught in field and then maintained in vials. Parasitoids were reared on a *Wolbachia*-free line of *D. melanogaster* originating from Ste Foy-lès-Lyon, France. Rearing and experiments were performed at 20°C, 12:12 light:dark cycle, and 70% relative humidity. *Drosophila* larvae and adults were fed standard diet and adult wasps were fed honey. Generation time is 28 days under these conditions.

Diagnostic Polymerase Chain Reaction

Specific polymerase chain reaction (PCR) assays were used to detect the three *Wolbachia* strains previously described in *A. tabida* (Vavre et al. 1999a). Based on the *Wolbachia wsp* gene sequences of the three strains (Genbank accession number AF124856, AF124857, AF124859), specific primers were designed for each of them: 5′-TGG TAT TAC AAA TGT AGC-3′ for wAtab1, 5′-ACC TAT AAG AAA GAC AAG-3′ for wAtab2 (172F in Zhou et al. 1998), and 5′-AAA GGG GAC TGA TGA TGT-3′ for wAtab3. All three forward primers were used with the same reverse primer: 5′-AAA AAT TAA ACG CTA CTC CA-3′ (691R in Zhou et al. 1998). These primers have not been designed to be specific for the *Wolbachia* subgroup to which they belong, but only to differentiate *Wolbachia* strains within *A. tabida*. All three specific PCR assays were performed as separate reactions on DNA isolated from a single wasp. ITS 2 primers were used to amplify insect nuclear DNA as a control to check for template DNA quality (forward primer: 5′-TTG CAG AGC TTG GAC TTG AA-3′; and reverse primer: 5′-CAT ATC TCC GCC ACC AGT AA-3′; Allemand et al. 2002). DNA extractions and PCR conditions were as described in Vavre et al. (2001), except that the annealing temperature used to detect wAtab3 was 55°C (instead of 52°C as for detection of wAtab1 and wAtab2).

Generating Insect Lines Having a Subset of Wolbachia Strains

Parasitoids were given rifampicin antibiotic (Hoechst, Strasbourg, Germany) during their larval stages, through *Drosophila* host larvae as previously described (Dedeine et al. 2001). Infested *D. melanogaster* larvae were fed a standard diet supplemented with low rifampicin concentrations (0.8 to 0.008 mg/g), far lower than the concentration required to completely eliminate *Wolbachia* infection (2 mg/g). Emerging, antibiotically treated *A. tabida* females were isolated and individually mated to completely cured males to avoid possible CI. Females were then allowed to oviposit for 48 h on about 300 *Drosophila* larvae, which is substantially more hosts than the potential total number of progeny per wasp female. Females were then stored individually in alcohol (95%) at −20°C until future molecular analysis. Lines were selected according to the infection status of their single foundress (i.e., the combination of the three *Wolbachia* strains they harbored), and maintained for several generations without antibiotics. At each generation, only a subset of lines was chosen and maintained to generate the next generation. Three daughters from each selected line were used to establish three new isofemale lines. At each generation, we also compared the proportion of sterile females (i.e., the ones that do not produce offspring) between lines founded in G0 by an antibiotically treated female and lines founded in G0 by an untreated female. After four generations of selection, we succeeded in establishing four lines harboring different bacterial combinations that proved stable over time. These lines were named according to their geographical origin (Pi, for Pierrefeu) and to the *Wolbachia* strains they harbor. According to this nomenclature, the triply infected line harboring all three *Wolbachia* strains are named Pi(123). Characterization and crosses of the established lines were done 12 generations after treatment to ensure that antibiotic treatment itself had no effects on host physiology and performance.

Crossing Experiments

To determine CI relationships between the established insect lines, individuals of different infection status were intercrossed. Because males emerge one or two days prior to females, all crosses performed were between three- to four-

day-old males and one- to two-day-old females. Emerged males and females were fed honey and kept separately at 20°C in optimal conditions until crosses. Twenty crosses of each modality were performed and in every case, matings were confirmed visually. Fertilized females were then individually isolated for 48 h in vials containing a group of L1-*Drosophila* larvae that had emerged from 150 eggs (this number of *Drosophila* hosts reduces both the risk of multiple infestations by parasitoid in a single *Drosophila* host and competition between developing *Drosophila* larvae). To increase the number of wasp offspring, a second identical 48-h infestation period was performed using the same conditions as above. In total, each female was allowed to oviposit on 300 host larvae over 96 h. At the end of development, numbers of male and female offspring wasps emerging from each vial were recorded. Infection status of both parents was checked at the end of the experiments to confirm that all individuals harbored the expected *Wolbachia* strains.

Several different series of crosses were performed to determine whether any of the *Wolbachia* strains induce CI. We performed two series (i.e., blocks) of such crosses where males harbored at least one or more *Wolbachia* strain(s) than females, and we compared the size and sex ratio of the offspring they produced. We also performed a series of crosses where males harbored at least one *Wolbachia* strain less than females to determine the role, if any, of nuclear genes in the incompatibility level between the insect lines. To compare size and sex ratio (sex ratios were arcsine-square-root transformed for analysis) of the offspring both within intraline crosses and within interline crosses, we used analysis of variance (ANOVA) with two factors of variation (i.e., cross modality and block). Comparisons between intra- and interline crosses were performed using the contrast method. The significance level of ANOVAs ($P = 0.05$) was adjusted following the Bonferroni procedure to correct for multiple analyses (Sokal and Rohlf 1995).

