ARTICLE

A Novel Technique for Removing *Wolbachia* Infections from *Aedes albopictus* (Diptera: Culicidae)

STEPHEN L. DOBSON[1] AND WANCHAI RATTANADECHAKUL

Department of Entomology, University of Kentucky, Lexington, KY 40546

J. Med. Entomol. 38(6): 844–849 (2001)

ABSTRACT Intracellular bacteria of the genus *Wolbachia* often behave as reproductive parasites by manipulating host reproduction to enhance the vertical transmission of infections. *Wolbachia* infections in *Aedes albopictus* (Skuse) cause a reproductive manipulation known as cytoplasmic incompatibility, which can reduce brood hatch. Because field populations of *Ae. albopictus* are naturally infected, studies of *Wolbachia*-induced effects on *Ae. albopictus* reproduction and fitness require that *Wolbachia* be artificially removed. Although simple techniques for clearing *Wolbachia* infections from other host insects have been developed, removal of *Wolbachia* bacteria from *Ae. albopictus* is difficult. Here we describe an improved method for removing *Wolbachia* infections *Ae. albopictus.* This method differs from earlier techniques in that it relies upon the tetracycline treatment of adults instead of larvae. We demonstrate that tetracycline treatment of adult *Ae. albopictus* can predictably generate uninfected individuals, simplify the procedure required for *Wolbachia* removal, and reduce the level of inbreeding required to produce uninfected lines.

KEY WORDS *Wolbachia pipientis*, *Aedes albopictus*, *Culex pipiens*, cytoplasmic incompatibility, tetracycline

OBLIGATE, INTRACELLULAR BACTERIA of the genus *Wolbachia* infect a diverse range of arthropods, including important insect pests (Werren et al. 1995, Jeyaprakash and Hoy 2000, Werren and Windsor 2000). The effects caused by *Wolbachia* infection were originally observed in *Culex pipiens* L., as a maternally inherited cytoplasmic factor that caused unidirectional incompatibility (Laven 1951). *Wolbachia* infections are now known to induce multiple reproductive manipulations that include cytoplasmic incompatibility (CI), feminization, male killing, and parthenogenesis (reviewed in O'Neill et al. 1997a, Werren 1997, Bourtzis and O'Neill 1998). Current *Wolbachia* research is focused on areas including the examination of mechanisms underlying the reproductive manipulations (Dobson and Tanouye 1996, Presgraves 2000), investigation of the potential applied use of *Wolbachia* to manipulate populations of medically and economically important invertebrates (Curtis and Sinkins 1998, Sinkins and O'Neill 2000, Taylor et al. 2000), and research into the role of *Wolbachia* in genetic conflict, host reproductive isolation, and speciation (reviewed in Rokas 2000, Wade 2001). Many aspects of this research require both infected and uninfected individuals.

Although some host populations naturally include infected and uninfected individuals, uninfected individuals are rare in other infected populations. These latter populations require the artificial removal of *Wolbachia* infection to establish uninfected (i.e., aposymbiotic) strains. Treatment with antibiotics and heat are commonly used for removing *Wolbachia* infection, with tetracycline treatment being the most frequently employed method (Tables 1 and 2). Removal of *Wolbachia* infections from mosquitoes have focused on rearing larvae in solutions of antibiotics (Table 2). Tetracycline concentrations below 0.05 mg/ml have been reported to successfully remove *Wolbachia* infections from *Cx. pipiens.* However, the removal of infections from *Aedes albopictus* (Skuse) has proven more difficult.

Here we describe a new technique for removing *Wolbachia* infection from *Ae. albopictus.* We have compared adult treatment with various larval treatments including heat treatments and a range of tetracycline concentrations. The tetracycline treatment of adults has proven to be the most simple and effective technique, resulting in higher rates of host survival and more predictable *Wolbachia* removal relative to alternative treatments. Adult tetracycline treatment permits the removal of infections from groups of individuals and thereby eliminates the requirement that uninfected strains be established from isofemale lines. The ability to establish aposymbiotic strains from multiple individuals will reduce experimental complications that result from inbreeding (Bordenstein and Werren 2000).

