Vertical Transmission of a *Drosophila* Endosymbiont Via Cooption of the Yolk Transport and Internalization Machinery

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ABSTRACT ***Spiroplasma*** **is a diverse bacterial clade that includes many vertically transmitted insect endosymbionts, including *Spiroplasma poulsonii*, a natural endosymbiont of *Drosophila melanogaster*. These bacteria persist in the hemolymph of their adult host and exhibit efficient vertical transmission from mother to offspring. In this study, we analyzed the mechanism that underlies their vertical transmission, and here we provide strong evidence that these bacteria use the yolk uptake machinery to colonize the germ line. We show that *Spiroplasma* reaches the oocyte by passing through the intercellular space surrounding the ovarian follicle cells and is then endocytosed into oocytes within yolk granules during the vitellogenic stages of oogenesis. Mutations that disrupt yolk uptake by oocytes inhibit vertical *Spiroplasma* transmission and lead to an accumulation of these bacteria outside the oocyte. Impairment of yolk secretion by the fat body results in *Spiroplasma* not reaching the oocyte and a severe reduction of vertical transmission. We propose a model in which *Spiroplasma* first interacts with yolk in the hemolymph to gain access to the oocyte and then uses the yolk receptor, Yolkless, to be endocytosed into the oocyte. Cooption of the yolk uptake machinery is a powerful strategy for endosymbionts to target the germ line and achieve vertical transmission. This mechanism may apply to other endosymbionts and provides a possible explanation for endosymbiont host specificity.**

IMPORTANCE **Most insect species, including important disease vectors and crop pests, harbor vertically transmitted endosymbiotic bacteria. Studies have shown that many facultative endosymbionts, including *Spiroplasma*, confer protection against different classes of parasites on their hosts and therefore are attractive tools for the control of vector-borne diseases. The ability to be efficiently transmitted from females to their offspring is the key feature shaping associations between insects and their inherited endosymbionts, but to date, little is known about the mechanisms involved. In oviparous animals, yolk accumulates in developing eggs and serves to meet the nutritional demands of embryonic development. Here we show that *Spiroplasma* coopts the yolk transport and uptake machinery to colonize the germ line and ensure efficient vertical transmission. The uptake of yolk is a female germ line-specific feature and therefore an attractive target for cooption by endosymbionts that need to maintain high-fidelity maternal transmission.**

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Virtually all terrestrial arthropod species harbor vertically transmitted microbial endosymbionts that play critical roles in the biology of their hosts. Many well-studied examples involve obligate bacterial endosymbionts (i.e., they are absolutely required for survival and reproduction) that supply their host with essential nutrients that are missing from its diet (1). On the other hand, facultative endosymbionts are not required for host development or host survival (2); these appear to be particularly common in insects, with most species harboring them (3). Many facultative endosymbionts manipulate the reproduction of their hosts in order to increase in frequency. Others increase the fitness of their hosts under certain conditions, for example, by protecting their hosts against different classes of parasites, and therefore might be useful tools to control insect vector-borne diseases (4). The best known facultative insect endosymbiont is *Wolbachia*, which is estimated to infect ~40% of terrestrial arthropod species. Recent work has shown that a number of other symbiont lineages, including *Spiroplasma*, are also common.

Spiroplasma bacteria are a members of the *Mollicutes* class, a wall-less eubacterial group related to Gram-positive bacteria. Initially discovered as the causative agents of important plant and insect diseases (5, 6), *Spiroplasma* bacteria are widely associated with arthropods, and an estimated 5 to 10% of all insect species are hosts, including 17 species of the genus *Drosophila* (7–9). *Spiroplasma* bacteria are especially diverse with respect to their modes of transmission. While some species are horizontally transmitted insect pathogens or commensals in the gut, many lineages exhibit transovarial vertical transmission from mother to offspring (10) and affect their hosts in diverse important ways. *Spiroplasma* bacteria have also been shown to confer resistance to macroparasites such as parasitoid wasps or nematodes on their hosts (11, 12). Endosymbiotic *Spiroplasma* bacteria are largely unable to survive outside their hosts, and although horizontal transmission between