Number of Oocytes Produced

Asobara tabida females produce most of their mature oocytes before they emerge as adults. To estimate oocyte load, newly emerged females were fed honey for five days to allow complete maturation of oocytes. Ovaries were dissected in a physiological saline solution, transferred into a neutral red solution for 5 min, and then gently crushed between a glass slide and cover-glass to disperse their contents. Total numbers of oocytes were counted using a video system assisted by computer.

RESULTS

Infection Status and Sterility of Untreated Females

The *Wolbachia* infection status of 15 untreated males and 46 untreated females was determined using a diagnostic PCR assay. Consistent with a previous study (Vavre et al. 1999a), all individuals were coinfected by the three *Wolbachia* strains wAtab1, wAtab2, and wAtab3. Nine of the 46 triply infected females (19.1%) produced no progeny, whereas all of the remaining 37 females were fertile and produced an average (± SD) of 106.2 (± 20.0) offspring. In a second experiment performed in same conditions, we found six sterile females on 27 (22.2%). However, the dissection of these six females showed that the number of oocytes they produced (263 ± 57 per female) was not significantly different than the number of oocytes produced by a group of 16 females of same age, but that were not allowed to oviposit (i.e., these females were introduced in vials without *Drosophila* host larvae; 257 ± 38; $F = 0.524$; df = 1, 20; $P = 0.785$). This last result indicates that the sterility of untreated triply infected females have not the same physiological origin compared to treated *Wolbachia*-free females that are incapable to produce any eggs (Dedeine et al. 2001). Together, these two experiments showed that under our experimental conditions, about 20% of untreated triply infected females do not produce progeny. Such female sterility had already been observed in *A. tabida* and apparently is due to a problem of females inherent to laboratory rearing conditions (M. Boulétreau, pers. obs.).

Infection Status and Sterility of Females during and after Antibiotic Treatment

To establish lines infected with a subset of *Wolbachia* strains, we carried out a selection protocol consisting of moderate antibiotic treatment for a single generation followed by four generations without treatment, which facilitates stochastic loss of *Wolbachia* during the recovering of *Wolbachia* density (Vavre et al. 2001). We then determined the infection status and sterility of females. As expected, all 27 females completely cured of the three *Wolbachia* strains were sterile. Since completely uninfected females are not informative for determining which of the three *Wolbachia* strains are involved in oogenesis, we studied sterility of antibiotically treated, but *Wolbachia*-infected females (i.e., partially cured females). In total, 36 such females in the first generation (G0) and 135 in subsequent generations (G1–G4) were studied for sterility. Eight (22.2%) and 28 (20.7%) females were found sterile in G0 and G1–G4, respectively. These proportions are not significantly different from the number of sterile untreated control females (19.1%), showing that antibiotic treatment itself did not increase sterility in G0 (exact Fisher test, $P = 0.820$). We also examined the relationship between the presence/absence of the three *Wolbachia* strains and sterility (Table 1). For females with or without strains wAtab1 or wAtab2, sterility remained around 20%, which is the same proportion of sterile females among untreated females. Thus, these data suggest that absence of these two strains within females does not increase sterility. In contrast, all females lacking the *Wolbachia* strain wAtab3 invariably were sterile. The most likely explanation for the complete correlation between sterility and the absence of wAtab3 is that this particular bacterial strain is the *Wolbachia* strain required by females for egg production. Moreover, because no relationship was found between the presence of either or both of the two other strains and sterility of females (see Table 1), it appears that only wAtab3 possesses the ability to act on host oogenesis. Consistent with this interpretation, a total of only eight females lacking wAtab3 were obtained, all of which were sterile, and only four combinations of the three *Wolbachia* strains could be maintained after line selection, all of which minimally harbor wAtab3: singly-infected Pi(3) line harbors wAtab3, doubly

TABLE 1. Rates of female sterility in presence/absence of the three *Wolbachia* strains. Values in parentheses represent the number of females tested. Only the G0 females were treated with antibiotics.

Wolbachia strain	Generation	Present	Absent	*P*-value (exact Fisher test)
wAtab1	G0	0.19 (26)	0.30 (10)	0.667
	G1–G4	0.20 (64)	0.21 (71)	1.000
wAtab2	G0	0.25 (32)	0.00 (04)	0.561
	G1–G4	0.24 (67)	0.18 (68)	0.406
wAtab3	G0	0.13 (32)	1.00 (04)	0.001
	G1–G4	0.18 (131)	1.00 (04)	0.002

infected Pi(13) line harbors wAtab1 and wAtab3, doubly infected Pi(23) line harbors wAtab2 and wAtab3, and the triply infected Pi(123) line harbors all three strains. Infection status of lines was checked every generation until G12, when infections were proved stable.

