APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2005, p. 3199–3204 Vol. 71, No. 6
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.6.3199–3204.2005

Characterization of *Wolbachia* Transfection Efficiency by Using Microinjection of Embryonic Cytoplasm and Embryo Homogenate

Zhiyong Xi and Stephen L. Dobson*

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

Received 27 September 2004/Accepted 29 December 2004

***Wolbachia* spp. are intracellular alpha proteobacteria closely related to *Rickettsia*. The maternally inherited infections occur in a wide range of invertebrates, causing several reproductive abnormalities, including cytoplasmic incompatibility. The artificial transfer of *Wolbachia* between hosts (transfection) is used both for basic research examining the *Wolbachia*-host interaction and for applied strategies that use *Wolbachia* infections to affect harmful insect populations. Commonly employed transfection techniques use embryonic microinjection to transfer *Wolbachia*-infected embryo cytoplasm or embryo homogenate. Although microinjections of both embryonic cytoplasm and homogenate have been used successfully, their respective transfection efficiencies (rates of establishing stable germ line infections) have not been directly compared. Transfection efficiency may be affected by variation in *Wolbachia* quantity or quality within the donor embryos and/or the buffer types used in embryo homogenization. Here we have compared *Wolbachia* bacteria that originate from different embryonic regions for their competencies in establishing stable germ line infections. The following three buffers were compared for their abilities to maintain an appropriate in vitro environment for *Wolbachia* during homogenization and injection: phosphate-buffered saline, *Drosophila* Ringer's buffer, and a sucrose-phosphate-glutamate solution (SPG buffer). The results demonstrate that *Wolbachia* bacteria from both anterior and posterior embryo cytoplasms are competent for establishing infection, although differing survivorships of injected hosts were observed. Buffer comparison shows that embryos homogenized in SPG buffer yielded the highest transfection success. No difference was observed in transfection efficiencies when the posterior cytoplasm transfer and SPG-homogenized embryo techniques were compared. We discuss the results in relation to intra- and interspecific *Wolbachia* transfection and the future adaptation of the microinjection technique for additional insects.**

Maternally transmitted *Wolbachia* spp. are within the alpha proteobacteria and widely infect invertebrates including nematodes, mites, spiders, and an estimated >20% of insect species (19, 20). *Wolbachia* infections induce a number of reproductive abnormalities, including cytoplasmic incompatibility (CI), parthenogenesis, feminization, and male killing. With the CI phenotype, *Wolbachia* disrupts the coordination of host pronuclei, resulting in karyogamy failure and embryo mortality (13).

The ability of *Wolbachia* to induce CI in its host has led to the proposal of strategies for controlling harmful insect populations. The strategies, including population replacement and population suppression (5), require the ability to generate novel *Wolbachia*-host associations via *Wolbachia* transfer (transfection). Transfection methods have also been used to facilitate studies of the *Wolbachia*-host interaction. For example, the interspecific transfer of *Wolbachia* between *Drosophila simulans* and *Drosophila melanogaster* has been used to demonstrate host effects on *Wolbachia* infection density and CI (3, 16).

Wolbachia transfection techniques have been developed for multiple insects including members of the orders Diptera, Lepidoptera, and Homoptera (3, 4, 9, 15–17). The methods used in the prior studies include the direct transfer of *Wolbachia*-infected embryonic cytoplasm and the transfer of infected embryo homogenate. With both techniques, the infected tissue is microinjected into the posterior end of early embryos, with a goal of infecting embryonic pole cells that will develop into germ tissues. Pole-cell infection is targeted, since this will develop into germ tissue and *Wolbachia* is maternally inherited. Thus, stable infection is thought to require that *Wolbachia* be established in the germ tissue that will develop into ovaries.

Although the transfer of embryonic cytoplasm is the most direct route for transfection, the use of homogenized embryos can be required for technical reasons including the physiology of donor embryos (4). For example, purification of *Wolbachia* following embryo homogenization can reduce complications associated with the microinjection of molecules and organelles from donor tissue that are detrimental to a distantly related recipient host. The use of homogenized tissue can allow the simultaneous transfer of multiple *Wolbachia* types by combining different insect tissues. *Wolbachia* enrichment from embryo homogenate can be used to facilitate transfection from small or weakly infected donor insects. Although microinjection of homogenized embryos can be technically advantageous and allows a broader application of *Wolbachia* transfection to include additional insect systems, its use has been limited in comparison with that of the cytoplasm transfer technique. Furthermore, there has not been a direct comparison of the two

* Corresponding author. Mailing address: Department of Entomology, University of Kentucky, S-225 Ag. Science Center North, Lexington, KY 40546. Phone: (859) 257-4902. Fax: (859) 323-1120. E-mail: sdobson@uky.edu.