Materials and Methods

Mosquito Strains, Experimental Crosses, and Maintenance. The Koh Samui strain of *Ae. albopictus* (*Koh;* Thailand, pre-1970) is infected with the *w*AlbA *Wolbachia* type (Sinkins et al. 1995). The Houston strain

[1] E-mail: sdobson@pop.uky.edu.

Table 1. Antibiotics and their effect on *Wolbachia* infection

Antimicrobial class/Target	Species tested	Effect on *Wolbachia*	Reference(s)
Nucleic Acid Synthesis Inhibitors			
RNA polymerase			
Rifampicin	*Aponagyrus diversicornis*	Effect	(Pijls et al. 1996)
	Muscidifurax uniraptor	Effect	(Zchori Fein et al. 2000)
	Trichogramma spp.	Effect	(Stouthamer et al. 1990)
DNA gyrase			
Ciprofloxacin	*Litomosoides sigmodontis*	No effect	(Hoerauf et al. 2000)
Protein Synthesis Inhibitors			
30S bacterial ribosome			
Tetracycline	Multiple infection types; See Table 2	Effect	(Yen and Barr 1971, Richardson et al. 1987, Stouthamer et al. 1990, Holden et al. 1993, Otsuka and Takaoka 1997)
Oxytetracycline	*Dirofilaria immitis*	Effect	(Genchi et al. 1998)
Minocycline	*Trichogramma cordubensis*	Effect	(Pintureau et al. 1999)
Gentamycin	*Trichogramma spp.*	No effect	(Stouthamer et al. 1990)
Streptomycin	In vitro	No effect	(O'Neill et al. 1997b)
	Ae. albopictus	No effect	(Kambhampati et al. 1993)
	Cx. pipiens	No effect	(Yen and Barr 1973)
Kanamycin	*Ae. albopictus*	No effect	(Kambhampati et al. 1993)
Spectinomycin	*Ae. albopictus*	No effect	(Kambhampati et al. 1993)
50S bacterial ribosome			
Chloramphenicol	See Table 2		
	Litomosoides sigmodontis	No effect	(Hoerauf et al. 2000)
Erythromycin	*L. sigmodontis*	No effect	(Hoerauf et al. 2000)
	Trichogramma spp.	No effect	(Stouthamer et al. 1990)
Enzyme Interference (antimetabolites)			
Sulphonamides	*Trichogramma spp.*	Effect	(Stouthamer et al. 1990)
	A. diversicornis	No effect	(Pijls et al. 1996)
Cell Wall (inhibit transpeptidation and cross wall formation)			
Penicillin G	*Trichogramma spp.*	No effect	(Stouthamer et al. 1990)
	In vitro	No effect	(O'Neill et al. 1997b)
	Cx. pipiens	No effect	(Yen and Barr 1973)
Ampicillin	*Ae. albopictus*	No effect	(Kambhampati et al. 1993)

(*Hou;* Texas 1986) is superinfected with both *w*AlbA and *w*AlbB *Wolbachia* types (Sinkins et al. 1995). UjuTet (*UjuT*) is an uninfected strain that was artificially generated by tetracycline treatment (Otsuka and Takaoka 1997). For maintenance, mosquitoes were reared using standard conditions at 28 ± 2°C, 75 ± 10% RH, and a photoperiod of 16:8 (L:D) h. Eggs were hatched in deoxygenated water and reared at low density in water augmented with liver powder. For adult maintenance and experimental crosses, a constant supply of 10% sucrose was provided to adults. Females were provided weekly with a mouse for blood feeding and eggs were collected weekly. Experimental crosses consisted of 10 two-day-old virgin females and males (20 mosquitoes total) placed in cages.

Polymerase Chain Reaction (PCR) Amplification. Infection type was monitored in both mosquito lines and crosses using *Wolbachia*-specific primers ("general *wsp* primers"; 81F and 691R) (Braig et al. 1998). For amplifications, ovaries or testes from individual mosquitoes were isolated and homogenized in 100 μl STE [0.1 M NaCl, 10 mM Tris HCl, and 1 mM EDTA (pH 8.0)]. Proteinase K was added to a final concentration of 0.4 mg/ml, and this mixture was incubated at 56°C for 1 h. Following heat inactivation at 95°C for 15 min, 1 μl of these samples were amplified in 50 mM KCl, 20 mM Tris HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 μM primers, and 1 U *Taq* DNA polymerase in a total volume of 20 μl. Samples were denatured for 3 min at 94°C, cycled 35 times at 94°C, 55°C and 72°C (1 min each), followed by a 10 min extension at 72°C using a PTC-200 Thermal Cycler (MJ Research, Watertown, MA). 10 μl of each amplification was electrophoresed on 1% agarose gels, stained with ethidium bromide and visualized under UV illumination.