Characterization of Lines

Wasps of the four lines with different combinations of *Wolbachia* strains, Pi(123), Pi(13), Pi(23), and Pi(3), were examined for egg production, infestation efficiency, and developmental success. Because all measures were performed 12 generations after antibiotic treatment, we can exclude the possibility of any direct effect of antibiotic treatment on host physiology and performance. Egg production of emerging females from each of the four lines did not differ significantly (Table 2; $F = 0.391$; df $=3, 56$; $P = 0.76$). Furthermore, the number of egg produced in all four lines fell to zero after curative antibiotic treatment. Previously, we have shown that toxic action of the antibiotic cannot explain the complete sterility of females, since even at high concentration individuals are not affected by treatment (Dedeine et al. 2001). Moreover, there were no significant differences in offspring production or progeny sex ratio of females from each of the four lines (Table 3A). Therefore, we conclude that the presence of the wAtab3 strain alone is sufficient for females to produce their full complement of mature oocytes, and that strains wAtab1 and wAtab2 do not affect numbers of oocytes produced.

Intra- and Interline Crosses

We performed a series of interline crosses to test whether either or both of the *Wolbachia* strains wAtab1 and wAtab2 are able to induce CI within *A. tabida*. First, crosses involving females that harbor one or more *Wolbachia* strains than males (no CI is expected in these crosses) were performed to determine whether any nuclear incompatibilities exist between lines as a result of the selection protocol (Table 4). No significant differences were observed between these inter- and intraline crosses both for offspring production ($F = 0.436$; df $= 1, 7$; $P = 0.390$) and sex ratio ($F = 0.849$; df $= 1, 7$; $P = 0.145$). To determine whether strains wAtab1 and wAtab2 are able to induce CI, we performed a series of crosses in which males harbored one or two *Wolbachia* strains that the females lacked (Table 3). For these crosses, we observed that offspring sex ratios were highly male biased compared to the intraline control crosses ($F = 80.790$; df $= 1, 9$; $P < 0.0001$), a pattern similar to other studies in haplodiploids where CI occurs (Breeuwer and Werren 1990; Breeuwer 1997; Vavre et al. 2000, 2001; Bordenstein et al. 2003). These results showed that both the strains wAtab1 and wAtab2 are able to induce CI. Furthermore, the male-biased sex ratio observed in crosses between Pi(13) males and Pi(3) females and between Pi(23) males and Pi(3) females, are not significantly different suggesting that *Wolbachia* strains wAtab1 and wAtab2 induce the same level of CI. Moreover, because reciprocal crosses between Pi(13) and Pi(23) are incompatible, we conclude that the two strains wAtab1 and wAtab2 are mutually incompatible with each other (i.e., bidirectionally incompatible).

Additionally, our data also show that total offspring production is significantly lower in incompatible crosses compared to compatible crosses ($F = 150.3$; df $= 1, 9$; $P < 0.0001$). This reduction in offspring production is due to a reduction in the numbers of daughters ($F = 266.2$; df $= 1, 9$; $P < 0.0001$); the numbers of sons remaining unchanged ($F = 0.314$; df $= 1, 9$; $P = 0.59$). Thus, the male-biased sex ratio observed in incompatible crosses apparently results from high female mortality, demonstrating that both *Wolbachia* strains wAtab1 and wAtab2 induce CI of the female mortality type. However, levels of CI induced by each strain appear to be similar but incomplete, since incompatible crosses involving either strain results in about 22% of fertilized eggs surviving and developing into adult females (see Table 3B).

TABLE 2. Numbers of oocytes (mean ± SD) produced by females of different infection status. Values in parentheses represent the number of females tested.

	Oocyte load	
Lines	Infected	Uninfected (Rifampicin cured)[1]
Pi(123)	254.2 ± 25.3 (15)	0.0 ± 0.0 (21)
Pi(13)	259.5 ± 23.3 (15)	0.0 ± 0.0 (19)
Pi(23)	247.9 ± 39.8 (15)	0.0 ± 0.0 (18)
Pi(3)	255.0 ± 27.2 (15)	0.0 ± 0.0 (20)

[1] Elimination of bacteria was verified by using a polymerase chain reaction assay (see Materials and Methods). To confirm infection status, a minimum of eight individuals per line was tested.

DISCUSSION

Previous research suggests that all individuals of the parasitoid wasp *A. tabida* harbor three different *Wolbachia* strains (Vavre et al. 1999a). Previous work has also shown

TABLE 3. Numbers of adult parasitoids emerging from intraline and and interline crosses where males harbor at least one *Wolbachia* strain that females lack (mean ± SD). Only the *F* for the factor of variation cross is indicated. The factor block and the interaction cross × block were either not significant or marginally significant, which was expected according to the variability of the measured traits. In only one case was a high interaction found between the factors ($F = 4.062$; df = 6,115; $P = 0.001$) for sex ratio in interline incompatible crosses.