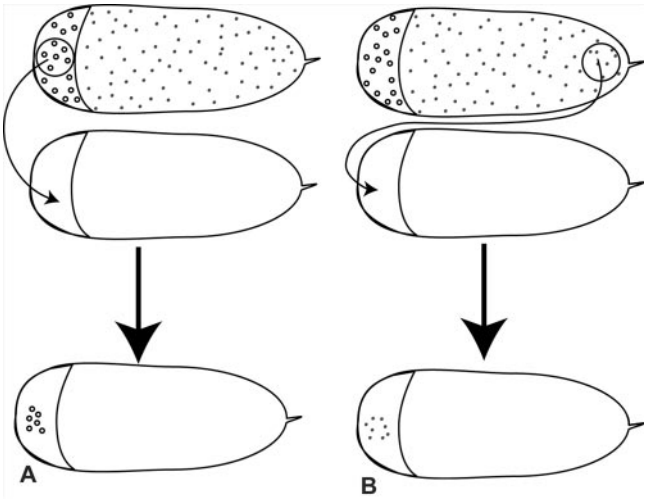

FIG. 1. *Wolbachia* embryonic transfer experiments with different cytoplasms. *Wolbachia* bacteria from the posterior (A) and anterior (B) of donor embryos are injected to the posterior ends of recipient embryos.

techniques for their transfection efficiencies (defined as the rates of establishing stable, maternally inherited *Wolbachia* infections).

A difference in transfection efficiency between the cytoplasm transfer and embryo homogenate transfer techniques can result from qualitative differences in the *Wolbachia* infections within the donor tissue. For example, all *Wolbachia* bacteria within an embryo may not be equally competent to establish germ line infection. A competency difference may result due to biological differences in *Wolbachia* or host factors. Injected polar plasm can form pole cells in the recipient embryos (8, 12, 14). An inability of *Wolbachia* to invade embryonic pole cells would limit transfection success to recipient embryos that were injected prior to pole-cell formation. In contrast, the injection of *Wolbachia* contained within pole plasm could subsequently form infected pole cells in the recipient that are derived from donor tissue. For *Wolbachia* in embryo homogenate, the buffer that provides the in vitro environment might play an important role in the maintenance of its infectivity, as has been found for *Rickettsia* (2).

Here we have compared the transfection efficiencies of *Wolbachia* bacteria originating from posterior and anterior cytoplasms. No difference was observed in the transfection success rates. Three buffers were compared by using homogenate injections. The results demonstrate an important effect of buffer type on transfection efficiency. Use of a sucrose-phosphate-glutamate solution (SPG buffer) resulted in a transfection success rate similar to that obtained using direct transfer of cytoplasm.

MATERIALS AND METHODS

***Drosophila* strains and embryo collection.** *Wolbachia*-infected *Drosophila simulans* Riverside (DSR) and the aposymbiotic DSRT strain were used in transfection studies (3). Flies were maintained at 25°C using standard *Drosophila* rearing conditions (1). For injection experiments, early embryos of DSR (donor) and DSRT (recipient) were collected every 30 min using agar plates with yeast paste. Following a water rinse, embryos were dechorionated in 50% bleach for 2 min. Dechorionated embryos were rinsed with water, aligned on an agar plate, and transferred to a glass slide with double-stick tape (Scotch, Model 666, St. Paul, MN). Recipient DSRT embryos were partially desiccated (18) and then covered with water-saturated Halocarbon oil 700 (Sigma-Aldrich Co., St. Louis, MO). Donor DSR embryos were not desiccated prior to covering with oil.

Embryo homogenization. Three buffers were used for embryo homogenization: phosphate-buffered saline (PBS) (130 mM NaCl, 7 mM $Na_2HPO_4 \cdot 2H_2O$, 3 mM $NaHPO_4 \cdot 2H_2O$, pH 7.0), *Drosophila* Ringer's buffer (182 mM KCl, 46

