Azufrado Canyon headwaters (Palestina fault zone). The missing 26 km^3 is interpreted to represent the cumulative volume of multiple, large rockslide avalanches of hydrothermally altered materials. On the eastern flank, the Lagunillas River canyon contains a similar feature 2.5 km long, 1 km wide, and 200 to 400 m deep. This depression also cuts into highly altered materials but does not show the obvious tectonic control exhibited on the north and northwest slopes. The processes that have been modeled may be partially responsible for recent sector collapse and eruptions at Ruiz.

The model proposed may explain the excess travel distance of volcanic debris avalanches (*28*). For modern examples (*29*) and many ancient deposits (*2*, *25*), the ratio of vertical drop (*H*) to travel length (*L*) for volcanic debris avalanches is much lower than the ratio for nonvolcanic deposits of similar volume. Edifice failure, related to the presence of extensive alteration materials, especially clays, along low-angle detachment planes and accompanied by the boiling of supercritical hydrothermal fluids is predicted to produce low-rigidity, perhaps partially fluidized (*29*) avalanches capable of traveling great distances.

Many ore deposits associated with acid sulfate alteration show features that suggest that rapid unroofing of the hydrothermal systems may have played an important role in ore formation (*30*). The presence of healed veins of calcite and anhydrite in ore deposits such as that in Lihir Island (Papua New Guinea) (*31*) corresponds to the brecciation and later deposition caused by the sudden decompression of the hydrothermal system. A large sector collapse apparently occurred so recently at Louise caldera on Lihir Island (*32*) that the debris avalanche deposit is preserved in this hydrothermal system, which is still depositing gold (*32*).

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33. We thank our colleagues, especially J. S. Hanor, M. L. Calvache, N. Pérez, and S. J. Schaefer. Supported by grants from NSF, Fondo Colombiano de Investigaciones Cientificas y Proyectos Especiales (COLCIENCIAS), and the Fulbright Association (S.N.W.) and from the Organization of American States and the American Association of University Women (D.L.L.). S. Selkirk and J. Bahamonde facilitated manuscript preparation.

17 December 1992; accepted 22 April 1993

Interspecific and Intraspecific Horizontal Transfer of *Wolbachia* in *Drosophila*

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Cytoplasmic incompatibility (CI) in *Drosophila simulans* is related to infection of the germ line by a rickettsial endosymbiont (genus *Wolbachia*). *Wolbachia* were transferred by microinjection of egg cytoplasm into uninfected eggs of both *D. simulans* and *D. melanogaster* to generate infected populations. Transinfected strains of *D. melanogaster* with lower densities of *Wolbachia* than the naturally infected *D. simulans* strain did not express high levels of CI. However, transinfected *D. melanogaster* egg cytoplasm, transferred back into *D. simulans*, generated infected populations that expressed CI at levels near those of the naturally infected strain. A transinfected *D. melanogaster* line selected for increased levels of CI expression also displayed increased symbiont densities. These data suggest that a threshold level of infection is required for normal expression of CI and that host factors help determine the density of the symbiont in the host.

Cytoplasmic incompatibility is a developmental defect described in a number of insect species (*1*). It is expressed when males that carry the bacterial endosymbiont *Wolbachia* are mated to females that lack the infection. Such a cross in *Drosophila* produces few, if any, viable offspring (*2*). However, the reciprocal cross—uninfected males mated to infected females—yields normal progeny counts, as do crosses within a given strain of infected individuals.

The nonreciprocal nature of CI indicates that the male's contribution is critical to CI expression. However, *Wolbachia* are not present in mature sperm; paternal transmission occurs only rarely, if at all (*3*). The microorganism in the adult male somehow renders the sperm incapable of successfully completing fertilization after entry into an uninfected egg cytoplasm (*4*, *5*). Compatible crosses involving infected females produce normal progeny counts regardless of the infection state of the male. Therefore, eggs derived from infected females somehow negate or rescue the action of *Wolbachia* on sperm.

Studies of CI have been hampered by the difficulty in culturing the symbiont outside of its host. To gain insight into the cellular and molecular basis of CI, we transinfected *Wolbachia* into uninfected *D. simulans* and *D. melanogaster* hosts and monitored their cellular distributions and CI expression with confocal microscopy and

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the polymerase chain reaction (PCR) using *Wolbachia*-specific primers (6).