Crosses (male × female)	No.	Males	Females	Males + Females	Sex ratio (freq. males)
A. Intraline crosses					
Pi(123) × Pi(123)	8	54.0 ± 21.2	127.6 ± 39.8	181.6 ± 55.1	0.276 ± 0.061
	8	64.1 ± 15.4	130.9 ± 26.4	195.0 ± 33.4	0.330 ± 0.059
Pi(13) × Pi(13)	8	60.8 ± 13.1	119.0 ± 36.2	179.8 ± 45.5	0.348 ± 0.062
	7	52.4 ± 16.2	94.9 ± 42.8	147.3 ± 55.8	0.372 ± 0.080
Pi(23) × Pi(23)	10	54.1 ± 21.6	109.2 ± 42.0	163.3 ± 59.8	0.325 ± 0.079
	7	72.6 ± 14.2	123.6 ± 25.7	196.1 ± 37.6	0.371 ± 0.033
Pi(3) × Pi(3)	10	68.6 ± 11.0	128.6 ± 22.1	197.2 ± 28.8	0.349 ± 0.039
	8	53.7 ± 21.6	96.5 ± 41.9	143.4 ± 60.7	0.375 ± 0.070
F (df = 3,62)		0.522	0.107	0.687	2.570
P		0.669	0.746	0.564	0.063
B. Interline incompatible crosses					
Pi(123) × Pi(13)	10	54.1 ± 16.0	34.4 ± 13.7	88.5 ± 22.1	0.612 ± 0.103
	6	60.7 ± 12.8	13.7 ± 5.3	74.3 ± 14.3	0.815 ± 0.069
Pi(23) × Pi(13)	10	53.7 ± 11.4	34.9 ± 27.0	88.6 ± 34.9	0.655 ± 0.176
	6	61.5 ± 16.7	16.7 ± 19.4	78.2 ± 27.6	0.820 ± 0.155
Pi(123) × Pi(23)	11	66.5 ± 17.5	46.2 ± 30.1	112.7 ± 28.2	0.622 ± 0.208
	6	55.2 ± 20.5	19.3 ± 17.1	74.5 ± 33.4	0.762 ± 0.126
Pi(13) × Pi(23)	9	62.4 ± 12.0	23.3 ± 30.7	85.8 ± 35.2	0.792 ± 0.197
	6	57.0 ± 12.6	31.3 ± 8.7	88.3 ± 17.2	0.647 ± 0.073
Pi(123) × Pi(3)	10	60.1 ± 15.4	15.7 ± 8.2	75.8 ± 20.4	0.799 ± 0.077
	10	54.6 ± 18.1	12.1 ± 14.2	66.7 ± 24.5	0.843 ± 0.142
Pi(13) × Pi(3)	10	60.8 ± 14.2	15.5 ± 22.3	76.3 ± 27.7	0.830 ± 0.163
	7	53.9 ± 20.9	33.9 ± 28.4	87.7 ± 40.7	0.637 ± 0.202
Pi(23) × Pi(3)	12	63.8 ± 08.5	40.9 ± 29.5	104.7 ± 32.9	0.655 ± 0.175
	7	56.0 ± 17.0	24.7 ± 7.3	80.7 ± 22.1	0.691 ± 0.078
F (df = 6,113)		0.157	1.597	1.300	1.695
P		0.987	0.155	0.264	0.129

that the presence of *Wolbachia* in *A. tabida* is specifically required for host oogenesis (Dedeine et al. 2001). However, until now the role of each of the three *Wolbachia* strains in host oogenesis was unknown. Our data show that only the *Wolbachia* strain wAtab3 is essential for oogenesis and that this strain is sufficient for the complete maturation of oocytes. Moreover, despite our findings that neither of the other two *Wolbachia* strains (wAtab1 and wAtab2) are required for oogenesis, crossing experiments indicate that both of these strains are capable of inducing partial CI (about 78%) of the female mortality type. The reason for the incomplete penetrance of CI is unknown. One possible explanation is that the males used in our crosses were too old (three to four days old), a factor known to reduce the level of CI in *Drosophila* (Reynolds and Hoffmann 2002; Reynolds et al. 2003). However, based on the biology of *Drosophila* parasitoids (reviewed in Carton et alc. 1986), we think that the age of males used in our experiments does not strongly differ from the age of males mating in natural populations. Indeed, *A. tabida* males emerge one or two days before females. Furthermore, it is assumed that males wait for females emerging from the same host aggregates to mate. Thus, we believe that the incomplete level of CI we observed in our crosses probably also occurs in nature. Because wAtab3 is always present in all individuals of both sexes, we cannot totally exclude that the presence of this strain is required by wAtab1 and wAtab2 to induce and/or rescue CI. However, such interbacterial strain interactions for CI expression have never been reported in other systems, suggesting that this possibility is unlikely. Additionally, because females lacking wAtab3 do not produce any progeny, we could not test whether wAtab3 is able to induce CI in addition to its role in host oogenesis.