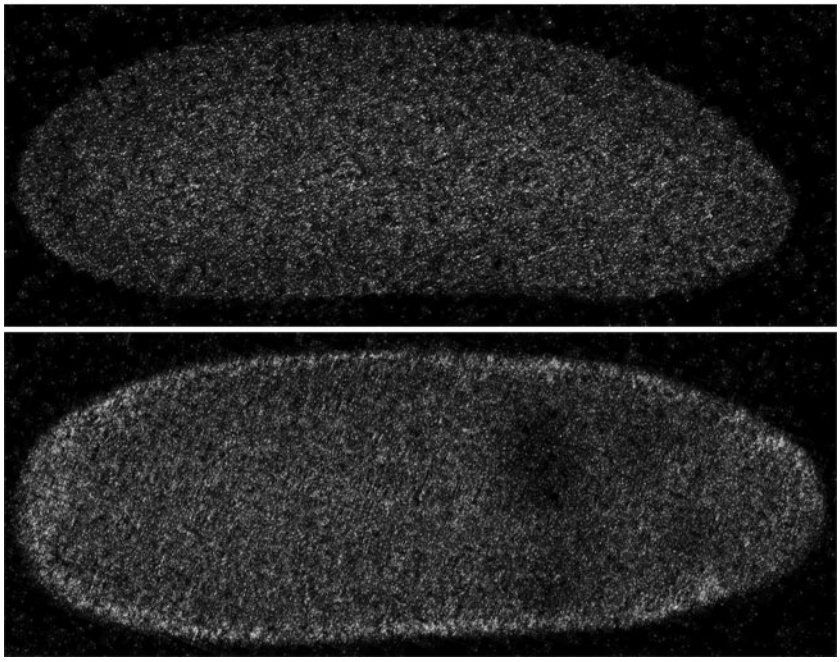

FIG. 2. Cortical distribution of *Wolbachia* bacteria in the early DSR embryo that are used as donors for transfection. Top, DSRT; bottom, DSR. The posterior ends of the embryos are orientated toward the left.

TABLE 1. *Wolbachia* infection in the anterior and posterior treatments

Experiment	Treatment	% Infection frequency (no. PCR positive/no. PCR tested) for:		% Transfection efficiency[a]
		G_0 adult	G_1 isofemale line	
1	Anterior	52.9 (9/17)	0 (0/14) 0 (0/13) 0 (0/13)	0 (0/3)
	Posterior	42.9 (9/21)	100.0 (3/3) 41.7 (5/12) 27.3 (3/11) 25.0 (3/12) 0 (0/12)	80 (4/5)
2	Anterior	88.9 (8/9)	33.3 (5/15) 0 (0/13)	50 (1/2)
	Posterior	94.7 (18/19)	76.9 (10/13) 14.3 (2/14) 0 (0/14) 0 (0/12) 0 (0/11) 0 (0/6)	33.3 (2/6)
	Anterior	83.3 (20/24)	50.0 (6/12) 27.3 (3/11) 8.3 (1/12) 0 (0/14) 0 (0/12) 0 (0/12)	50.0 (3/6)
	Posterior	65.6 (21/32)	85.7 (12/14) 50.0 (6/12) 38.5 (5/13) 0 (0/12) 0 (0/12) 0 (0/12) 0 (0/11) 0 (0/11) 0 (0/11)	33.3 (3/9)

[a] Numbers of stably infected lines/numbers of infected G_0 isofemale lines are given in parentheses.

mM NaCl, 3 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.2), and SPG buffer (218 mM sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, and 4.9 mM L-glutamate, pH 7.2). The three buffers were selected because of their prior use in *Wolbachia* transfection with cell lines, *Drosophila*, and nematodes (4, 6, 10). For homogenization, approximately 30 μl of dechorionated eggs was rinsed with distilled water, transferred to a new tube, and rinsed with 0.5 ml buffer. Eggs were transferred into 1 ml fresh buffer in a Dounce tissue grinder (Fisher Scientific, Pittsburgh, PA) and briefly homogenized (~10 strokes at room temperature with the tight-fitting B-type pestle). The homogenate was transferred to a 1.5-ml tube and centrifuged at 300 × *g* for 5 min to remove large debris. The supernatant was transferred into a separate tube and centrifuged at 12,000 × *g* for 10 min to pellet the *Wolbachia* cells. The supernatant was removed, leaving a pellet in ~50 μl, which was resuspended by pipetting. Debris was cleared from the suspension by centrifuging at 300 × *g* for 3 min. The supernatant was then transferred into a clean tube at 25°C until used for injection (<5 h).

Embryonic microinjection. *Wolbachia* extract or embryo cytoplasm from *Drosophila simulans* (DSR) was microinjected into a DSRT embryo by standard techniques (3, 18). The needles (TW100F-4; World Precision Instruments, Inc., Sarasota, FL) were pulled using a micropipette puller (Model P-87; Sutter Instrument Co., Novato, CA). Injection was conducted with an IM 300 microinjector (Narishige Scientific Instrument Lab., Tokyo, Japan). For the posterior and anterior treatments, cytoplasm was withdrawn from donor embryos using the microinjector. Approximately 5% of the embryo cytoplasm was withdrawn from the posterior and anterior ends of the embryo (Fig. 1). Cytoplasm withdrawal was repeated sequentially with approximately 10 donor embryos and then immediately used to inject recipient DSRT embryos. Donor and recipient embryos were manipulated prior to pole-cell formation, which occurs ~90 min after oviposition. The injection volume was determined empirically during injections. We assumed that the ideal injection volume would "reinflate" the desiccated recipient egg but not overpressurize the egg, which would result in significant cytoplasm outflow following needle removal. There was significant variation during injections due to differences in the desiccations of recipient eggs and slight variations in needle shape. After injection, slides with aligned embryos under oil were incubated at 21°C and 100% rH. Eggs were observed frequently so that hatching larvae could be quickly transferred onto instant *Drosophila* medium (Carolina Biological Supply Co., Burlington, NC). Subsequently, flies were moved to 25°C and reared as described above. Virgin females resulting from injected embryos (generation 0 [G_0]) were isolated with four DSRT males to establish isofemale lines.