Tetracycline and Heat Treatments. All attempts to remove *Wolbachia* infection using larval and adult treatments began with superinfected mosquitoes. Initial larval tetracycline treatments were conducted as previously described (Otsuka and Takaoka 1997). In brief, eggs were hatched and first instars placed into an unbuffered tetracycline solution [5.0 mg/ml; pH 2.7] for 24 h. Larvae were then transferred to water without tetracycline and reared to adulthood.

Three alternative larval treatments employed lower tetracycline concentrations, were buffered to pH 7, and varied the duration of the tetracycline treatment. In the first two treatment types, first instars were treated similar to the Otsuka method, but larvae were reared for 24 h in either 1.0 or 2.5 mg/ml tetracycline solutions (pH 7). In a third treatment type, first instars were reared for 6 d in 2.5 mg/ml tetracycline (pH 7) and then transferred to water without tetracycline to complete development.

In an additional treatment, first instars were reared at 32°C either without tetracycline or in a 2.5 mg/ml tetracycline solution (pH 7). Due to high mortality, the tetracycline solution was diluted with water to 1.25

Table 2. Treatments and effects on *Wolbachia* infections in mosquitoes

Treatment type and species treated	Larval treatment level	Effect on host survival	Effect on *Wolbachia* infection	Infection detection method	Reference(s)
Tetracycline					
Aedes albopictus	5.0 mg/ml	[b]	[d]	[h, i]	(Otsuka and Takaoka 1997)
	0.33 mg/ml	[c]	[e]	[h]	(Kambhampati et al. 1993)
Ae. kesseli	≤2.0 mg/ml	[b]	[e]	[h]	(Trpis et al. 1981)
Ae. polynesiensis	≤2.0 mg/ml	[b]	[e]	[h]	(Trpis et al. 1981)
Ae. tafahi	0.017 mg/ml	[c]	[f]	[g]	(Wright and Wang 1980)
Ae. polynesiensis	0.017 mg/ml	[c]	[d]	[g]	(Wright and Wang 1980)
Armigeres subalbatus	0.25 mg/ml	[c]	[d]	[h, i]	(Jamnongluk et al. 2000)
Culex pipiens pipiens	≤0.05 mg/ml	[b]	[d]	[g, h, i]	(Yen and Barr 1973, Portaro and Barr 1975, Awahmukalah and Brooks 1985, Guillemaud et al. 1997)
Cx. quinquefasciatus	≤20 mg/ml	[b]	[d]	[h]	(Suenaga 1992)
	0.025 mg/ml	[b]	[d]	[g, h]	(Curtis et al. 1983)
Cx. pipiens molestus	≤20 mg/ml	[b]	[d]	[h]	(Suenaga 1993)
Chloramphenicol					
Ae. albopictus	0.33 mg/ml	[c]	[f]	[h]	(Kambhampati et al. 1993)
Ae. kesseli	≤2.0 mg/ml	[b]	[e]	[h]	(Trpis et al. 1981)
Ae. polynesiensis	≤2.0 mg/ml	[b]	[e]	[h]	(Trpis et al. 1981)
Cx. pipiens pipiens	≤1.0 mg/ml	[b]	[f]	[g, h]	(Yen and Barr 1973)
Heat Treatment					
Ae. kesseli	32.5°C	[b]	[e]	[h]	(Trpis et al. 1981)
Ae. polynesiensis	33°C	[b]	[e]	[h]	(Trpis et al. 1981)
Ae. polynesiensis	33°C	[b]	[d]	[g, h]	(Wright and Wang 1980)
Ae. tafahi	33°C	[a]	NA	NA	(Wright and Wang 1980)
Tetracycline & Heat					
Ae. tafahi	0.017 mg/ml at 30°C	[a]	NA	NA	(Wright and Wang 1980)