Host-*Wolbachia* interactions show an impressive range of phenotypes, including pathogenesis, different types of reproductive parasitism (i.e., CI, male-killing, parthenogenesis, and feminization) and mutualism (i.e., increasing or being obligatory for host survival and/or fecundity; Werren 1997; Stouthamer et al. 1999; Bandi et al. 2001; Dedeine et al. 2003; McGraw and O'Neill 2004). However, the contribution of host and bacterial factors responsible for this diversity of phenotypes remains controversial, with some results supporting involvement of host factors (Fujii et al. 2001; Sasaki et al. 2002) and other results supporting involvement of bacterial factors only (Moret et al. 2001). Clearly the nature of the *Wolbachia*-induced phenotype more likely depends both on host and bacterial factors and on the interaction between them. In the triply infected wasp *A. tabida*, we found intra-individual coexistence of one obligatory *Wolbachia* strain, which is required for oogenesis, with two facultative CI-inducing strains. Thus, within a given nuclear background (i.e., each insect individual), different genotypes of *Wolbachia* express different phenotypes. The fact that only the wAtab3 strain is essential for female reproduction indicates that its action on oogenesis requires some specific factor(s)

TABLE 4. Numbers of adult parasitoids emerging from intraline and interline crosses where females harbor at least one *Wolbachia* strain that males lack (mean ± SD). Only the *F* for the factor of variation cross is indicated. The factor block and the interaction cross × block were either not significant or marginally significant, which was expected according to the variability of the measured traits.

Crosses (male × female)	No.	Males	Females	Males + Females	Sex ratio (freq. males)
A. Intraline crosses					
Pi(123) × Pi(123)	9	61.8 ± 19.7	105.3 ± 33.1	167.1 ± 46.8	0.370 ± 0.062
Pi(13) × Pi(13)	6	61.2 ± 17.1	86.2 ± 11.2	147.3 ± 15.8	0.414 ± 0.052
Pi(23) × Pi(23)	7	60.0 ± 12.2	106.7 ± 20.3	166.7 ± 31.2	0.360 ± 0.025
Pi(3) × Pi(3)	10	62.7 ± 13.3	100.0 ± 21.8	162.7 ± 30.5	0.386 ± 0.058
F (df = 3,28)		0.048	0.991	0.476	1.259
P		0.986	0.415	0.701	0.307
B. Interline compatible crosses					
Pi(3) × Pi(123)	7	61.8 ± 7.9	122.6 ± 18.2	184.4 ± 23.2	0.336 ± 0.031
Pi(13) × Pi(123)	6	67.5 ± 17.1	109.3 ± 36.9	176.8 ± 32.2	0.393 ± 0.126
Pi(23) × Pi(123)	7	60.7 ± 9.5	113.0 ± 22.0	173.7 ± 28.5	0.351 ± 0.038
Pi(3) × Pi(13)	7	59.7 ± 16.4	109.6 ± 20.5	169.3 ± 34.0	0.350 ± 0.040
Pi(3) × Pi(23)	8	65.5 ± 13.6	106.5 ± 24.0	172.0 ± 36.1	0.382 ± 0.035
F (df = 4,30)		0.415	0.459	0.244	1.064
P		0.797	0.765	0.911	0.391

that neither wAtab1 nor wAtab2 strains have. This conclusion is consistent with the general idea that the bacterial genotype can strongly contribute to determine the type of phenotype induced by a given host-*Wolbachia* interaction.

One remaining question is whether the ability of the *Wolbachia* strain wAtab3 to act on oogenesis has arisen within the *A. tabida* lineage and is thus unique to this species, or whether this ability to act on oogenesis represents an ancestral character of wAtab3, in which case the same function may be or have been induced in another host species. Currently, there are no potential candidates of *Wolbachia* strains inducing such a phenotype excluding *Wolbachia* in filarial nematodes. Indeed, in these particular hosts, *Wolbachia* are also required for essential host functions including embryogenesis, growth, and fertility (reviewed in Bandi et al. 2001). One could argue that the *A. tabida* lineage may have acquired wAtab3 from filariae by an interspecific horizontal transfer of the bacteria. However, there is strong evidence against this hypothesis. Indeed, phylogenetic data indicate that the *Wolbachia* lineages of nematodes (clades C and D) and arthropods (clades A and B, including the strain wAtab3) are strongly differentiated and diverged about 100 million years ago (Bandi et al. 1998). Werren et al. (1995) and Vavre et al. (1999a) reported that the three strains of *Wolbachia* in *A. tabida* belong to the clade A of *Wolbachia* based on *FtsZ* and *wsp* gene sequences, respectively. Using individuals from the Pi(3) line, which are singly infected by wAtab3, we confirmed these results by sequencing both genes of the wAtab3 strain (results not shown). It appears that wAtab3 does not share a recent common ancestor with any nematode *Wolbachia*, but instead belongs to a clade known only to infect arthropods. Thus, the special role of *Wolbachia* bacteria in affecting host physiology has occurred independently in the two *Wolbachia* lineages.