Fluorescence in situ hybridization. Embryos were dechorionated as described above and fixed in 4% formaldehyde-PBS (50:50) for 10 min. After that, embryos were rinsed six times with PBT (0.1% Tween 20 in PBS). Hybridization was conducted following the manufacturer's instructions (GeneDetect, Bradenton, FL) with the buffer containing 100 ng probes at 37°C overnight. The following two fluorescein isothiocyanate 5′-end-labeled 16S rRNA gene *Wolbachia* probes (synthesized by Sigma-Genosys Ltd., Haverhill, United Kingdom) were used: *w*Ri 603 (5′-ACCAGATAGACGCCTTCGGCC-3′) and *w*Ri 409 (5′-CTTCTG TGAGTACCGTCATTATC-3′). *w*Ri 603 was designed from 16S rRNA genes of *Wolbachia* from DSR; *w*Ri 409 was used in the previous studies (11). After hybridization, embryos were mounted on slides in Vectashield medium (Vector Laboratories, Burlingame, CA) and observed using a TCS NT confocal microscope (Leica Microsystems UK Ltd.).

PCR screening for infection status. General wsp primers (81F/691R) were used for *Wolbachia* detection as previously described (22). Following the production of G_1 pupae, G_0 females were sacrificed for use in PCR assays. G_0 females testing negative for *Wolbachia* infection were discarded along with their progeny. G_0 males were PCR assayed for *Wolbachia* infection at the same time as the females. Following eclosion and mating, approximately 12 G_1 females were isolated in media vials to establish isofemale lines. Following the production of G_2 pupae, G_1 females were assayed for *Wolbachia* infection using PCR. G_1 females that tested negative for *Wolbachia* infection were discarded along with their progeny.

Cytoplasmic incompatibility crossing assays and statistical analysis. Tests were performed at 25°C. Three virgin 5-day-old females were mated with two virgin 4-day-old males in a *Drosophila* medium vial for 2 days. Flies were then transferred into containers fitted with yeast-covered apple juice plates. After 24 h, the plates were removed. Egg hatch was assessed >36 h after oviposition. CI is calculated as the percentage of egg mortality. Statistical comparisons of infection status were conducted using chi-square or Fisher's exact tests, depending upon sample size. Kruskal-Wallis analysis was used in statistical comparisons of egg mortality levels (CI levels). All statistical comparisons were performed using SAS version 8.0 (SAS Institute, Cary, N.C.).

RESULTS

Prior studies of *Drosophila* embryos have shown a higher density of *Wolbachia* in the peripheral egg cytoplasm (3). To directly characterize *Wolbachia* infection levels in the *D. simulans* strain used here, a fluorescence in situ hybridization technique was used to visualize *Wolbachia* in embryos. Consistent with the prior reports, higher levels of *Wolbachia* infection were observed in the cortical regions, and *Wolbachia* bacteria are present in both the anterior and the posterior areas (Fig. 2).

To examine for a difference in the transfection efficiencies of posterior and anterior cytoplasms, a strategy diagramed in Fig. 1 was used. Anterior or posterior cytoplasm of infected DSR donor embryos was microinjected into the posterior of uninfected embryos (DSRT). Injected individuals surviving to adulthood (G_0 adults) were examined for *Wolbachia* infection by PCR. No difference (chi-square result, 0.751; df = 1; $P > 0.3$) was observed in the frequencies of G_0 *Wolbachia* infection in a comparison of the anterior (75.0% ± 19.4%; three experiments; Table 1) and posterior (67.4% ± 26.0%; three experiments) treatments.

