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INVERTEBRATE MICROBIOLOGY

Mechanisms of Horizontal Cell-to-Cell Transfer of *Wolbachia* spp. in *Drosophila melanogaster*

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ABSTRACT *Wolbachia* is an intracellular endosymbiont present in most arthropod and filarial nematode species. Transmission between hosts is primarily vertical, taking place exclusively through the female germ line, although horizontal transmission has also been documented. The results of several studies indicate that *Wolbachia* spp. can undergo transfer between somatic and germ line cells during nematode development and in adult flies. However, the mechanisms underlying horizontal cell-to-cell transfer remain largely unexplored. Here, we establish a tractable system for probing horizontal transfer of *Wolbachia* cells between *Drosophila melanogaster* cells in culture using fluorescence *in situ* hybridization (FISH). First, we show that horizontal transfer is independent of cell-to-cell contact and can efficiently take place through the culture medium within hours. Further, we demonstrate that efficient transfer utilizes host cell phagocytic and clathrin/dynamin-dependent endocytic machinery. Lastly, we provide evidence that this process is conserved between species, showing that horizontal transfer from mosquito to *Drosophila* cells takes place in a similar fashion. Altogether, our results indicate that *Wolbachia* utilizes host internalization machinery during infection, and this mechanism is conserved across insect species.

IMPORTANCE Our work has broad implications for the control and treatment of tropical diseases. *Wolbachia* can confer resistance against a variety of human pathogens in mosquito vectors. Elucidating the mechanisms of horizontal transfer will be useful for efforts to more efficiently infect nonnatural insect hosts with *Wolbachia* as a biological control agent. Further, as *Wolbachia* is essential for the survival of filarial nematodes, understanding horizontal transfer might provide new approaches to treating human infections by targeting *Wolbachia*. Finally, this work provides a key first step toward the genetic manipulation of *Wolbachia*.

KEYWORDS *Drosophila*, *Wolbachia*, endocytosis, entry, horizontal, invasion, phagocytosis, transfer, transmission

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Wolbachia spp. are intracellular bacteria that are transmitted through the female germ lines of arthropods and filarial nematodes (1, 2). In arthropods, *Wolbachia* spp. function as either a mutualist or a parasite, while in filarial nematodes, *Wolbachia* spp. are essential for host survival. Efficient maternal transmission of *Wolbachia* cells in *Drosophila melanogaster* requires their localization to the posterior cortex of the developing embryo, as this is the future site of the germ line (3). In filarial nematodes, *Wolbachia* cells undergo a precise pattern of migration during host development that involves not only asymmetric mitotic segregation but also the invasion of germ line precursors from somatic cells (4). Thus, the ability of *Wolbachia* spp. to undergo cell-to-cell transfer plays an important role in maintaining vertical transmission (5).

While *Wolbachia* spp. are primarily vertically transmitted, horizontal transmission between arthropods has also been documented in nature (6–8). In these cases, the simplest routes of transmission appear to be the hemolymph or the gut, as *Wolbachia* bacteria present in these tissues can easily exit the host through excretion or injury and come into contact with an uninfected host (9). Support for this route comes from previous studies that found that purified *Wolbachia* can remain viable in an extracellular environment and infect mosquito cell lines, ovaries, and testes when cocultured (10, 11). Indeed, *Wolbachia* cells injected into the hemolymph of an uninfected fly can navigate to the germ line after crossing multiple somatic tissues not only in *Drosophila* (12, 13) but also in parasitoid wasps (14). It remains unclear how *Wolbachia* achieves this, as it must traverse a number of membrane and extracellular matrix barriers.

Insight into the mechanisms driving horizontal *Wolbachia* transmission will likely come from work on the well-studied mechanisms by which other pathogenic bacteria invade host cells, which can be categorized as mechanisms that utilize or alter internalization processes, such as pinocytosis, phagocytosis, and endocytosis (15). Pinocytosis involves the invagination of specialized plasma membrane regions to form pockets that allow for the nonspecific entry of extracellular particles (16). Phagocytosis involves the formation of membrane protrusions, driven by actin rearrangements, to engulf large receptor-bound particles (17). However, the use of host cellular pathways for invasion often requires active manipulation by the microbe. Bacterial entry via modification of host cellular machinery is known to be accomplished via two general mechanisms, the clathrin-dependent "zipper method" and the bacterial effector-dependent "trigger method" (18). In the zipper method, bacteria bind to receptors on the cell surface that induce actin extensions of the membrane through a clathrin-dependent pathway and serve to engulf the cell. Bacteria that utilize the trigger method synthesize type III secretion systems through which they secrete effector proteins to restructure the host cytoskeleton in order to facilitate attachment and invasion (18–20). In addition, invasive microbes may also up- or downregulate host cellular signaling pathways to disable host defenses and increase their own survival (21, 22). While viruses primarily utilize the same pathways to enter host cells, some enveloped viruses can enter through passive membrane fusion by simply blending their host-derived envelope with the plasma membrane of a new host cell (23). Within the host cell, *Wolbachia* bacteria are encompassed by a self-derived membrane and an outer host-derived membrane (24, 25), which potentially play a role in horizontal transfer by membrane fusion.

Given these possibilities, we sought to identify the mechanisms by which *Wolbachia* bacteria are horizontally transferred and to establish a useful system for the further study of this interesting phenomenon.