[a] Severe; no successful eclosion to adulthood.
[b] Moderate to high; many of the treated individuals failed to eclose.
[c] Not reported.
[d] *Wolbachia* infection cleared from one or more treated individuals.
[e] Effect on compatibility of interspecific crosses; unknown if this reflects a reduction in the *Wolbachia* infection levels.
[f] *Wolbachia* not cleared via treatment.
[g] Infection status confirmed using electron microscopy.
[h] Infection status confirmed using crossing experiments.
[i] Infection status confirmed using PCR.

mg/ml at day 4. Eclosed adults were reared at 28°C. Similar treatments were attempted using 35°C, but a line was not established due to high larval mortality and the failure of eggs from the few treatment survivors to hatch.

Tetracycline treatment of adults was accomplished by introducing a solution of tetracycline dissolved in 10% sucrose (pH 7) into the cage. Adults were continually exposed to the tetracycline solution. An alternative source of sucrose was not provided when adults were being tetracycline-treated. Buffering of tetracycline solutions was accomplished using a 1 M solution of unbuffered Tris (pH 11). Attempts to buffer larval rearing pan solutions of 5.0 mg/ml tetracycline to a more neutral pH resulted in the precipitation of the tetracycline.

To directly compare tetracycline treatment of larvae versus adults, first instar *Hou* were divided into two treatment types (three replications/treatment; ≈1,000 larvae/replication). In the larval treatments (TL), larvae were reared using the previously described Otsuka method. In the adult treatments (TA), larvae were reared through eclosion without tetracycline, and then adults were given a 1-mg/ml tetracycline solution in sucrose as described above.

Results

Larval Treatments. Initial attempts to remove *Wolbachia* infection from a superinfected *Ae. albopictus* strain by repeating a previously described procedure (Otsuka and Takaoka 1997) were unsuccessful. In each case, rearing of larvae in 5.0 mg/ml tetracycline resulted in high mortality. This high mortality is likely to have resulted at least in part from the acidity of the tetracycline solution (pH 2.7). The rare survivors produced few eggs with low hatching rates. However, by repeating the procedure twice to treat ≈3,000 larvae, 14 females survived to adulthood. The treated female adults (F_1) were mated to uninfected males (*UjuT*), blood-fed, and then placed individually into oviposition cups in an attempt to establish isofemale lines. Following oviposition, females were PCR-tested for *Wolbachia* infection. *Wolbachia*-specific primers failed to amplify in two of the 14 F_1 females. Only one of these two females produced eggs. These eggs were hatched, and the resulting progeny (F_2) were tested for *Wolbachia* infection via PCR. Only one of 14 F_2 females failed to amplify. All of the F_3 and F_4 progeny resulting from this line tested positive for *Wolbachia* infection via PCR. Thus, we were unable to generate

Table 3. Average percent egg hatch in F_3 crosses of tetracycline-treated lines

	HT1 crosses		TA crosses		TL crosses	
A	*Hou* × *HT1*	84.3 ± 10.0; 4 (1,289)	*Hou* × TA	93.0 ± 1.9; 4 (3,606)	*Hou* × TL	91.8 ± 4.0; 4 (4,621)
B			*Koh* × TA	83.1 ± 15.7; 4 (2,039)	*Koh* × TL	0.0 ± 0.1; 4 (3,805)
C	*UjuT* × *HT1*	85.7 ± 5.2; 4 (2,766)	*UjuT* × TA	80.0 ± 12.1; 4 (1,851)	*UjuT* × TL	0.0 ± 0.0; 4 (3,111)
D	*HT1* × *Hou*	0.0 ± 0.0; 6 (4,858)	TA × *Hou*	0.2 ± 0.3; 3 (2,826)	TL × *Hou*	76.9 ± 24.1; 3 (7,080)
E			TA × *Koh*	8.5 ± 1.0; 3 (3,162)	TL × *Koh*	91.0 ± 5.9; 3 (3,191)
A	*HT1* × *UjuT*	89.7 ± 9.5; 5 (2,114)	TA × *UjuT*	89.3 ± 5.7; 3 (2,845)	TL × *UjuT*	92.7 ± 2.4; 3 (3,151)

Average ± SD; Number of replicate cages (total eggs counted).