Large quantities of *Wolbachia* in the egg and embryo (4) suggested that egg cytoplasm would provide a rich source of the bacteria for microinjection experiments and for biochemical isolation of the symbiont. We first attempted intraspecific transfer between the naturally infected *D. simulans* strain and a *D. simulans* strain that had been treated with tetracycline to remove the bacteria (7). The tetracycline-treated flies were shown to be free of microbial infection by PCR amplification of DNA from ovary tissue and by 4,6-diamino-2-phenylindole (DAPI) fluorescent examination of the eggs as described (4, 6). Injected eggs were allowed to hatch, and individual females were used to establish stock lines. The second generation was tested for the presence of *Wolbachia* by DAPI fluorescence and PCR. Additional generations were reared and assayed to ensure that the infection was stable. PCR assays of ovary tissue confirmed that stable infections had been established (Fig. 1A). Each infected line was then screened for the expression of CI in test crosses (Table 1) (8). Identical experiments were performed in interspecific transfer experiments with *D. melanogaster* eggs serving as hosts for infected *D. simulans* egg cytoplasm.

One transinfected *D. simulans* line (DSR/DSRT; Table 1) displayed high percentages of unidirectional CI compared to uninfected females in test crosses (Table 1, cross a; 74% egg mortality), although at lower percentages than control incompatible crosses (Table 1, cross i; 97% egg mortality). However, by generation 9 the level of expression of CI had increased to 87% egg mortality in incompatible test crosses (Table 1, cross e).

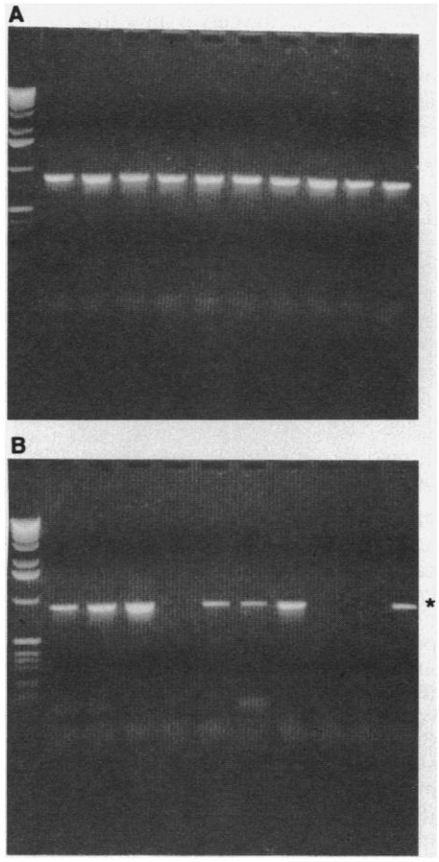

Fig. 1. Polymerase chain reaction analysis of *D. simulans* and transinfected *D. melanogaster*. (**A**) *Drosophila simulans*, transinfected strain, DSR*. All ten individuals assayed were positive. (**B**) Transinfected line DM*7. Each lane shown represents PCR products from single female ovaries selected at random from each stock. Primers specific for the *Wolbachia* 16*S* ribosomal DNA (*6*) were used in PCR to amplify DNA. The asterisk points to the PCR product obtained. Insect ovary tissue was dissected into 50 μl of STE [100 mM NaCl, 10 mM tris-HCl (pH 8.0), and 1 mM EDTA (pH 8.0)], homogenized, and incubated with 2 μl of proteinase K (10 mg/ml) for 30 min at 37°C followed by incubation for 5 min at 95°C. Samples were briefly centrifuged in a microcentrifuge and 1 μl of the supernatant was used as the template in subsequent PCR reactions. Polymerase chain reaction conditions included 2.5 mM $MgCl_2$, all four deoxynucleotide triphosphates (each at 200 μM), and 400 nM primer. A temperature profile of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min was used for 35 cycles. Appropriate controls with the use of naturally uninfected or tetracycline-treated lines showed that PCR amplification occurred only in lines that were known to be carrying the symbiont.

Table 1. Cytoplasmic incompatibility cross results for the transinfected lines of *D. simulans* and *D. melanogaster*. Cytoplasmic incompatibility is reported as the percentage egg mortality ± SE. The ratio of eggs to females uses mean numbers; the number of females only is in parentheses. Abbreviations: DSR, *D. simulans*, Riverside strain, naturally infected; DSRT, *D. simulans*, Riverside strain treated with tetracycline; DSRT*, transinfected tetracycline-treated *D. simulans*; DM, *D. melanogaster*, naturally uninfected; DM*/DSRT*, transinfected *D. melanogaster* and *D. simulans* (Riverside) strains, respectively.