RESULTS

Horizontal transfer of *Wolbachia* is independent of cell-to-cell contact. Previous studies established that *Wolbachia* extracted from infected mosquito cell lines can enter uninfected cells and tissues when cocultured (10, 11). By extracting *Wolbachia* from *Drosophila* JW18 and LDW1 cells infected with the *w*Mel strain and coculturing this extract with doxycycline-cured JW18 (JW18-DOX) or LDW1 (LDW1-DOX) cells for 1 to 24 h, we confirmed this phenomenon in *Drosophila* (Fig. 1A and B). That is, free *Wolbachia* cells entering uninfected JW18-DOX cells were observed through fixed fluorescence imaging (Fig. 1A). In addition, the early and late stages of free *Wolbachia* cell entry into LDW1-DOX cells were observed using electron microscopy (Fig. 1B). These observations included contact between free *Wolbachia* cells and the host cell membrane and integration of *Wolbachia* into the host cytoplasm following entry in a vacuole.

While significant, these experiments did not reflect the *in vivo* environment of *Wolbachia* spp., where they must transfer between living cells. Thus, to determine if *Wolbachia* can horizontally transfer between intact *Drosophila* cells, we cocultured uninfected S2 cells and *Wolbachia*-infected JW18 cells on the same surface (Fig. 1C).

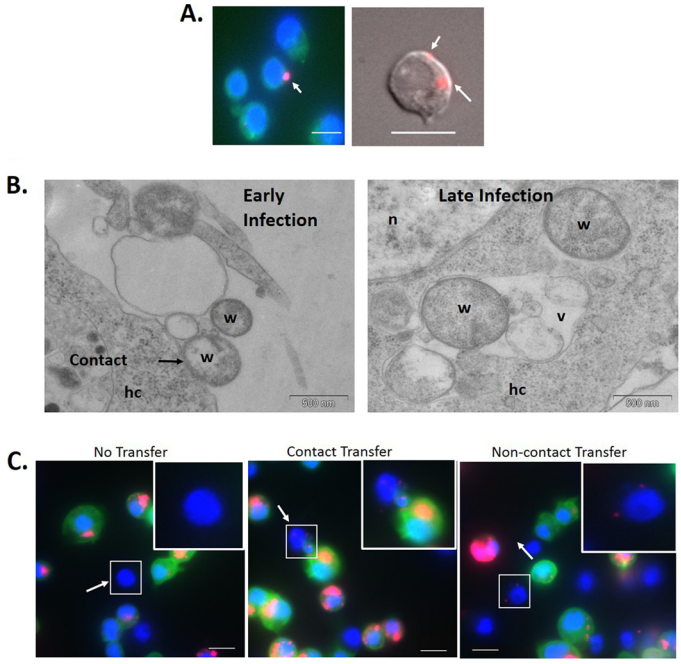

FIG 1 Horizontal transfer of *Wolbachia* bacteria between *Drosophila* cells. (A) *Wolbachia* bacteria extracted from infected JW18 cells were added to JW18-DOX cells and incubated for 24 h. (B) *Wolbachia* bacteria extracted from infected LDW1 cells were added to LDW1-DOX cells and incubated for 1 h. (C) Uninfected *Drosophila* S2 cells and *Wolbachia*-infected JW18 cells were cocultured on a glass coverslip for 24 h. *Wolbachia* infections in previously uninfected cells can be seen with FISH (A) and DIC (C) imaging or electron microscopy (B) to determine if horizontal transfer of infection took place. Results are typical of the multiple fields of view examined. Red, *Wolbachia*; blue, nuclei stained with DAPI; green, GFP-Jupiter (JW18 only). hc, host cell; n, nucleus; v, vesicle; w, *Wolbachia*. Bar, 10 μm.

JW18 cells carry GFP-Jupiter, a tubulin binding protein, which allows for the distinction of originally infected and uninfected cells by visualization of green fluorescent protein-tagged microtubules (26). Within 24 h of coculturing, transfer of *Wolbachia* from JW18 to previously uninfected S2 cells was readily apparent (Fig. 1C). While some S2 cells remained uninfected, many in close proximity to infected JW18 cells became infected, perhaps through cell-to-cell contact. We also observed that S2 cells that were not adjacent to JW18 cells became infected. These results suggest that *Wolbachia* can transfer horizontally from cell to cell in culture. Thus, our next goal was to determine if this phenomenon required contact between infected and uninfected cells.

To address this issue, we utilized a transwell system in which infected JW18 cells and uninfected S2 cells were seeded in chambers separated by a polyester membrane that allowed for the sharing of culture medium and passage of bacteria but prevented contact between larger eukaryotic cells (Fig. 2A) (see Materials and Methods). In these assays, transfer of *Wolbachia* infection was also observed, similar to when cells were cultured on the same surface (Fig. 2B and C). The proportion of newly infected cells after 6 h of coculturing was 43% ($n = 56$). After the cells were cocultured for 1 day, this number decreased slightly to 26% ($n = 90$). A similar number, 22% ($n = 93$), was observed after 2 days of coculturing. The infection rate then rose to 54% by 3 days of coculturing. As a control, JW18-DOX cells were used in place of infected cells in the transwell assay; no *Wolbachia* infections were detected in the S2 cells. Significantly, *Wolbachia* infections acquired through coculture in a transwell localized within the host cell (see Fig. S1 in the supplemental material) and were present and abundant 21 days after infection. Thus, the horizontally transferred *Wolbachia* was stably maintained through multiple division cycles (Fig. 3). These results strongly suggest that *Wolbachia* can horizontally transfer between infected and uninfected cells in culture, and this ability does not require cell-to-cell contact.