TABLE 2. CI level of transfected lines (G_5)

Cross		% Egg mortality (average ± SD)	No. of eggs/cross (average ± SD)	No. of crosses
Female	Male[a]			
DSRT	Posterior	91.6 ± 3.6	102 ± 83	8
DSRT	Anterior	94.6 ± 6.2	135 ± 88	8
DSRT	Ringer's	94.1 ± 6.4	75 ± 34	7
DSRT	SPG	92.1 ± 4.0	113 ± 33	5
DSRT	DSR	86.3 ± 9.7	124 ± 55	13
DSRT	DSRT	9.6 ± 7.0	116 ± 62	11
DSR	Posterior	15.7 ± 7.8	87 ± 47	6
DSR	Anterior	14.0 ± 12.8	99 ± 49	6
DSR	Ringer's	9.5 ± 5.4	83 ± 17	5
DSR	SPG	14.1 ± 5.7	93 ± 18	6
DSR	DSR	11.2 ± 8.3	102 ± 65	12

[a] Description indicates the type of injection.

PCR detection of *Wolbachia* in G_0 females may not reflect stable transfection. For example, G_0 PCR detection could result from a somatic infection or an artifact associated with the microinjection technique and PCR detection. To determine which lines represented stable transfections, females from G_1 isofemale lines were PCR assayed. As shown in Table 1, no difference (Fisher's exact test, $P > 0.7$) in the transfection efficiency was observed between the anterior (33.3% ± 28.9%; three experiments) and posterior (48.9% ± 27.0%; three experiments) treatments. As additional confirmation of stable transfection, approximately three G_2 females were PCR tested from each isofemale line. All of the G_2 females were positive for infection. Subsequently, randomly selected lines from both the anterior and the posterior treatments have been maintained. A PCR assay at G_{19} demonstrates that the isofemale lines continue to be infected.

As confirmation that PCR-positive transfected lines represent *Wolbachia* infections capable of inducing CI, crosses of transfected lines were used to determine CI levels in the G_5 generation. The results are shown in Table 2. Low egg mortality (<16%) was observed in the compatible crosses (i.e., crosses of DSR females and the DSRT × DSRT cross). No difference in egg mortality was observed between the compatible crosses (Kruskal-Wallis; df = 5; $P > 0.2$). In contrast, more than 90% mortality resulted from crosses of uninfected DSRT females with the transfected males. The high egg mortality is similar to that observed in the incompatible cross between DSRT females and DSR males and is consistent with expectations of CI. Although the egg mortality was higher in crosses of DSRT females with transfected males relative to that in control crosses of DSRT females and DSR males, the difference was not significant (Kruskal-Wallis; df = 4; $P > 0.06$).

As shown in Table 3, similar egg hatch rates (chi-square result, 0.043; df = 1; $P > 0.8$) were observed in the anterior (28.3% ± 7.2%) and posterior (33.0% ± 4.7%) treatments. In contrast, a higher larval survivorship (chi-square result, 10.812; df = 1; $P < 0.001$) was observed in the posterior treatment (55.3% ± 6.5%) than in the anterior treatment (34.9% ± 6.6%). No difference was observed in pupal survivorships (Fisher's exact test; $P > 0.2$) or sex ratios (chi-square result, 0.344; df = 1; $P > 0.5$).

To characterize the effect of buffer type on *Wolbachia* transfection success, three buffers were compared: PBS, Ringer's, and SPG buffer. The frequencies of G_0 *Wolbachia* infection in the surviving adults were similar (chi-square result, 2.339; df = 2; $P > 0.3$) among the three buffer treatments (Table 4). However, the transfection efficiencies differed significantly between the buffer types. Specifically, the transfection efficiency with SPG is significantly higher than those with Ringer's (Fisher's exact test; $P < 0.01$) and PBS (Fisher's exact test; $P < 0.005$). Transfection efficiencies did not differ between the Ringer's and PBS buffers (Fisher's exact test; $P > 0.9$). Lower larval survivorship (chi-square result, 15.256; df = 2; $P < 0.0006$) and pupal survivorship (chi-square result, 10.513; df = 2; $P < 0.006$) were observed with the PBS buffer relative to the other buffer treatments (Table 5). The SPG and Ringer's buffer treatments did not differ in their larval survivorship (chi-square result, 0.119; df = 1; $P > 0.7$) or pupal survivorship (chi-square result, 1.016; df = 1; $P > 0.3$) rates. No effect was observed due to buffer type on egg hatch rates (chi-square result, 5.711; df = 2; $P > 0.05$) or sex ratios (chi-square result, 2.292; df = 2; $P > 0.3$).

Our results demonstrate that with the use of the SPG buffer, transfection efficiencies can be obtained that are similar to that of the posterior cytoplasm transfer technique. No difference (Fisher's exact test, $P > 0.2$) was observed in the comparison of transfection efficiencies of the posterior (48.9% ± 27.0%; described above) and SPG (69.2%; Table 4) treatments. No difference in survivorship (chi-square result, 1.866; df = 1; $P > 0.1$) was observed between the posterior (7.6%; 36 females/474 injected eggs; Table 3) and SPG (10.8%; 37 females/342 injected eggs; Table 5) treatments.