A, Positive controls to test fecundity of treated males and females. Crosses were expected to be compatible regardless of infection type resulting from treatments (Dobson et al. 2001). B, Crosses were expected to be incompatible unless the treatment removed either the *wAlbB* infection or both infections from males. C, Crosses were expected to be incompatible unless the treatment removed both infections from males. D, Crosses were expected to be compatible unless the treatment removed one or more infections from females. E, Crosses were expected to be compatible unless the treatment removed either the *wAlbA* or both infections from females.

an aposymbiotic line using the previously described technique (Otsuka and Takaoka 1997).

Additional unsuccessful attempts to remove *Wolbachia* infection were made by varying the concentration and duration of the larval tetracycline treatment. The use of lower concentrations of tetracycline resulted in higher mosquito survival, but survivors and their progeny tested positive for *Wolbachia* infection via PCR ($n = 39$). In addition, to PCR assays, individuals from the treated lines were crossed with individuals of known infection types. Crosses demonstrated that treated females were compatible with single-infected *Koh* males (96.4% hatching). Based on previously described crossing results (Dobson et al. 2001), this crossing result is consistent with treated females harboring either the *w*AlbA infection or both infection types. Crosses of treated males were incompatible with both uninfected (*UjuT;* 0.0% hatching) and single-infected females (*Koh;* 2.0% hatching). This crossing result is consistent with treated males harboring both the *w*AlbA and *w*AlbB infection types (Dobson et al. 2001). Thus, both the PCR and crossing results demonstrated that the larval tetracycline treatments failed (i.e., that the treated females, males, and their progeny remained superinfected).

Attempts to clear *Wolbachia* infection using heat, using heat combined with tetracycline, and by repeating tetracycline treatment for sequential generations were also unsuccessful. PCR assays demonstrated that adults surviving heat and heat/tetracycline treatments were all infected ($n = 8$). In an attempt to repeat tetracycline treatment for sequential generations, larvae (F_1) were treated using the Otsuka method. The few resulting F_2 larvae were then treated with 2.5 mg/ml tetracycline. However, no F_2 individuals survived in this experiment.

Adult Treatments. Due to the inability to remove *Wolbachia* infection via treatment of larvae, we attempted to treat adults with tetracycline. Initially, both larvae and adults were treated. Larvae were reared through eclosion in 1.25 mg/ml tetracycline (pH 7), and the surviving adults were supplied with a 0.5-mg/ml tetracycline solution in sucrose. Although *Wolbachia* infections were detected by PCR in some of the treatment survivors, by establishing isofemale lines from uninfected individuals, an uninfected line was obtained in the F_5 generation (designated as the "*HT1*" line). PCR tests of the *HT1* line (F_5) demonstrated that 12/12 tested females were uninfected. Crosses of the F_5 individuals showed that the *HT1* line was unidirectionally incompatible with infected males (Table 3).

In a second experiment directly comparing the tetracycline treatment of larvae (TL) and adults (TA), the adult treatments consistently resulted in higher survival of treated individuals and improved rates of *Wolbachia* removal. Nine or fewer F_1 individuals survived in each of the three larval tetracycline treatments (TL; 0.8 ± 0.2% survival). In contrast, more than 680 F_1 individuals survived in each of the three adult tetracycline treatments (TA; 78.5 ± 15.8% survival). F_1 females were mated with males from the same treatment group, and F_2 eggs were collected and hatched. The hatching rates were determined for a subset of the F_2 eggs. Hatching rates for the F_2 eggs from the larval (TL) and adult (TA) tetracycline treatments were 77.8 ± 12.0% and 9.3 ± 4.5% respectively. PCR amplifications of both F_1 and F_2 females demonstrated that survivors of the TL and TA treatments were infected and uninfected, respectively. F_3 females and males were crossed with strains of known infection type, and the hatching rates were determined (Table 3). Based on hatching rates previously determined for crosses between uninfected, single-infected, and superinfected individuals (Dobson et al. 2001), the F_3 crosses confirmed that the TL and TA individuals were superinfected and uninfected, respectively. Each of the replications was maintained separately through the F_5 generation. PCR assays of the F_5 generation again demonstrated that all tested TL females ($n = 22$) were infected and that all TA females ($n = 20$) were uninfected.