Donor/host strain	Eggs injected (*n*)	Infected isofemale lines (*n*)	Generation tested (post-infection)	Cross (female × male)	Eggs/ females	Mean egg mortality (%)	Comparison (*P*)
DSR/DSRT	34	1	2	a: DSRT × DSRT*	44 (9)	74.1 ± 11.4	a versus b (<0.001)
				b: DSRT × DSRT	40 (10)	11.1 ± 3.28	a versus i (0.049)
			4	c: DSRT × DSRT*	38 (8)	78 ± 8.0	c versus d (0.001)
				d: DSRT × DSRT	35 (9)	25.4 ± 10.7	c versus i (0.02)
			9	e: DSRT × DSRT*	26 (9)	86.7 ± 3.8	e versus f (<0.001)
				f: DSRT × DSRT	21 (9)	26.9 ± 5.4	e versus i (0.02)
DM*7/DSRT	28	1	4	g: DSRT × DSRT*	54 (10)	75.3 ± 7.7	g versus h (0.001)
				h: DSRT × DSRT	50 (10)	18.3 ± 8.1	h versus i (0.02)
				i: DSRT × DSR	33 (10)	97.3 ± 2.0	g versus i (0.02)
DSR/DM	34	3	2	j: DM × DM*7	64 (10)	26.4 ± 9.3	j versus m (0.08)
				k: DM × DM*8	37 (10)	17.8 ± 4.8	k versus m (0.11)
				l: DM × DM*9	63 (10)	15.3 ± 9.5	l versus m (0.5)
				m: DM × DM	39 (10)	8.5 ± 2.7	
			3†	n: DM × DM*7	51 (15)	35 ± 4.4	n versus q (0.005)
			3‡	n′: DM × DM*7	47 (6)	15 ± 4.2	n′ versus q (0.027)
			4	o: DM × DM*7	72 (10)	21.7 ± 11.3	o versus q (0.28)
				p: DM × DM*8	60 (9)	6.9 ± 3.1	p versus q (0.22)
				q: DM × DM	55 (4)	1.0 ± 0.60	

†PCR-positive males. ‡PCR-negative males.

Three *D. melanogaster* lines (DM*7, DM*8, and DM*9) were established by the interspecific transfer of *D. simulans* cytoplasm into *D. melanogaster* eggs (Table 1). The state of infection was monitored by PCR; an example for line DM*7 is shown (Fig. 1B) in which seven out of ten (70%) individuals assayed by PCR were scored positive (PCR-positive). These lines were tested for CI in generation 2 (Table 1, crosses j, k, and l). All three of the lines had elevated levels of egg mortality when compared to control crosses (Table 1). Because 30% of the individuals in line DM*7 were uninfected (Fig. 1B), we therefore retested line DM*7 for CI and assayed males for the presence of *Wolbachia*. This allowed the removal of *Wolbachia*-negative males from the analysis. Of the 25 males tested, 18 were PCR-positive and produced egg mortality rates of 35% (Table 1, cross n), whereas matings that involved the remaining seven PCR-negative males produced reduced egg mortality (Table 1, 15%, cross n′). These results indicate that a significant degree of heterogeneity exists in the levels of infection in these transinfected lines. Individuals in which *Wolbachia* can be detected by PCR analysis are capable of generating the CI phenotype but at a much lower level than in the original host, *D. simulans*. By generation 4, line DM*8 showed a decrease in egg mortality to a level near that of the wild type (Table 1, cross p). We subsequently retested these lines at generation 8 with PCR and DNA fluorescence and found that both lines DM*8 and DM*9 had lower levels of infection. Eventually, line DM*8 lost the infection completely (9). However, line DM*7 appeared to maintain CI levels similar to those measured in the previous generations.

The low, but statistically significant, levels of CI in these transinfected lines are similar to those previously determined for naturally infected *D. melanogaster* taken from the field (*10*) and could indicate that (i) host factors function in the expression of the CI phenotype or (ii) a threshold level of infection is needed for the expression of CI. These two possibilities are not mutually exclusive. Host factors in *D. melanogaster* may serve to increase the threshold level in this organism.