Another important unanswered question is how *A. tabida* became dependent on wAtab3 for egg production, since this is a function that the insect was obviously able to accomplish before the acquisition of wAtab3. Different hypotheses have already been suggested on this issue (Charlat and Merçot 2001; Dedeine et al. 2001, 2003). One hypothesis is that the strain wAtab3 produces a toxic molecule in host females that is transmitted to all offspring via the egg cytoplasm, which can only be rescued by female offspring inheriting wAtab3. In instances where the offspring females inherit wAtab3, in wAtab3-infected daughters, this *Wolbachia* produces a second molecule, a kind of antidote that neutralizes the toxin. Conversely, in wAtab3-uninfected daughters, the toxin is not neutralized by the antidote and thus can act on the host to specifically inhibit oogenesis (Charlat and Merçot 2001; Dedeine et al. 2003).

Initially called ''sterilization of aposymbiotic sisters'' (Charlat and Merçot 2001), we propose to rename this hypothesis ''sterilization of aposymbiotic daughters'' (SAD), a name that more accurately describes the observed phenotype. The mechanism of SAD phenotype may be similar to certain postsegregation distorting phenotypes such as those induced by endosymbionts in some *Paramecia* or by selfish nuclear loci in the flour beetle *Tribolium castaneum* (for an excellent introduction of these phenotypes see Werren and O'Neill 1997). Interestingly, however, while SAD protects wAtab3 from inefficient transmission, this phenotype does not explain how the *Wolbachia* strain inducing SAD initially spread in host populations. Indeed, SAD results in a relative disadvantage of infected females compared to uninfected females because the former produce some proportion of sterile daughters (i.e., all daughters that fail to inherit *Wolbachia*). Consequently, it is unlikely that a *Wolbachia* strain inducing only the SAD phenotype will increase in frequency in host populations. One possibility, therefore, is that wAtab3 was initially ''hitchhiked'' by CI induced by wAtab1 and/or wAtab2 or that, in addition to inducing the SAD phenotype, wAtab3 also induced CI.

Another hypothesis that could explain how *A. tabida* females became dependent on wAtab3 for oogenesis is that, once completely infected by wAtab3, the host *A. tabida* accumulated irreversible modifications in one or more nuclear gene essential for oogenesis. This model assumes that wAtab3 strain had the special capability to act on host oogen-

esis, and that this action interfered with the oogenetic control of the host. We envision two nonexclusive evolutionary mechanisms by which the acquisition of wAtab3 may have modified host genes involved in oogenesis.

First, the acquisition of wAtab3 may have resulted in functional redundancy for genes involved in oogenesis (i.e., both partners are genetically able to act on oogenesis). Thus, if wAtab3 carried a gene(s) capable of acting on oogenesis, then the host copy may have been free of selective constraints and thus accumulated one or more deleterious mutations to the extent that the insect now completely depends on the gene copy from wAtab3 for successful oogenesis. Additionally, because redundancy of a physiological function is costly, selection would have favor wAtab3-infected mutant females (i.e., dependent on wAtab3 for oogenesis) relatively to wAtab3-infected nonmutant females, thus driving the dependence on wAtab3 for oogenesis in populations. A recent study on *D. melanogaster* supports this possibility in *A. tabida* (Starr and Cline 2002). In this study, authors showed that the *Wolbachia* strain wDmel restores fertility to mutant females prevented from making eggs by protein-coding lesions in *Sex-lethal* (*Sxl*), the master regulator gene of sex determination. Importantly, this result obtained in *D. melanogaster* clearly supports two assumptions of our model on *A. tabida*: (1) a mutation in a single host nuclear gene can lead to the specific inhibition of oogenesis; and (2) *Wolbachia* infection can phenotypically restores this nuclear defect. For this reason, as suggested by Starr and Cline (2002), we agreed that the situation of mutant females in *D. melanogaster* would mimic the naturally occurring situation observed in *A. tabida*. However, this analogy presents important limits. For example, wDmel and wAtab3 are not related in the *Wolbachia* A-clade (Vavre et al. 1999a), suggesting that, even though the ability to act on oogenesis seems to be share between these two *Wolbachia*, the effective expression of this trait required intimate species-specific host-*Wolbachia* interactions.

A second possible mechanism by which the acquisition of wAtab3 may have modified host genes is that the presence of wAtab3 resulted in the fixation of new alleles for certain nuclear genes that were functional only in the presence of this *Wolbachia* strain. For instance, if the infection affected the level of expression of host gene(s) involved in oogenesis (i.e., over- or underexpression), then selection may have afforded an advantage to those females harboring new allelic forms that under or overexpressed these gene(s) to return to the initial level of expression. Consequently, if in presence of these new alleles, the optimal level of expression of host gene(s) required the presence of wAtab3, their fixation in host populations would have led to the dependence of females on wAtab3 to produce eggs. Such host adaptation in response of *Wolbachia* infection has recently been documented in the two-spotted spider mite, *Tetranychus urticae* (Vala et al. 2003). In this species, in which *Wolbachia* bias the offspring sex ratio toward females, a compensatory host mechanism has evolved to counteract the action of *Wolbachia*.