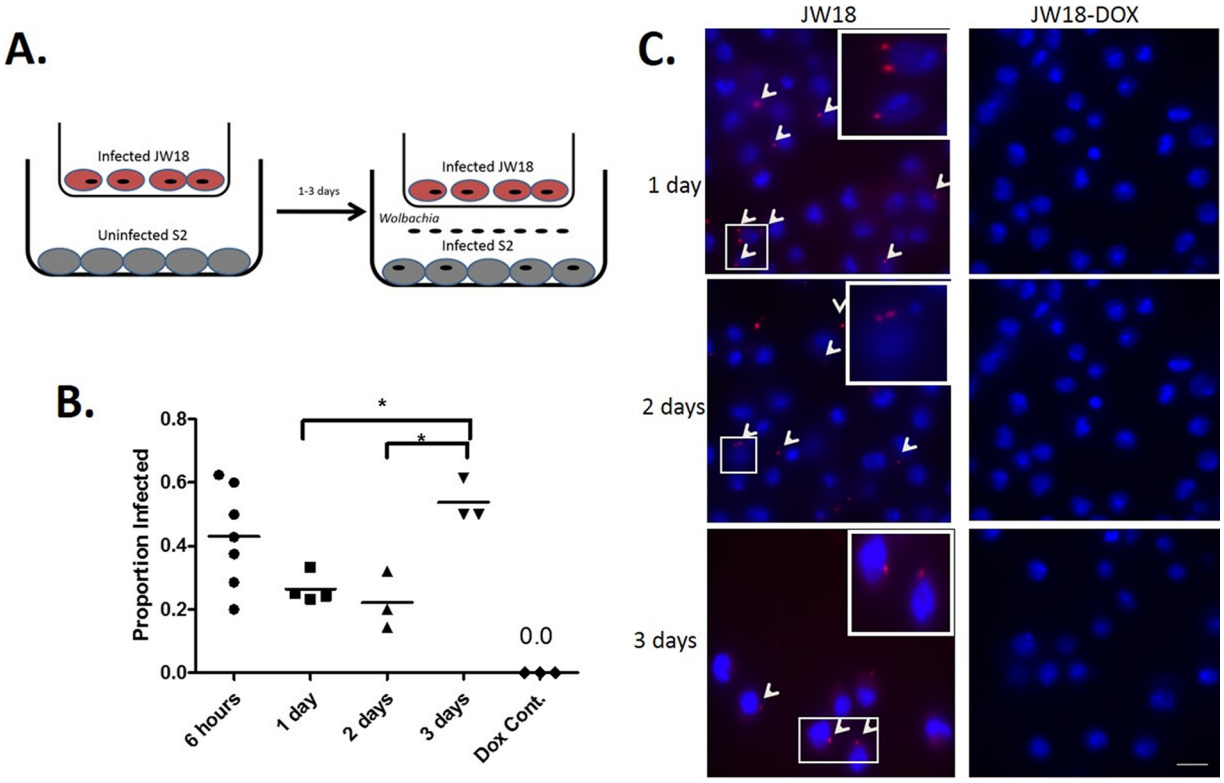

FIG 2 Horizontal transfer of *Wolbachia* bacteria between *Drosophila* cells separated in a transwell. (A) Uninfected *Drosophila* S2 cells were seeded beneath *Wolbachia*-infected JW18 cells in a transwell insert. (B) After coculture for 6 h or 1, 2, or 3 days, new *Wolbachia* infections in previously uninfected S2 cells were visualized by FISH in 3 to 7 fields of view for each group. S2 cells plated underneath doxycycline-cured JW18 cells (JW18-DOX) served as a negative control for FISH staining. Data are presented as proportion of infected cells ± SEM and were analyzed by one-way analysis of variance (ANOVA), followed by Newman-Keul's multiple-comparison test ($F = 5.31$; $R^2 = 0.551$; $df = 16$). Differences were deemed significant when the *P* value was <0.05 (indicated by an asterisk above the bracket). (C) Representative images for 1- to 3-day time points. Red, *Wolbachia* (arrowheads); blue, nuclei stained with DAPI. Bars, 10 μm.

Horizontal transfer of *Wolbachia* uses host clathrin and dynamin. We next sought to investigate the mechanisms involved in the horizontal transfer of *Wolbachia*. Given that many intracellular bacteria enter host cells by engaging components of the endocytic pathway, we hypothesized that this might also hold true for *Wolbachia*. We tested this hypothesis by inhibiting host cell dynamin, a GTPase necessary for the pinching and intracellular release of a variety of endocytic vesicles, using the small-molecule inhibitor dynasore (27). We then analyzed cell-to-cell transfer rates between

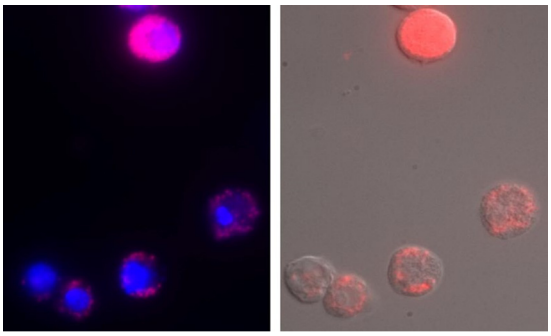

FIG 3 Long-term *Wolbachia* infection in *Drosophila* S2 cells after coculture with infected JW18 cells in a transwell chamber. Uninfected *Drosophila* S2 cells were seeded beneath *Wolbachia*-infected JW18 cells in a transwell insert. After coculture for 3 days, the transwell insert containing infected cells was removed, and new medium was added to the previously uninfected S2 cells. S2 cells were then cultured for an additional 18 days (21 days total), and *Wolbachia* infections were visualized by FISH and DIC. Red, *Wolbachia*; blue, nuclei stained with DAPI.