To investigate these possibilities and the nature of the polymorphism observed in our transinfected lines, we measured symbiont levels in infected eggs using a quantitative confocal microscopy assay (Fig. 2). This assay was also useful in determining the subcellular localization of the symbiont. More than 90% of the *Wolbachia* were distributed within the cortical regions, 5 to 10 μm from the egg surface. The remaining 10% were found in the interior of the egg, mostly surrounding nuclei; some were also seen free in the cytoplasm (*11*). An example of the localization of bacteria in the egg cortex is shown (Fig. 2A), as well as the entire distribution for the naturally infected strain and two transinfected lines (Fig. 2B). Comparisons of the curves show that (i) the distribution of the symbiont was similar in all three strains and (ii) each strain had different numbers of the symbiont (best seen in a comparison of peak heights). A more accurate quantitative measure of symbiont densities is given (Fig. 2C) in which average numbers of *Wolbachia* in a defined volume element are plotted (Fig. 2). The correlation between symbiont levels in the egg and the degree of CI expression is also shown (Fig. 2C).

In addition to a lower overall density of bacteria in transinfected lines, confocal observations of transinfected *D. melanogaster*

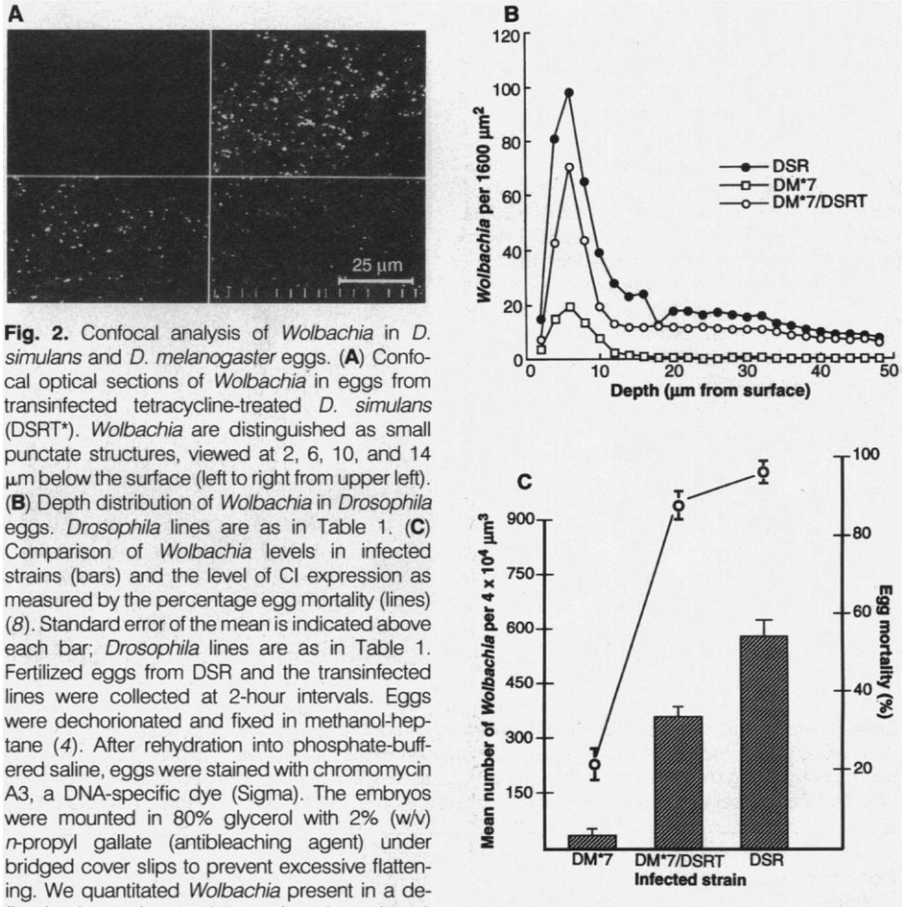

Fig. 2. Confocal analysis of *Wolbachia* in *D. simulans* and *D. melanogaster* eggs. (**A**) Confocal optical sections of *Wolbachia* in eggs from transinfected tetracycline-treated *D. simulans* (DSRT*). *Wolbachia* are distinguished as small punctate structures, viewed at 2, 6, 10, and 14 μm below the surface (left to right from upper left). (**B**) Depth distribution of *Wolbachia* in *Drosophila* eggs. *Drosophila* lines are as in Table 1. (**C**) Comparison of *Wolbachia* levels in infected strains (bars) and the level of CI expression as measured by the percentage egg mortality (lines) (*8*). Standard error of the mean is indicated above each bar; *Drosophila* lines are as in Table 1. Fertilized eggs from DSR and the transinfected lines were collected at 2-hour intervals. Eggs were dechorionated and fixed in methanol-heptane (*4*). After rehydration into phosphate-buffered saline, eggs were stained with chromomycin A3, a DNA-specific dye (Sigma). The embryos were mounted in 80% glycerol with 2% (w/v) *n*-propyl gallate (antibleaching agent) under bridged cover slips to prevent excessive flattening. We quantitated *Wolbachia* present in a defined volume element (40 μm by 40 μm by 48 μm) by collecting *z* axis images at 2-μm intervals; these were stored in a Zeiss LSM (laser scanning microscope) computer, and the number of bacteria from each depth was tabulated. Optical sections were collected with a Zeiss LSM microscope using a ×40 oil objective, with a numerical aperture of 1.0. At least 50 eggs were counted for each line. Data were then plotted as a function of depth beneath the egg surface (A) or the total number of bacteria was plotted as a bar graph (C). The level of CI expression for each line was determined as described in Table 1 (*8*) and plotted as a line graph in (C).