Discussion

Here we describe an improved means for removing *Wolbachia* from *Ae. albopictus.* Previous attempts at clearing infections from mosquitoes have focused on the tetracycline treatment of larvae and have shown that higher concentrations are required to remove *Wolbachia* infection from *Ae. albopictus* relative to other mosquitoes (Table 2). We demonstrate that tetracycline concentrations below 5 mg/ml fail to remove *Wolbachia* infection from *Ae. albopictus* larvae

and that tetracycline concentrations of 5 mg/ml cause high larval mortality and fail to remove infections from a majority of the surviving individuals.

Potential reasons for the ineffectiveness of tetracycline treatment in *Ae. albopictus* include the resistance of *Wolbachia* to tetracycline and the detoxification of the antibiotic in the host before it reaches the *Wolbachia* (Kambhampati et al. 1993). However, previous work with an in vitro infection shows that the *w*AlbB *Wolbachia* type from *Ae. albopictus* is susceptible to tetracycline (O'Neill et al. 1997b). We have also successfully cleared the *w*AlbB infection from an in vitro culture with concentrations of tetracycline as low as 1 μg/ml (data not shown). This suggests that *Wolbachia* infections in *Ae. albopictus* larvae are at least partially protected from the effects of tetracycline by their host.

Although low mortality was observed in F_1 individuals in the adult tetracycline treatments, F_2 egg hatching rates were reduced relative to untreated *Hou* crosses (Dobson et al. 2001). One potential explanation for these low hatching rates is the differential removal of *Wolbachia* infections from females and males. For example, low hatching would occur if the infection were cleared more readily from females relative to males (e.g., from increased consumption of the sucrose/tetracycline solution in females relative to males). Alternatively, tetracycline treatment of adults may directly reduce fecundity.

Tetracycline can be fed to *Ae. albopictus* adults either as a solution in blood or in sucrose. We have examined sucrose solutions as the simplest method. Because only females blood feed, a blood/tetracycline mixture will treat only females. A sucrose/tetracycline solution will treat both sexes, because both females and males feed on sugar solutions introduced into cages.

Tetracycline treatment of adults simplifies *Wolbachia* removal from *Ae. albopictus* by decreasing mosquito mortality associated with tetracycline treatment, by predictably generating uninfected individuals, and by eliminating the requirement of establishing isofemale lines to produce aposymbiotic lines. Avoiding isofemale lines is adventageous since *Ae. albopictus* females do not oviposit well when isolated (Wright and Pal 1967). Avoiding isofemale lines also permits generation of genetically similar infected and aposymbiotic strains, which will be useful for examining possible fecundity benefits associated with *Wolbachia* infections in *Ae. albopictus* (Dobson et al. 2001). Previous *Ae. albopictus* crossing experiments have suggested possible fecundity benefits associated with *Wolbachia* infection (Dobson et al. 2001). However, this prior study was complicated by differing host genetic backgrounds. Thus, the fecundity advantage observed in infected hosts may have reflected differences in the *Wolbachia* infection type or host genetic background (Stolk and Stouthamer 1996, Bordenstein and Werren 2000). Additional research should include variation of the tetracycline treatment method reported here, with the goal of generating an *Ae. albopictus* strain that is single-infected with the *w*AlbB infection. A strain single-infected with *w*AlbB is not currently available and will be useful for elucidating reproductive effects that are attributable to the *w*AlbB infection (Dobson et al. 2001).

Acknowledgments

We thank Scott O'Neill and Yasushi Otsuka for supplying the mosquito strains used in these experiments and John Webb for his assistance in maintaining the mosquito colonies and conducting experiments. We also thank Eric Marsland for his comments and improving this manuscript. This research was supported in part by United States Department of Agriculture NRICGP grant #9902683. This is publication 01-08-1 of the University of Kentucky Agricultural Experiment Station.

References Cited

Awahmukalah, D.S.T., and M. A. Brooks. 1985. Search for a sensitive stage for aposymbiosis induction in *Culex pipiens pipiens* by antibiotic treatment of immature stages. J. Invertebr. Pathol. 45: 54–59.