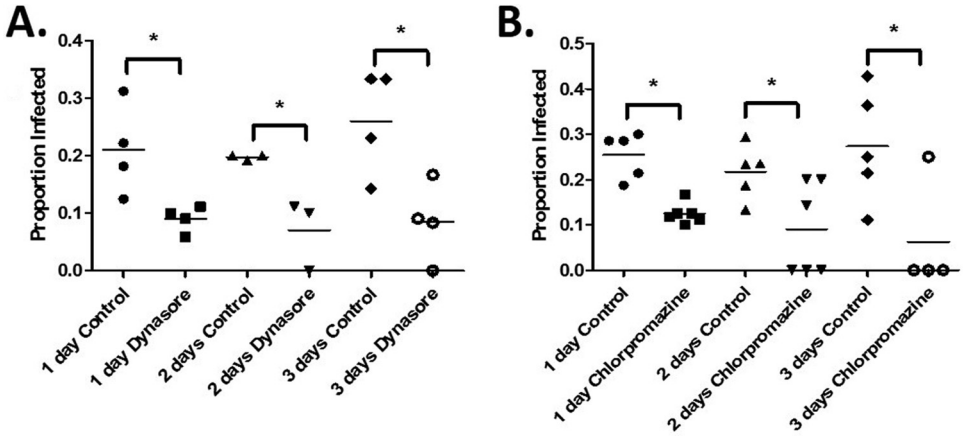

FIG 4 Horizontal transfer of *Wolbachia* is clathrin mediated. (A) Uninfected *Drosophila* S2 cells were pretreated with 80 μM dynasore or DMSO (control) for 1 h prior to seeding *Wolbachia*-infected *Drosophila* JW18 cells in a transwell insert above them. After being cocultured for 1, 2, or 3 days, new *Wolbachia* infections in previously uninfected S2 cells were visualized by FISH in 3 to 7 fields of view for each group. Data are presented as proportion of infected cells $\pm$ SEM and were analyzed by *t* test to determine differences between control and dynasore-treated groups at each time point ($t = 2.96$, $df = 6$ at 1 day; $t = 3.58$, $df = 4$ at 2 days; $t = 3.05$, $df = 6$ at 3 days). Differences were deemed significant when the *P* value was <0.05 (indicated by an asterisk above the bracket). (B) Uninfected *Drosophila* S2 cells were pretreated with 10 μM chlorpromazine or DMSO (control) for 1 h prior to seeding *Wolbachia*-infected *Drosophila* JW18 cells in a transwell insert above them. After being cocultured for 1, 2, or 3 days, new *Wolbachia* infections in previously uninfected cells were visualized by FISH and analyzed as described for panel A ($t = 5.73$, $df = 9$ at 1 day; $t = 2.44$, $df = 9$ at 2 days; $t = 2.51$, $df = 7$ at 3 days).

infected JW18 and uninfected S2 cells in our transwell assay. As predicted, treatment with dynasore significantly reduced the efficiency of cell-to-cell transfer relative to dimethyl sulfoxide (DMSO)-treated controls (Fig. 4A). After 1 day, the infection rate in untreated control cells was 21% ($n = 105$), compared to 9% ($n = 47$) in dynasore-treated cells. We observed a similar pattern after 2 days of dynasore treatment, with infection decreasing from 20% in controls ($n = 66$) to 7% in dynasore-treated groups ($n = 42$). Dynasore produced the strongest effect after 3 days of treatment, reducing infection from 26% in controls ($n = 45$) to 8% in treated cells ($n = 46$). The incomplete inhibition of horizontal transmission by dynasore suggests that *Wolbachia* spp. employ additional mechanisms of internalization (see below).

Nevertheless, these experiments demonstrate that *Wolbachia* spp. use dynamin for horizontal transfer into new host cells. Using dynamin, *Wolbachia* cells entered through a clathrin-dependent mechanism. To test this, we used chlorpromazine to inhibit host clathrin (28, 29), a coat protein involved in the formation of vesicles. Similar to dynamin inhibition, inhibition of clathrin reduced infection from 25% in controls ($n = 75$) to 12% after 1 day of treatment ($n = 64$) (Fig. 4B). After 2 days of treatment, the infection rate decreased from 22% in controls ($n = 95$) to 9% in treated cells ($n = 35$). As with dynasore, chlorpromazine produced the strongest effect after 3 days of treatment, reducing infection from 27% ($n = 45$) to 6% ($n = 17$). These results suggest that *Wolbachia* spp. utilize clathrin-mediated endocytosis pathways for entry during horizontal cell-to-cell transfer. We also used the inhibitors genistein and filipin to test the involvement of caveolin (29). In these experiments, caveolin inhibition did not inhibit cell-to-cell transfer (J. E. Pietri, unpublished data).

Host cells internalize *Wolbachia* via engulfment. Finding that clathrin and dynamin are involved in *Wolbachia* uptake prompted us to examine the interaction between *Wolbachia* spp. and host cells at the ultrastructural level. Transmission electron microscopy (TEM) reveals that *Wolbachia* uptake by host cells appears to be accomplished by engulfment via extensions of the cytoplasm similar to those of phagocytic pseudopodia (Fig. 5A and B). The bacteria were observed in contact with putative clathrin-coated pits (Fig. 5C and D), which in some cases were associated with pseudopodia (Fig. 5D). Internalization via membrane fusion may also contribute to