TABLE 3. Survival rates from anterior and posterior treatments

Experiment	Treatment	% Survival to:			Sex ratio (female/total)
		Hatch (larvae/eggs)	Pupation (pupae/larvae)	Eclosion (adult/pupae)	
1	Anterior	30.9 (51/165)	37.3 (19/51)	89.5 (17/19)	58.8 (10/17)
	Posterior	24.6 (48/195)	47.9 (23/48)	95.7 (22/23)	50.0 (11/22)
2	Anterior	20.2 (33/163)	27.3 (9/33)	100.0 (9/9)	55.6 (5/9)
	Posterior	33.0 (33/100)	57.6 (19/33)	100.0 (19/19)	42.1 (8/19)
3	Anterior	33.8 (68/201)	39.7 (27/68)	88.9 (24/27)	33.3 (8/24)
	Posterior	32.4 (58/179)	60.3 (35/58)	94.3 (33/35)	51.5 (17/33)
Average of three experiments ± SD	Anterior	28.3 ± 7.2	34.9 ± 6.6	92.8 ± 6.2	49.2 ± 13.9
	Posterior	33.0 ± 4.7	55.3 ± 6.5	96.7 ± 8.0	47.9 ± 5.1

TABLE 4. *Wolbachia* infection in different buffer treatments

Treatment	% Infection frequency for:		% Transfection efficiency[c]
	G_0[a]	G_1[b]	
PBS	55.8 (24/43)	0 (0/3)	0 (0/8)
		0 (0/11)	
		0 (0/6)	
		0 (0/10)	
		0 (0/13)	
		0 (0/7)	
		0 (0/14)	
		0 (0/9)	
Ringer's	67.4 (31/46)	17.6 (3/17)	10 (1/10)
		0 (0/22)	
		0 (0/19)	
		0 (0/18)	
		0 (0/3)	
		0 (0/15)	
		0 (0/18)	
		0 (0/15)	
		0 (0/13)	
		0 (0/10)	
SPG	69.4 (50/72)	100.0 (12/12)	69.2 (9/13)
		100.0 (15/15)	
		37.5 (6/16)	
		31.3 (5/16)	
		31.3 (5/16)	
		25.0 (3/12)	
		20.0 (3/15)	
		8.1 (13/16)	
		7.7 (1/13)	
		0 (0/15)	
		0 (0/15)	
		0 (0/14)	
		0 (0/16)	

[a] Numbers of PCR-positive G_0 adults/numbers of PCR-tested G_0 adults are given in parentheses.

[b] Each row represents a G_1 isofemale line established from an infected G_0 female; rows show the percent G_1 infection frequency for each isofemale line (number of PCR-positive G_1 females/number of PCR-tested G_1 females).

[c] Numbers of stably transfected lines and numbers of infected G_0 lines are shown in parentheses.

DISCUSSION

Here we show that *Wolbachia* bacteria from both anterior and posterior embryo cytoplasms are competent for establishing stable infection in *D. simulans*. The anterior and posterior treatments resulted in similar frequencies of G_0 PCR-positive individuals. G_0 PCR-positive individuals demonstrate that *Wolbachia* DNA persisted in <1-h-old embryos into adulthood, suggesting the presence of viable *Wolbachia* bacteria and not of an artifact of residual *Wolbachia* DNA. This was confirmed via PCR assays of the G_1 and subsequent generations, demonstrating vertical inheritance. Crossing test results were consistent with expectations for *Wolbachia*-induced CI in the transfected lines. No differences in CI levels were observed between the anterior, posterior, and buffer treatments.

PCR-positive G_1 individuals were consistently observed to transmit the *Wolbachia* infection to offspring. This has implications for subsequent transfection efforts. While intensive PCR screening is required in G_0 and G_1 to identify infected lines, we observed it to be unusual to lose the infection from infected G_1 lines. Thus, screening efforts may be reduced following the recognition of G_1-positive lines. In contrast, PCR-positive G_0 females frequently resulted in uninfected G_1 lines. Possible explanations could be that the G_0 positives represent a PCR artifact (residual *Wolbachia* DNA), that G_0 infection is limited to somatic tissue, or that ovaries are partially or weakly infected.

Although both anterior and posterior injections resulted in transfected lines, considerable variation was observed between replicate experiments (Table 1). A possible explanation for the variability includes variation in needle shape, which changes during injections due to small breaks. The volume of material injected also changes unpredictably due to clogging of the needle. Thus, constant adjustment is needed during sequential injections.