Table 2. Results of the CI selection assay. The parental strain was DM*7 as reported in Table 1. Isofemale lines were established and male progeny tested (*15*). Cytoplasmic incompatibility is reported as mean percentage egg mortality.

Assay generation	Isofemale lines crossed (*n*)	Eggs counted (*n*)	Mean % egg mortality (SE)	Range % egg mortality
1	52	44,381	18 (1.4)	2 to 44
2	40	18,497	34 (2.9)	1 to 61
3	37	15,409	28 (2.1)	7 to 72
4	54	15,696	41 (3.7)	0.7 to 92
5	42	11,932	49 (3.7)	6 to 99
6	17	4,067	83 (3.2)	57 to 100

eggs revealed that a large percentage of the eggs were devoid of *Wolbachia* (*12*). This heterogeneity may reflect a similar heterogeneity in ovarial stem cells that are either uninfected or have amounts of *Wolbachia* too small for proper transmission into the oocyte. Low bacterial densities in these cells would in turn produce a proportion of uninfected cystoblasts as a result of a stochastic loss of bacteria during mitosis. The production of uninfected gamete cells in the female is likely to be paralleled in males, which raises the possibility that a proportion of the males in this population produce spermatids that contain amounts of *Wolbachia* insufficient to elicit CI. Therefore, both density and segregation phenomena act to produce low levels of CI expression in these transinfected *D. melanogaster* lines (*13*).

That host factors in *D. melanogaster* can influence the level of bacterial infection is supported by our observations that cytoplasm taken from the transinfected strains of *D. melanogaster* could be used to successfully reinfect an uninfected strain of *D. simulans* (Table 1, DM*7/DSRT). The expression of CI in this strain equaled the amounts seen in the DSR/DSRT transinfection experiments (for example, cross a compared with cross g, Table 1). *Wolbachia* levels in this line are also significantly increased compared to the transinfected *D. melanogaster* line (Fig. 2, B and C), as expected if host factors influence symbiont density and expression of CI.

To investigate the influence of host factors in our *D. melanogaster* lines, we developed a kinship selection scheme originally designed to select for increased levels of CI in mosquitoes (*14*). Isofemale lines were reared and male progeny tested in single pair matings to uninfected females (*15*). Isofemale lines that displayed increased levels of CI expression were then used to generate new isofemale lines, and the CI crosses were repeated. The results of this selection strategy are shown (Table 2). Each successive generation except generation 3 reached a higher mean level of CI expression; by generation 6, the mean CI level was >80%. These results suggest that we have selected for those individual females with increased levels of *Wolbachia* or for natural variations in the genetic background of the host that allowed the symbiont to expand in numbers, or both (*16*).

Recent studies of the classification and phylogeny of microorganisms from a diverse range of host insect species that display CI have shown that they are all closely related (*6*, *17*), and microbial phylogeny does not appear to correlate with CI crossing type (*18*). Therefore, the divergence of *Wolbachia* may not fully explain differences in the expression of the CI phenotypes observed between species and populations. This variability may instead reflect differences in host-symbiont interactions specific for each species.

The importance of host-symbiont interactions is highlighted by our observations that the transfer of the microorganism elicits different levels of CI in *D. simulans* and *D. melanogaster* hosts. The observation that *Wolbachia* can be grown in an alternative host (*D. melanogaster*) and then reintroduced back into its original host with a concomitant recovery of the characteristic CI phenotype provides strong evidence that *Wolbachia* are the etiologic agents of incompatibility and lends credence to previous studies that relied on indirect antibiotic treatments to demonstrate *Wolbachia* involvement in CI. Our studies also provide the incentive to perform further interspecific transinfection experiments with cytoplasms from other, more distantly related species known to display CI. The study of CI in *D. simulans* and *D. melanogaster* promises to provide new insights into the cellular mechanisms of host-symbiont interactions in insects, and perhaps other symbiotic systems, and to further our understanding of host factors that affect the expression of CI.