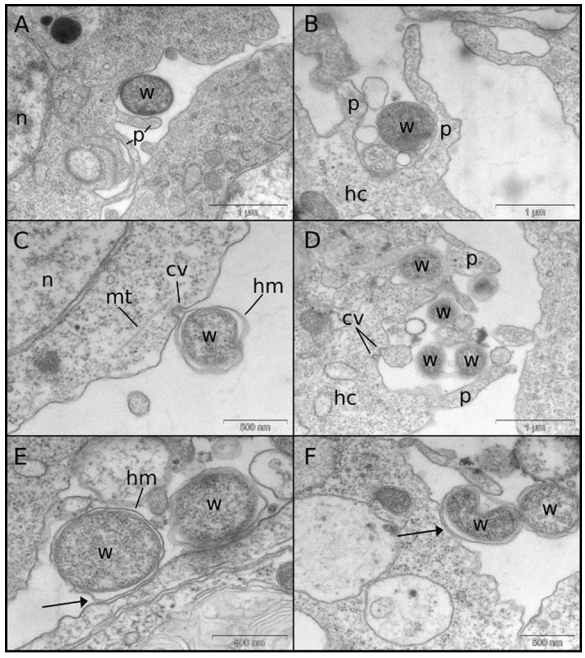

FIG 5 Transmission electron micrographs of LDW/JW18 cells exposed to *Wolbachia* bacteria from cell lysates or infected JW18 cells. (A and B) Wolbachia bacteria are frequently seen surrounded by phagocytic pseudopodium-like extensions of the host cell. (C and D) *Wolbachia* bacteria can be seen contacting what appear to be clathrin-coated pits, sometimes coinciding with pseudopodia (D). (E and F) The host-derived membrane surrounding the *Wolbachia* double membrane can be seen in close contact with the host cell membrane (arrows). cv, clathrin vesicle; hc, host cell; hm, host membrane; mt, microtubules; n, nucleus; p, pseudopodia; w, *Wolbachia*.

transfer rates, as the host-derived membrane of extracellular *Wolbachia* was often seen in close contact with the host membrane (Fig. 5E and F).

Horizontal transfer of *Wolbachia* takes place efficiently between cells of divergent hosts. Having implicated components of the host endocytic and phagocytic pathways in horizontal transfer, we sought to determine if a species barrier to horizontal transfer exists. We predicted that if this were the case, horizontal transfer of *Wolbachia* between cells of different insect species would be reduced or inhibited altogether. We examined this possibility by analyzing horizontal transfer rates between infected C6/36 cells from the mosquito *Aedes albopictus* and uninfected *Drosophila* S2 cells in our transwell assay (Fig. 6A). Despite *Wolbachia* infection rates in C6/36 and *Drosophila* JW18 cells being equal (Fig. 7), cell-to-cell transfer of *Wolbachia* from these cells to *Drosophila* S2 cells was somewhat lower. The proportion of newly infected cells after 1 day of coculturing was a mere 6% and decreased to 4% on day 2. Although new infections increased to 35% after 3 days of coculturing, this rate was lower than that observed between *Drosophila* cells (Fig. 2B), suggesting that while horizontal transfer takes places between different species, it may be less efficient. To rule out the effect of differences in *Wolbachia* exocytosis rates in mosquito and *Drosophila* cells, we pretreated JW18-DOX cells with dynasore and incubated them with crude *Wolbachia* preparations derived from fly or mosquito cells. In these experiments, *Wolbachia* infection rates in cells treated with *Wolbachia* bacteria derived from mosquito cells and with *Wolbachia* bacteria derived from *Drosophila* cells were not different, regardless of pretreatment (Fig. 6B). That is, within 24 h of incubation with *Wolbachia* bacteria from *Drosophila* cells, 60% of previously uninfected cells became infected ($n = 63$). This proportion was reduced to 14% by pretreating the cells with dynasore ($n = 75$). Similarly, when *Wolbachia* bacteria from *A. albopictus* cells were used, 71% ($n = 63$) of previously uninfected cells became infected. After pretreatment of the cells with dynasore, infection was almost completely blocked, as only 6% of the cells became

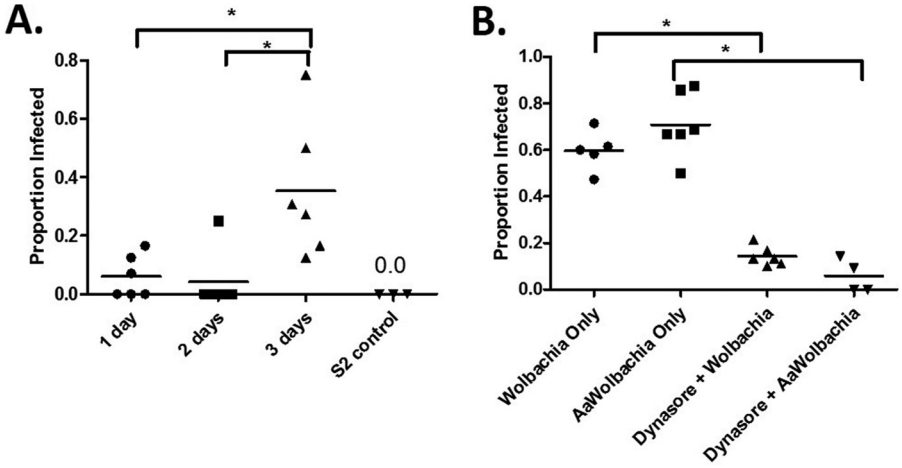

FIG 6 Horizontal transfer of *Wolbachia* bacteria takes places between mosquito and *Drosophila* cells. (A) Uninfected *Drosophila* S2 cells were seeded beneath *Wolbachia*-infected *A. albopictus* cells (C6/36) in a transwell insert. After being cocultured for 1, 2, or 3 days, new *Wolbachia* infections in previously uninfected cells were visualized by FISH in 6 fields of view for each group. S2 cells plated in the absence of C6/36 cells served as a control for FISH staining. Data are presented as proportion of infected cells ± SEM and were analyzed by one-way ANOVA, followed by Newman-Keuls multiple-comparison test to determine differences between time points ($F = 7.78$, $R^2 = 0.509$, df = 17). Values were deemed significant when $P < 0.05$ (indicated by an asterisk above the bracket). (B) Pretreatment of JW18-DOX cells with dynamin prior to the addition of crude *Wolbachia* preparations from infected *Drosophila* JW18 cells or mosquito C6/36 cells (*AaWolbachia*) for 24 h resulted in a reduced ability of *Wolbachia* bacteria to invade cells. Data are presented as proportion of infected cells ± SEM and were analyzed by one-way ANOVA, followed by Newman-Keuls multiple-comparison test to determine differences between groups ($F = 61.4$, $R^2 = 0.912$, df = 20). Values were deemed significant when the *P* value was <0.05 (indicated an asterisk above the bracket).

infected ($n = 55$). Thus, reliance of *Wolbachia* spp. on components of the endocytic pathway for cell-to-cell transfer appears to be conserved across species.