Both of two proposed mechanisms by which *A. tabida* females have become genetically dependent on wAtab3 for oogenesis (i.e., accumulation of deleterious mutations or fixation of new allelic forms) assume that the frequency of wAtab3 was high in the population before the spread of the host genetic modifications. For this reason, an important issue is to determine how wAtab3 strain initially spread in host populations. Our study shows that both wAtab1 and wAtab2 induce CI in *A. tabida*. Thus, as proposed for the phenotype SAD, one possibility is that CI induced by wAtab1 and wAtab2 initially drives wAtab3 in populations by hitchhiking, or that, in addition to acting on oogenesis, wAtab3 itself initially induced CI. The possibility of dual actions of a particular *Wolbachia* strains is not unrealistic given the results from recent studies. For example, in *D. melanogaster*, wDmel induces CI in addition to restore oogenesis defect of *Sxl*-mutant females (Reynolds and Hoffmann 2002). Also, in the mosquito *Aedes albopictus*, *Wolbachia* infection is associated with both CI and increased host fecundity (Dobson et al. 2002, 2004).

Another possibility is that the action of wAtab3 on oogenesis initially increased the egg production of wAtab3-infected females, increasing their fecundity compared to those of wAtab3-uninfected females in populations. Such a mutualistic *Wolbachia* that increases fecundity without apparent other effect on host have been documented in the parasitoid wasp *Trichogramma bourarachae* (Vavre et al. 1999b).

While we cannot distinguish among these alternative scenarios, we should point out that the three hypotheses (i.e., SAD phenotype and modification of host genome by deleterious mutations or by fixation of new allelic forms) are not mutually exclusive. However, one major difference between SAD phenotype and the two other hypotheses is that the SAD phenotype does not involve any genetic modifications of the nuclear *A. tabida* genome, thus potentially allowing the opportunity of *A. tabida* to eliminate wAtab3 through evolution of resistance. In contrast, because the two other hypotheses do invoke such genetic modification(s), it is likely that the dependence of *A. tabida* on wAtab3 is definitive in these models, allowing this *Wolbachia* strain to persist in this host lineage for a long period of coevolution.

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Literature Cited

Allemand, R., C. Lemaitre, F. Frey, M. Boulétreau, F. Vavre, G. Nordlander, J. Van Alphen, and Y. Carton. 2002. Phylogency of six African *Leptopilina* species (Hymenoptera: Cynipoidea, Figitidae), parasitoids of *Drosophila*, with description of three new species. Ann. Soc. Entomol. Fr. 38:319–332.

Bandi, C., T. J. C. Anderson, C. Genchi, and M. L. Blaxter. 1998. Phylogeny of *Wolbachia* in filarial nematodes. Proc. R. Soc. Lond. B. 265:2407–2413.

Bandi, C., A. J. Trees, and N. W. Brattig. 2001. *Wolbachia* in filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases. Vet. Parasitol. 98: 215–238.