Bordenstein, S. R., and J. H. Werren. 2000. Do *Wolbachia* influence fecundity in *Nasonia vitripennis*? Heredity 84: 54–62.

Bourtzis, K., and S. L. O'Neill. 1998. Wolbachia infections and arthropod reproduction. Bioscience 48: 287–293.

Braig, H. R., W. Zhou, S. L. Dobson, and S. L. O'Neill. 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis.* J. Bacteriol. 180: 2373–2378.

Curtis, C. F., D. S. Ellis, P. E. Doyle, N. Hill, B. D. Ramji, L. W. Irungu, and H. Townson. 1983. Susceptibility of aposymbiotic *Culex quinquefasciatus* to *Wucheria bancrofti.* J. Invertebr. Pathol. 41: 214–223.

Curtis, C. F., and S. P. Sinkins. 1998. Wolbachia as a possible means of driving genes into populations. Parasitology. 116(suppl.): S111–115.

Dobson, S., and M. Tanouye. 1996. The paternal sex ratio chromosome induces chromosome loss independently of *Wolbachia* in the wasp *Nasonia vitripennis.* Dev. Genes Evol. 206: 207–217.

Dobson, S. L., E. J. Marsland, and W. Rattanadechakul. 2001. *Wolbachia*-induced cytoplasmic incompatibility in single- and superinfected *Aedes albopictus* (Diptera: Culicidae). J. Med. Entomol. 38: 382–387.

Genchi, C., L. Sacchi, C. Bandi, and L. Venco. 1998. Preliminary results on the effect of tetracycline on the embryogenesis and symbiotic bacteria (*Wolbachia*) of *Dirofilaria immitis.* An update and discussion. Parassitologia 40: 247–9.

Guillemaud, T., N. Pasteur, and F. Rousset. 1997. Contrasting levels of variability between cytoplasmic genomes and incompatibility types in the mosquito *Culex pipiens.* Proc. R. Soc. Lond. [Biol.] 264: 245–251.

Hoerauf, A., L. Volkmann, C. Hamelmann, O. Adjei, I. B. Autenrieth, B. Fleischer, and D. W. Buttner. 2000. Endosymbiotic bacteria in worms as targets for a novel chemotherapy in filariasis. Lancet 355: 1242–3.

Holden, P. R., P. Jones, and J. F. Brookfield. 1993. Evidence for a *Wolbachia* symbiont in *Drosophila melanogaster.* Genet. Res. 62: 23–29.

Jamnongluk, W., P. Kittayapong, and S. L. O'Neill. 2000. *Wolbachia* infection and expression of cytoplasmic incompatibility in *Armigeres subalbatus* (Diptera: Culicidae). J. Med. Entomol. 37: 53–57.

Jeyaprakash, A., and M. A. Hoy. 2000. Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. Insect Mol. Biol. 9: 393–405.

Kambhampati, S., K. S. Rai, and S. J. Burgun. 1993. Unidirectional cytoplasmic incompatibility in the mosquito, *Aedes albopictus.* Evolution 47: 673–677.

Laven, H. 1951. Crossing experiments with *Culex* strains. Evolution 5: 370–375.

O'Neill, S. L., A. A. Hoffmann, and J. H. Werren. 1997a. Influential passengers: inherited microorganisms and arthropod reproduction. Oxford University Press, Oxford.

O'Neill, S. L., M. Pettigrew, S. P. Sinkins, H. R. Braig, T. G. Andreadis, and R. B. Tesh. 1997b. *In vitro* cultivation of *Wolbachia pipientis* in an *Aedes albopictus* cell line. Insect Mol. Biol. 6: 33–39.

Otsuka, Y., and H. Takaoka. 1997. Elimination of *Wolbachia pipientis* from *Aedes albopictus.* Med. Entomol. Zool. 48: 257–260.

Pijls, J.W.A.M., H. J. van Steenbergen, and J.J.M. van Alphen. 1996. Asexuality cured: The relations and differences between sexual and asexual *Apoanagyrus diversicornis.* Heredity 76: 506–513.