DISCUSSION

In our study, we documented the horizontal transfer of *Wolbachia* bacteria between *Drosophila* cells in culture and demonstrate that this process occurs through components of the host phagocytic and endocytic pathways. As such, our work directly demonstrates horizontal transfer of *Wolbachia* bacteria between cells while identifying a potential mechanism.

Our finding that horizontal transfer takes place between infected and uninfected cells when cultured together (Fig. 1) or separated by transwells (Fig. 3) suggests that cell-to-cell contact is not required to achieve efficient transfer. Nonetheless, cell-to-cell contact may play some role in horizontal transfer, as we observed several instances of *Wolbachia* bacteria transferring between cells in direct contact with each other (Fig. 1C). However, a large proportion of horizontally acquired infections can be accounted for by transfer through the culture medium (Fig. 2). *Wolbachia* spp. can achieve a >50% infection rate through this route, implicating it as the prevalent mechanism for horizontal transfer. Nonetheless, a fair proportion of bacteria invading through this method may not survive, as reduced infection levels between 6 and 24 h in our transwell assay suggest that perhaps some horizontally acquired *Wolbachia* bacteria are digested or killed by the host cell.

Transfer through the culture medium likely takes place via uptake after *Wolbachia* bacteria are exocytosed from infected cells. The release of *Wolbachia* bacteria after cell lysis may make some minor contribution to horizontal transmission. However, it is unlikely that these infrequent cell death events account for the high rates of infection transfer observed in our short-term assays, given that infected JW18 cells can be maintained in culture without passaging for 7 to 10 days without notable cell lysis occurring (J. E. Pietri, unpublished data).

Our experiments using dynasore and chlorpromazine to block dynamin and clathrin

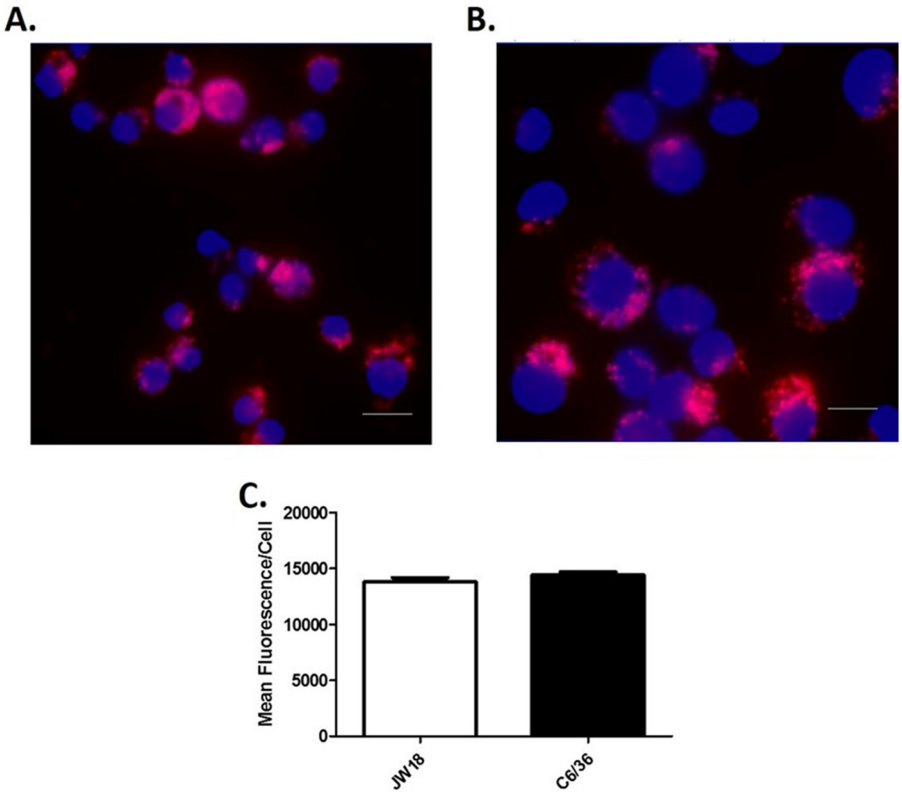

FIG 7 *Wolbachia* infection in *Drosophila* and *A. albopictus* cells. Cells were seeded on glass coverslips for 24 h and subsequently fixed with 8% paraformaldehyde for detection of *Wolbachia* by FISH (red) in *Drosophila* JW18 cells (A) and *A. albopictus* C6/36 cells (B). DAPI was used as a counterstain for cell nuclei (blue). Bar, 10 μm. (C) *Wolbachia* infection in JW18 and C6/36 cells was quantified by measurement of red fluorescence intensity. Data were analyzed by *t* test, and no significant differences between the two groups were found ($P = 0.223$, $t = 1.25$, $df = 18$).

activity in uninfected cells reveal the particular pathways of endocytosis coopted by *Wolbachia* spp. after their release from infected cells (Fig. 4A). Reduced horizontal transfer following inhibition of dynamin and clathrin, but not caveolin, argues against the possibility that *Wolbachia* bacteria enter cells exclusively through a process such as passive membrane fusion. Further, while *Drosophila* S2 and JW18 cells are passively phagocytic to some extent, the use of clathrin and dynamin in transfer suggests a bacterially induced mode of entry, such as the zipper method (18). However, clathrin has been reported to be involved in some forms of phagocytosis in *Drosophila* (e.g., references 30 to 32), preventing us from excluding this as a mechanism of uptake with these data alone.