Comparison of anterior and posterior cytoplasm injections did not show a significant difference in *Wolbachia* transfection success (Table 1). However, interpretation is complicated by the experimental variability described in the preceding paragraph. Furthermore, the transfection efficiencies as defined in Tables 1 and 4 can be biased by the number of G_1 isofemale lines obtained. For example, some G_0 females produced fewer than seven G_1 daughters. The number of replicate experiments described here is sufficient to detect obvious differences, such as the observed effect of buffer type. While an obvious difference between the anterior and posterior treatments was not observed, additional replicates might identify more subtle effects. For example, although not statistically significant, there is a trend for higher levels of vertical inheritance between G_0 and G_1 in the posterior treatments (G_1 isofemale frequency; Table 1).

As a difference between the anterior and posterior treatments was not observed, we hypothesized that mixing anterior and posterior cytoplasms via embryo homogenization should not be detrimental to transfection efficiency. However, the preparation of embryo homogenate could reduce *Wolbachia* viability. Therefore, three homogenization buffers were examined for their effects on *Wolbachia* viability as measured by transfection efficiencies. The results demonstrate significant differences between the buffers, with SPG resulting in the highest transfection efficiency (69.2%). In contrast, embryo homogenization in PBS or Ringer's buffer leads to complete or partial loss of *Wolbachia* infectivity.

The SPG buffer used here was originally designed and optimized for *Rickettsia* (2). Thus, we hypothesized that the environment provided by SPG buffer would also be suitable to maintain *Wolbachia* in vitro because of similar metabolic properties shared by *Rickettsia* and *Wolbachia* (21). A possible reason for the success of the SPG buffer may be that glutamate is important for *Wolbachia* survival in vitro. Prior characterization of the *w*Mel *Wolbachia* genome suggests that *Wolbachia* obtains much of its energy from amino acids (21). Limited carbohydrate metabolism in *Wolbachia* suggests that the su-

TABLE 5. Survival rates with different buffer treatments

Treatment	% Survival to:			Sex ratio (female/total)
	Hatch (Larvae/eggs)	Pupation (Pupae/larvae)	Eclosion (Adult/pupae)	
PBS	32.1 (143/446)	18.8 (84/143)	51.2 (43/84)	41.9 (18/43)
Ringer's	29.9 (90/301)	22.9 (69/90)	66.7 (46/69)	34.8 (16/46)
SPG	38.3 (131/342)	30.1 (103/131)	73.8 (76/103)	48.7 (37/76)

crose in SPG may also be important to *Wolbachia* survival in vitro (21). The possibility that SPG buffer increases the infectivity of germ cells cannot be excluded.

The larval survival rate was significantly higher in the posterior treatment relative to that in the anterior treatment. This is likely due to differing cytoplasmic components. For example, bicoid and nanos occur along opposite gradients in embryos and are important in anterior and posterior development. Prior work shows that manipulation of the gradient via transfer of bicoid or nanos can corrupt normal development and result in mortality (7, 8). Thus, increased mortality in the anterior treatment may have resulted from misplaced morphogens in the embryo.

Wolbachia transfection between different invertebrate species, generating novel infection types, has been used to understand mechanisms of reproductive manipulations and other host-bacterium interactions. Furthermore, *Wolbachia* transfection is required for the applied strategies that use *Wolbachia* infections to affect populations of insect pests and disease vectors. Here we have demonstrated that *Wolbachia* bacteria from both anterior and posterior embryo cytoplasms are competent for establishing infection. Comparison also demonstrates that the SPG buffer provides an appropriate in vitro environment for *Wolbachia*, resulting in a transfection success rate comparable to that obtained by the direct transfer of infected embryonic cytoplasm. An ability to utilize homogenized embryos as a source of *Wolbachia* in transfections is expected to simplify future transfection attempts, especially with donor insects that are small or weakly infected.

ACKNOWLEDGMENTS

This work was supported by an NIH grant (NIH-AI-51533).

We thank Subba Reddy Palli for assistance with fluorescence in situ hybridization, John Webb and Lok-Sze Cecilia for the help with injection, and Yan Xie for the statistical analysis.

This is publication 04-08-181 of the University of Kentucky Agricultural Experiment Station.