Pintureau, B., L. Chapelle, and B. Delobel. 1999. Effects of repeated thermic and antibiotic treatments on a *Trichogramma* (Hym., Trichogrammatidae) symbiont. J. Appl. Entomol. 123: 473–483.

Portaro, J. K., and R. A. Barr. 1975. "Curing" *Wolbachia* infections in *Culex pipiens.* J. Med. Entomol. 12: 265.

Presgraves, D. C. 2000. A genetic test of the mechanism of *Wolbachia*-induced cytoplasmic incompatibility in *Drosophila.* Genetics 154: 771.

Richardson, P. M., W. P. Holmes, and G. B. Saul II. 1987. The effect of tetracycline on non-reciprocal cross incompatibility in *Mormoniella vitripennis (Nasonia vitripennis).* J. Invertebr. Pathol. 50: 176–183.

Rokas, A. 2000. *Wolbachia* as a speciation agent. Trends Ecol. Evol. 15: 44–45.

Sinkins, S. P., H. R. Braig, and S. L. O'Neill. 1995. Wolbachia superinfections and the expression of cytoplasmic incompatibility. Proc. R. Soc. Lond. [Biol.] 261: 325–330.

Sinkins, S. P., and S. L. O'Neill. 2000. *Wolbachia* as a vehicle to modify insect populations, pp. 271–287. *In* A. M. Handler and A. A. James [eds.], Insect transgenesis: methods and applications. CRC, Boca Raton, FL.

Stolk, C., and R. Stouthamer. 1996. Influence of a cytoplasmic incompatibility-inducing *Wolbachia* on the fitness of the parasitoid wasp *Nasonia vitripennis.* Proc. Exp. Appl. Entomol. N E V Amst. 7: 33–37.

Stouthamer, R., R. F. Luck, and W. D. Hamilton. 1990. Antibiotics cause parthenogenic *Trichogramma* (Hymenoptera: Trichogrammatidae) to revert to sex. Proc. Natl. Acad. Sci. USA 87: 2424–2427.

Suenaga, O. 1992. Treatment of *Wolbachia pipientis* infection with tetracycline hydrochloride and the change of cytoplasmic incompatibility in a strain of *Culex pipiens quinquefasciatus* from Bangkok Thailand. Trop. Med. 34: 15–19.

Suenaga, O. 1993. Treatment of *Wolbachia pipientis* infection with tetracycline hydrochloride and the change of cytoplasmic incompatibility in a Nagasaki strain of *Culex pipiens molestus.* Trop. Med. 35: 105–110.

Taylor, M., J. C. Bandi, A. M. Hoerauf, and J. Lazdins. 2000. *Wolbachia* bacteria of filarial nematodes: a target for control? Parasitol. Today 16: 179–80.

Trpis, M., J. B. Perrone, M. Reisseg, and K. L. Parker. 1981. Control of cytoplasmic incompatibility in the *Aedes scutellaris* complex. J. Hered. 72:313- 317.

Wade, M. J. 2001. Infectious speciation. Nature (Lond.) 409: 675–677.

Werren, J. H. 1997. Biology of *Wolbachia.* Annu. Rev. Entomol. 42: 587–609.

Werren, J. H., D. Windsor, and L. R. Guo. 1995. Distribution of *Wolbachia* among neotropical arthropods. Proc. R. Soc. Lond. [Biol.] 262: 197–204.

Werren, J. H., and D. M. Windsor. 2000. *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? Proc. R. Soc. Lond. [Biol.] 267: 1277–1285.

Wright, J. D., and B. T. Wang. 1980. Observations on Wolbachiae in mosquitoes. J. Invertebr. Pathol. 35: 200–208.

Wright, J. W., and R. Pal. 1967. Genetics of insect vectors of disease. Elsevier, New York.

Yen, J. H., and A. R. Barr. 1971. New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens* L. Nature 232: 657–658.

Yen, J. H., and A. R. Barr. 1973. The etiological agent of cytoplasmic incompatibility in *Culex pipiens.* J. Invertebr. Pathol. 22: 242–250.

Zchori Fein, E., Y. Gottlieb, and M. Coll. 2000. *Wolbachia* density and host fitness components in *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). J. Invertebr. Pathol. 75: 267–272.

Received for publication 4 January 2001; accepted 19 June 2001.