It is possible that *Wolbachia* spp. use an active mode, such as the zipper method, and a passive method, such as phagocytosis, for uptake, as is the case for several other invasive bacteria. For instance, *Chlamydia* spp. can specifically trigger phagocytosis for entry into in HeLa cells, as demonstrated by experiments comparing the internalization rate of this bacterium with those of *Escherichia coli* and polystyrene beads (33). However, in the same cell type (i.e., HeLa cells), and in human endometrial gland epithelial cells, *Chlamydia* can be observed in coated pits and vesicles, indicative of endocytosis (34). Similarly, *Listeria* has been shown to enter cells through multiple mechanisms depending on the cell type being invaded. For instance, traditional phagocytosis and a formin-dependent phagocytosis-like process (35) have been demonstrated in vascular endothelial cells, while a clathrin-mediated process (33) appears to be critical in HeLa cells.

In addition, we suggest that *Wolbachia* bacteria may bind to a variety of host cell receptors to gain entry into host cells. This is consistent with results of studies of other

invasive intracellular bacteria, which demonstrate that while the machinery for endocytosis is often conserved, a variety of receptors can be used. For instance, although *Listeria* and *Neisseria* both enter through clathrin-coated pits (33–38), *Listeria* utilizes the hepatocyte growth factor receptor (met) (38), while *Neisseria* uses the asialoglycoprotein receptor (ASGP-R) (37). Similarly, microorganisms may make use of the same receptors but achieve entry through different mechanisms. For example, both *Salmonella* and *Candida* bind to the epidermal growth factor receptor (EGFR) (39, 40), but they make use of phagocytosis and clathrin-mediated pathways, respectively (18, 41). The receptor(s) that *Wolbachia* spp. bind prior to entry remain undetermined. However, the conservation of horizontal transfer across species suggests that this receptor and its ligand(s) may be highly conserved, as *Wolbachia* derived from the C6/36 and JW18 cells used as *Wolbachia* donors in our experiments harbored *w*AlbB and *w*Mel, respectively.

The processes of phagocytosis and endocytosis are intrinsically linked to the actin cytoskeleton (42). Intriguingly, a number of microbes rely on host actin for invasion and are able to manipulate its structure through the use of secreted effectors (19). The same appears to be true for *Wolbachia*, which was recently shown to rely on host actin for efficient maternal transmission (43). *Wolbachia* also encodes a secreted effector, WD0830, which interacts with the host cytoskeleton (44). This is particularly important, as it suggests that the horizontal transfer process may not be passive and host driven but, rather, induced by *Wolbachia* spp. through the secretion of effector proteins that drive cytoskeletal changes for engulfment. This mode of transfer might explain cortical actin rearrangements that are associated with *Wolbachia* migration during filarial nematode development (4).

Differences in *Wolbachia* exocytosis rates may play some role in controlling horizontal transmission, as entry of *Wolbachia* extracted from mosquito cells from crude extractions was not inhibited compared to *Wolbachia* extracted from *Drosophila* cells (Fig. 6B), despite our transwell assay in which lower rates of horizontal transfer were found (Fig. 6A). It is unlikely that the reduced titer in mosquito cells plays a role in this discrepancy between assays, as infection levels in mosquito cells were equal to those in *Drosophila* cells (Fig. 7). Likewise, genotype-specific differences in bacterial surface factors are likely not involved given the different strains of *Wolbachia* harbored by JW18 and C6/36 cells. However, differences in recipient cell properties, such as the presence or absence of particular receptors, may contribute to differences in the efficiency of horizontal transfer and should be explored further.

Ultimately, the results of our work significantly advance our understanding of how *Wolbachia* is transmitted both vertically and horizontally. During early embryogenesis in filarial nematodes, *Wolbachia* segregates exclusively to the lineage producing the hypodermal chords, somatic tissues that provide nutrients to developing germ line cells. Occupation of the germ line for eventual vertical transmission requires cell-to-cell transfer from the chords (4). The relevance of somatic to germ line cell-to-cell transfer for vertical transmission is further illuminated by images of *Wolbachia*-infected oocytes from recently captured wild *Drosophila* (45). Egg chambers were identified in which *Wolbachia* was not present in many of the early developing oocytes, but all of the mature oocytes were infected. The absence of *Wolbachia* early in oogenesis is likely a direct result of its failure to segregate to the differentiating daughter cell during germ line stem cell division. The fact that these empty oocytes eventually become infected suggests that *Wolbachia* bacteria present in the surrounding somatic follicle cells eventually enter the oocyte using cell-to-cell transfer as a backup mechanism to ensure vertical transmission (46).

Our findings also shed some light on possible routes of horizontal transmission of *Wolbachia* infection in nature. Previous work showed that *Wolbachia* bacteria in the hemolymph of adult flies can migrate to the germ line across multiple somatic tissues (12). This is likely mediated by cell-to-cell transfer between various tissues and suggests that *Wolbachia* which enters a new host through the gut or a wound may use cell-to-cell transfer to establish both a somatic and stable (germ line) infection.

While more specifics regarding the mechanisms of horizontal transfer remain to be

uncovered, our transwell fluorescence *in situ* hybridization (FISH) assay is a simple and tractable system for further probing cell exit and entry of *Wolbachia* bacteria, as it allows for the separate manipulation of recipient (uninfected) and donor (infected) cells while providing several advantages over antibody-based staining by increasing specificity and reducing background fluorescence. Our system is also highly biologically relevant, as *Wolbachia* bacteria that infect through this method can achieve proper localization inside the host cell (Fig. 1; see also Fig. S1 in the supplemental material) and also appear highly stable, surviving for at least 21 days (Fig. 3).