Bordenstein, S. R., J. J. Uy, and J. H. Werren. 2003. Host genotype

determines cytoplasmic incompatibility type in the haplodiploid genus *Nasonia*. Genetics 164:223–233.
Bourtzis, K., H. R. Braig, and T. L. Karr. 2003. Cytoplasmic incompatibility. Pp. 217–246 *in* K. Bourtzis and T. Miller, ed. Insect symbiosis. CRC Press, Boca Raton, FL.
Breeuwer, J. A. J. 1997. *Wolbachia* and cytoplasmic incompatibility in the spider mites *Tetranychus urticae* and *T. turkestani*. Heredity 79:41–47.
Breeuwer, J. A. J., and J. H. Werren. 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two species. Nature 346:558–560.
Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience, New York.
Carton, Y., M. Boulétreau, J. J. M. van Alphen, and J. C. van Lenteren. 1986. The *Drosophila* parasitic wasps. Pp. 347–394 *in* M. Ashburner, H. L. Carson, and J. N. Thompson, ed. Genetics and biology of *Drosophila*. Academic Press, London.
Charlat, S., and H. Merçot. 2001. Did *Wolbachia* cross the border? Trends Ecol. Evol. 16:540–541.
Dedeine, F., F. Vavre, F. Fleury, B. Loppin, M. E. Hochberg, and M. Boulétreau. 2001. Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. Proc. Natl. Acad. Sci. USA 98:6247–6252.
Dedeine, F., C. Bandi, M. Boulétreau, and L. H. Kramer. 2003. Insights into *Wolbachia* obligatory symbiosis. Pp. 267–282 *in* K. Bourtzis and T. Miller, ed. Insect symbiosis. CRC Press, Boca Raton, FL.
Dobson, S. L., E. J. Marsland, and W. Rattanadechakul. 2002. Mutualistic *Wolbachia* infection in *Aedes albopictus*: accelerating cytoplasmic drive. Genetics 160:1087–1094.
Dobson, S. L., W. Rattanadechakul, and E. J. Marsland. 2004. Fitness advantage and cytoplasmic incompatibility in *Wolbachia* single- and superinfected *Aedes albopictus*. Heredity 93: 135–142.
Douglas, A. E. 1994. Symbiotic interactions. Oxford Univ. Press, New York.
———. 1998. Nutritional interactions in insect-microbial symbiosis: aphids and their symbiotic bacteria Buchnera. Annu. Rev. Entomol. 43:17–37.
Frank, S. A. 1998. Dynamics of cytoplasmic incompatibility with multiple *Wolbachia* infections. J. Theor. Biol. 192:213–218.
Fujii, Y., D. Kageyama, S. Hoshizaki, H. Ishikawa, and T. Sasaki. 2001. Transfection of *Wolbachia* in Lepidoptera: the feminizer of the adzuki bean borer *Ostrinia scapulalis* causes male killing in the Mediterranean flour moth *Ephestia kuehniella*. Proc. R. Soc. Lond. B 268:855–859.
Gottlieb, Y., and E. Zchori-Fein. 2001. Irreversible thelytokous reproduction in *Muscidifurax uniraptor*. Entomol. Exp. Appl. 100: 271–278.
Margulis, L., and R. Fester. 1991. Symbiosis as a source of evolutionary innovation. MIT Press, Cambridge, MA.
McGraw, E. A., and S. L. O'Neill. 2004. *Wolbachia pipientis*: intracellular infection and pathogenesis in *Drosophila*. Curr. Opin. Microbiol. 7:67–70.
Mira, A., and N. A. Moran. 2002. Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. Microbial Ecol. 44:137–143.
Moret, Y., P. Juchault, and T. Rigaud. 2001. *Wolbachia* endosymbiont responsible for cytoplasmic incompatibility in a terrestrial crustacean: effects in natural and foreign hosts. Heredity 86: 325–332.
Mouton, L., H. Henri, M. Boulétreau, and F. Vavre. 2003. Strain-specific regulation of intracellular *Wolbachia* density in multiply infected insects. Mol. Ecol. 12:3459–3465.
Reynolds, K. T., and A. A. Hoffmann. 2002. Male age, host effects and the weak expression or non-expression of cytoplasmic incompatibility in *Drosophila* strains infected by maternally transmitted *Wolbachia*. Genet. Res. Camb. 80:79–87.
Reynolds, K. T., L. J. Thomson, and A. A. Hoffmann. 2003. The effects of host age, host nuclear background and temperature on phenotypic effects of the virulent *Wolbachia* strain *popcorn* in *Drosophila melanogaster*. Genetics 164:1027–1034.
Sasaki, T., T. Kubo, and H. Ishikawa. 2002. Interspecific transfer of *Wolbachia* between two Lepidopteran insects expressing cytoplasmic incompatibility: a *Wolbachia* variant naturally infecting *Cadra cautella* causes male killing in *Ephestia kuehniella*. Genetics 162:1313–1319.
Sokal, R. R., and F. J. Rohlf. 1995. Biometry. 3rd ed. W. H. Freeman, New York.
Starr, D. J., and T. W. Cline. 2002. A host-parasite interaction rescues *Drosophila* oogenesis defects. Nature 418:76–79.
Stouthamer, R., J. A. J. Breeuwer, and G. D. D. Hurst. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. 53:71–102.
Vala, F., T. van Opijnen, J. A. J. Breeuwer, and M. W. Sabelis. 2003. Genetic conflicts over sex ratio: mite-endosymbiont interactions. Am. Nat. 34:254–266.
van Alphen, J. J. M., and A. R. M. Janssen. 1982. Host selection by *Asobara tabida* Nees (Braconidae: Alysiinae), a larval parasitoid of Drosophilidae. Neth. J. Zool. 32:215–231.
Vavre, F., F. Fleury, D. Lepetit, P. Fouillet, and M. Boulétreau. 1999a. Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. Mol. Biol. Evol. 12: 1711–1723.
Vavre, F., C. Girin, and M. Boulétreau. 1999b. Phylogenetic status of a fecundity-enhancing *Wolbachia* that does not induce thelytoky in *Trichogramma*. Insect Mol. Biol. 8:67–72.
Vavre, F., F. Fleury, J. Varaldi, P. Fouillet, and M. Boulétreau. 2000. Evidence for female mortality in *Wolbachia*-mediated cytoplasmic incompatibility in haplodiploid insects: epidemiologic and evolutionary consequences. Evolution 54:191–200.
Vavre, F., F. Dedeine, M. Quillon, P. Fouillet, F. Fleury, and M. Boulétreau. 2001. Within-species diversity of *Wolbachia*-induced cytoplasmic incompatibility in haplodiploid insects. Evolution 55:1710–1714.
Werren, J. H. 1997. Biology of *Wolbachia*. Annu. Rev. Entomol. 42:587–609.
Werren, J. H., and S. L. O'Neill. 1997. The evolution of heritable symbionts. Pp. 1–41 *in* S. L. O'Neill, A. A. Hoffmann, and J. H. Werren, ed. Influential passengers: inherited microorganisms and arthropod reproduction. Oxford Univ. Press, Oxford, U.K.
Werren, J. H., and D. M. Windsor. 2000. *Wolbachia* infection frequencies in insects: evidence of global equilibrium. Proc. R. Soc. Lond. B 262:197–204.
Werren, J. H., W. Zhang, and L. R. Guo. 1995. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proc. R. Soc. Lond. B 261:55–71.
Zhou, W., F. Rousset, and S. L. O'Neill. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp sequences. Proc. R. Soc. Lond. B 265:509–515.

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