REFERENCES

1. **Ashburner, M.** 1989. *Drosophila*, a laboratory handbook. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Bovarnick, M. R., J. C. Miller, and J. C. Snyder.** 1950. The influence of certain salts, amino acids, sugars, and proteins on the stability of rickettsiae. J. Bacteriol. **59:**509–522.
3. **Boyle, L., S. L. O'Neill, H. M. Robertson, and T. L. Karr.** 1993. Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. Science **260:**1796–1799.
4. **Braig, H. R., H. Guzman, R. B. Tesh, and S. L. O'Neill.** 1994. Replacement of the natural *Wolbachia* symbiont of *Drosophila simulans* with a mosquito counterpart. Nature **367:**453–455.
5. **Dobson, S. L.** 2003. Reversing *Wolbachia*-based population replacement. Trends Parasitol. **19:**128–133.
6. **Dobson, S. L., E. J. Marsland, Z. Veneti, K. Bourtzis, and S. L. O'Neill.** 2002. Characterization of *Wolbachia* host cell range via the in vitro establishment of infections. Appl. Environ. Microbiol. **68:**656–660.
7. **Driever, W., V. Siegel, and C. Nusslein-Volhard.** 1990. Autonomous determination of anterior structures in the early *Drosophila* embryo by bicoid morphogen. Development **109:**811–820.
8. **Gilbert, S. F.** 1997. Developmental biology, 5th ed. Sinauer Associates, Inc., Sunderland, Massachusetts.
9. **Giordano, R., S. L. O'Neill, and H. M. Robertson.** 1995. *Wolbachia* infections and the expression of cytoplasmic incompatibility in *Drosophila sechellia* and *D. mauritiana*. Genetics **140:**1307–1317.
10. **Hartmann, N., H. Stuckas, R. Lucius, W. Bleiss, F. Theuring, and B. H. Kalinna.** 2003. Trans-species transfer of *Wolbachia*: microinjection of *Wolbachia* from *Litomosoides sigmodontis* into *Acanthocheilonema viteae*. Parasitology **126:**503–511.
11. **Heddi, A., A. M. Grenier, C. Khatchadourian, H. Charles, and P. Nardon.** 1999. Four intracellular genomes direct weevil biology: nuclear, mitochondrial, principal endosymbiont, and *Wolbachia*. Proc. Natl. Acad. Sci. USA **96:**6814–6819.
12. **Illmensee, K., and A. P. Mahowald.** 1974. Transplantation of posterior polar plasm in *Drosophila*. Induction of germ cells at the anterior pole of the egg. Proc. Natl. Acad. Sci. USA **71:**1016–1020.
13. **Lassy, C. W., and T. L. Karr.** 1996. Cytological analysis of fertilization and early embryonic development in incompatible crosses of *Drosophila simulans*. Mech. Dev. **57:**47–58.
14. **Mahowald, A. P., K. Illmensee, and F. R. Turner.** 1976. Interspecific transplantation of polar plasm between *Drosophila* embryos. J. Cell Biol. **70:**358–373.
15. **McGraw, E. A., D. J. Merritt, J. N. Droller, and S. L. O'Neill.** 2002. *Wolbachia* density and virulence attenuation after transfer into a novel host. Proc. Natl. Acad. Sci. USA **99:**2918–2923.
16. **Poinsot, D., K. Bourtzis, G. Markakis, C. Savakis, and H. Mercot.** 1998. *Wolbachia* transfer from *Drosophila melanogaster* into *D. simulans*: host effect and cytoplasmic incompatibility relationships. Genetics **150:**227–237.
17. **Riegler, M., S. Charlat, C. Stauffer, and H. Mercot.** 2004. *Wolbachia* transfer from *Rhagoletis cerasi* to *Drosophila simulans*: investigating the outcomes of host-symbiont coevolution. Appl. Environ. Microbiol. **70:**273–279.
18. **Santamaria, P.** 1986. Injecting eggs, p. 159–173. *In* D. B. Roberts (ed.), *Drosophila*, a practical approach. IRL Press Ltd., Oxford, United Kingdom.
19. **Werren, J. H., and D. M. Windsor.** 2000. *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? Proc. R. Soc. London Ser. B **267:**1277–1285.
20. **Werren, J. H., W. Zhang, and L. R. Guo.** 1995. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proc. R. Soc. London Ser. B **261:**55–63.
21. **Wu, M., L. V. Sun, J. Vamathevan, M. Riegler, R. Deboy, J. C. Brownlie, E. A. McGraw, W. Martin, C. Esser, N. Ahmadinejad, C. Wiegand, R. Madupu, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, A. S. Durkin, J. F. Kolonay, W. C. Nelson, Y. Mohamoud, P. Lee, K. Berry, M. B. Young, T. Utterback, J. Weidman, W. C. Nierman, I. T. Paulsen, K. E. Nelson, H. Tettelin, S. L. O'Neill, and J. A. Eisen.** 16 March 2004, posting date. Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: a streamlined genome overrun by mobile genetic elements. PLoS Biol. **2:**E69. [Online.] http://biology.plosjournals.org.
22. **Zhou, W., F. Rousset, and S. O'Neill.** 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. Proc. R. Soc. London Ser. B **265:**509–515.