MATERIALS AND METHODS

Cell culture and infections. Stocks of uninfected *Drosophila* S2 cells, *Wolbachia*-infected *Drosophila* JW18 cells (26), *Wolbachia*-infected *A. albopictus* C6/36 cells, and doxycycline-cured JW18 (JW18-DOX) cells were maintained in Shields and Sang M3 insect medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Gibco) at a temperature of 24 to 26°C. We also created an additional immortalized cell line from primary cultures of *Wolbachia*-infected *D. melanogaster* bearing red fluorescent protein (RFP)-histone (47) and green fluorescent protein (GFP)-Jupiter (48). This cell line is called LDW1.

JW18 and LDW1 cells are naturally infected with the *w*Mel strain of *Wolbachia* (26), while C6/36 cells were artificially infected with the *w*AlbB strain from Aa23 cells, as previously described (49). For FISH assays, cells were seeded on glass coverslips in untreated 6-well polystyrene plates (Costar). In transwell assays, uninfected cells were seeded in the same manner, while infected cells were seeded on polyester transwell membrane inserts with a pore size of 3.0 μm (Costar). For assays of dynamin inhibition, uninfected cells on coverslips in the bottom transwell were treated with 80 μM dynasore (27) or an equal volume of DMSO (control) for 1 h, and the medium was then changed prior to seeding infected cells on the top well or prior to adding crude *Wolbachia* preparations directly to the culture medium for an additional 24 h. For assays of clathrin inhibition, uninfected cells on coverslips in the bottom transwell were treated with 10 μM chlorpromazine (28) for 1 h, and the medium was changed prior to seeding infected cells on the top well. Crude *Wolbachia* extracts were prepared by running infected cells in culture medium through 5.0-μm filter spin columns (Millipore) for lysis to release *Wolbachia* bacteria and remove large cellular debris.

Primary neuroblast cell culture and infections. *Drosophila* stocks homozygous for neuroblast-specific GAL4 expression (OK371, as identified in reference 50) and CD-ChRFP (2) under an upstream activation sequence (UAS) promoter (Bloomington stock 27391) were crossed. Third-instar larvae were collected for brain dissection and primary culture (51), modified to exclude antibiotics from all reagents except for the Shields and Sang medium used to wash the cells, which contained 1:1,000 penicillin-streptomycin. The brain homogenate was plated on concanavalin A-coated glass coverslips as described above and incubated at 25°C overnight. Neuroblasts were tested for cell-to-cell transfer as described above.

Passive uptake of fluorescently labeled dextran. S2 cells and neuroblasts were incubated with 20 mg/ml 1:1,000 fluorescently labeled dextran (molecular weight, 40,000) overnight at 25°C. Culture medium with beads was aspirated, and cells were processed for detection of *Wolbachia* bacteria by using FISH (see next section).

FISH detection of *Wolbachia*. *Wolbachia* detection by FISH was performed 1, 2, or 3 days after coculture of uninfected and infected cells and 24 h after the addition of crude *Wolbachia* preparations to cured cells. Cells on glass coverslips were fixed with 8% paraformaldehyde for 20 min at room temperature, washed 3 times with phosphate-buffered saline (PBS), and treated with prehybridization buffer for 90 min at room temperature. The prehybridization buffer consisted of 50% deionized formamide by volume, 4× saline sodium citrate (SSC), 0.5× Denhardt's solution, 0.1 M dithiothreitol (DTT), and 0.1% Tween 20 in deionized water. After prehybridization, cells were hybridized overnight at 37°C in hybridization buffer (prehybridization buffer minus detergent) containing 500 nM *Wolbachia* W2 fluorescent DNA probe (5-CTTCTGTGAGTACCGTCATTATC-3) (Bioresearch Technologies) (52). After hybridization, cells were washed 3 times with 1× SSC plus 0.1% Tween 20, 3 times with 0.5× SSC, and 3 times with PBS to remove any free *Wolbachia* bacteria on the slide. The last step of each wash series was performed at 42°C to eliminate nonspecific binding of the FISH probe. Slides were then mounted and stained using Vectashield fluorescent mounting medium with DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories).

Microscopy and image analysis. All fluorescence and differential interference contrast (DIC) imaging was performed on a Leica DMI 6000 inverted wide-field microscope under equal exposure times and conditions. For quantitation of *Wolbachia* infection during coculture over time, 3 to 7 fields (technical replicates) for each group from 3 independent experiments (biological replicates) were scored for the proportion of cells displaying red puncta in the ImageJ cell counter tool (http://imagej.nih.gov/ij/). Only cells with *Wolbachia* puncta in close association with the nucleus were scored as infected to reduce the number of false-positive infections from *Wolbachia* bacteria on the slides outside the cell, despite them being negligible. Counts from each field were plotted as the proportion infected per field of view ± standard error of the mean (SEM) and were pooled for analysis by one-way ANOVA followed by Newman-Keuls multiple-comparison test or by *t* test to determine differences in infection over time and between the treated and untreated groups. For electron microscopy, samples were fixed with 2% glutaraldehyde and 0.5% paraformaldehyde in 0.075 M cacodylate buffer and postfixed with 2% osmium

tetroxide. Samples were dehydrated through a graded series of ethanol and embedded in epoxy resin. Ultrathin (70-nm) sections (Ultracut UC6, Leica) were collected on Formvar/carbon-coated copper grids. Sections were then poststained with aqueous 4% uranyl acetate and lead citrate. All samples were observed in a Tecnai 12 (FEI, The Netherlands) transmission electron microscope at 80 kV equipped with a 1K-by-1K-resolution Keen View camera.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03425-16.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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