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7. Transinfected lines were generated by microinjection of egg cytoplasm. Basic procedures of egg collection, manipulation, and microinjections were those described by Ashburner [M. Ashburner, Drosophila, *A Laboratory Manual* (Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989), pp. 228–229]. Embryos from a naturally infected strain of *D. simulans* taken from Riverside, CA and designated DSR (*2*) were collected hourly and dechorionated by hand. Dechorionated embryos were lined up on rubber cement–coated cover slips and covered with mineral oil. Only eggs younger than nuclear cycle 9 were selected for transfer. We removed cytoplasm from eggs by inserting into the posterior end a finely pulled glass needle that had previously been filled with sterile Ringer solution to facilitate cytoplasmic uptake and prevent clogging. Cytoplasm was removed only from the most posterior end of the embryo and injected into similarly prepared *D. simulans* (previously treated to remove bacteria) (*4*) designated DSRT or *D. melanogaster* (naturally uninfected) fertilized eggs. Injections were into the posterior end only (estimated injection volume was less than 5% of total egg volume). After injection, cover slips that contained injected eggs were placed on grape juice agar plates and incubated in a humid chamber for 24 hours. We removed hatched larvae to a cornmeal-molasses food vial after gently removing the oil. Eclosed flies were sexed, females placed into a cornmeal vial with males, and stocks established. The infection status of the lines was monitored approximately every other generation by PCR assay and DAPI staining (*4*, *6*).
8. Transinfected lines were tested for CI as follows. Ten food vials that contained one virgin female and two males were set up as listed in Table 1. After 1 to 2 days, flies were transferred into bottles fitted with end caps containing grape juice agar. After 24 hours, the caps were removed and the eggs counted. Unhatched eggs were then counted 36 hours later. Cytoplasmic incompatability expression levels are listed in Table 1 as the percentage egg mortality—that is, the ratio of unhatched to total eggs. Control crosses were simultaneously performed as listed in Table 1 and compared to CI crosses. Standard Student's *t* test analyses were performed (Table 1).
9. By generation 8, *Wolbachia* were undetectable in line DM*8 as monitored by PCR and DAPI fluorescence (L. Boyle, S. L. O'Neill, H. M. Robertson, T. L. Karr, data not shown).
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11. The distribution of *Wolbachia* during the early cleavage stages of development was examined with the use of monoclonal antibodies to *Wolbachia* and visualized with a confocal microscope (T. L. Karr and H. Kose, unpublished results).
12. *Wolbachia* levels in 50 eggs were determined from the transinfected strains and compared to levels in the naturally infected *D. simulans* strain. These measurements revealed that approximately 40% of the *D. melanogaster* strains were devoid of the symbiont compared to <2% in the naturally infected *D. simulans* strain (L. Boyle and T. L. Karr, unpublished data).
13. A similar correlation between bacterial density and the expression of CI during recovery of infection after antibiotic treatment has been observed in the parasitic wasp *Nasonia vitripennis* (J. A. J. Breeuwer and J. Werren, *Genetics*, in press).
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15. Screens to isolate lines of *D. melanogaster* that displayed elevated levels of CI expression were performed as follows: Starting from a partially incompatible population (strain DM*7), isofemale lines were established in individual food vials. Male progeny was then tested for the level of CI expression in a standard CI cross. Ten single pair matings were set up between a male offspring and a virgin uninfected female. Flies were removed and egg counts recorded 36 to 48 hours later. A final egg count was made 36 to 48 hours later and recorded. The ratio of hatched-to-unhatched eggs was recorded, and the percentage egg mortality was calculated. Lines that displayed the highest levels of CI were established, and another round of selection ensued.
16. In recent experiments, bacterial densities in eggs from the selected lines were measured at generations 4 and 6 with the DAPI fluorescence assay. More than 95% of eggs contained *Wolbachia* in amounts greater than those found in the parental line (L. Boyle and T. L. Karr, unpublished results).
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19. Supported by U.S. Department of Agriculture research grant AG 91-37302-6766 and in part by NSF research grant DMB 88-18889. T.L.K. is especially grateful to the Beckman Institute for Advanced Science and Technology for monetary and facility support.

4 December 1992; accepted 9 March 1993