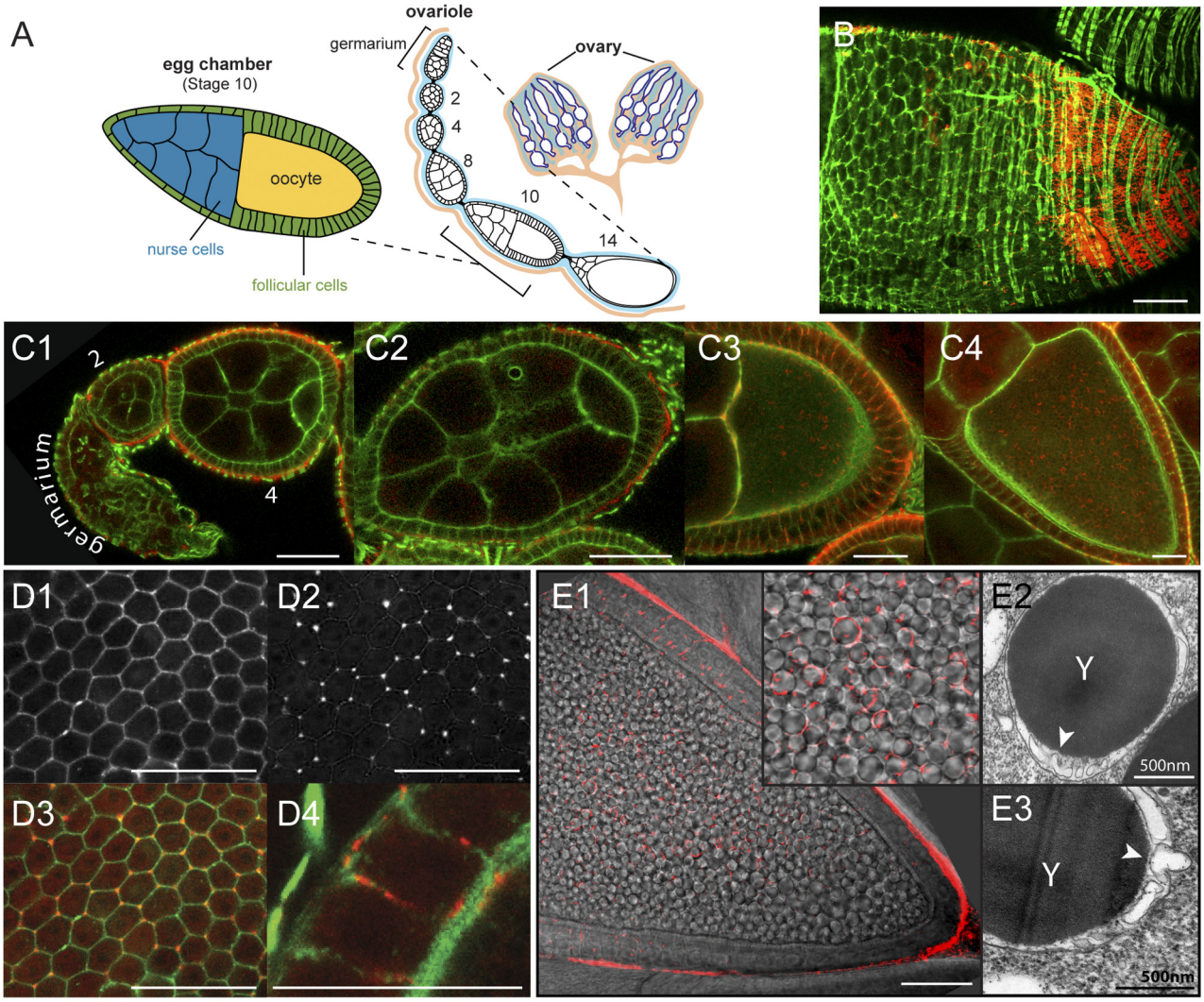

FIG 1 *Spiroplasma* colonization of the germ line. (A) Drawing showing the structure of an egg chamber, ovarioles. and ovary of *Drosophila*. The ovariole is surrounded by a muscular epithelium (light blue). Development of egg chambers progresses along the length of the ovariole, beginning with stem cell division in the germarium, which eventually becomes vitellogenic (accumulates yolk) and is termed the vitellarium, after which dumping of nurse cell contents into the oocyte occurs. Vitellogenic stage 10 egg chambers (left) are characterized by an outer layer of columnar follicle cells (green), an expanded oocyte (yellow), and nurse cells (blue). (B) Exterior surface of a vitellogenic stage 10 egg chamber. *Spiroplasma* bacteria are stained red (immunostaining with anti-*Spiroplasma* antibody), and cortical actin is stained green (with phalloidin), and this is the case for all subsequent images. (C) *Spiroplasma* localization in a series of egg chambers that represents the progression from germarium to vitellogenic oocytes. Germarium, stage 2, and stage 4, C1; stage 6, C2; stage 9, C3; stage 10, C4. (D) Actin staining of cell boundaries (D1) and *Spiroplasma* bacteria (D2) are merged (D3, D4) to show that *Spiroplasma* passes between follicle cells. D1 to D3 are transverse sections, and D4 is a longitudinal section. (E1) Bright-field image overlaid with fluorescent *Spiroplasma* staining reveals the localization of *Spiroplasma* bacteria in relation to yolk granules. In all of the fluorescence microscopy images, the scale bar represents 25 μm. (E2, E3) Electron micrograph showing localization of *Spiroplasma* bacteria (arrow) in relation to yolk granules (Y). In E1, *Spiroplasma* bacteria are contained between the yolk granule and the vesicular membrane. In E2, *Spiroplasma* bacteria can be seen partially penetrating the vesicular membrane. *Spiroplasma* cells could be identified in yolk granules on the basis of their size and morphology and the absence of such cells in yolk granules of flies without *Spiroplasma* bacteria.

hosts can occur, it is rare (9). Colonization of new hosts occurs almost entirely by vertical transmission from mother to offspring, which must therefore occur with high fidelity.

The mechanism utilized by *Spiroplasma* bacteria to achieve vertical transmission has not yet been established. In this paper, we analyze the vertical transmission of *Spiroplasma poulsonii* strain MSRO (referred here to as *Spiroplasma*) in its natural host, *Drosophila melanogaster* (13). We provide genetic evidence for an interaction between *Spiroplasma* bacteria and the host yolk transport and uptake machinery. More specifically, we show that mutations that disrupt either oocyte yolk uptake or yolk secretion by the fat body severely impair the efficiency of vertical *Spiroplasma* transmission.

RESULTS

***Spiroplasma* bacteria colonize the vitellogenic oocyte.** The *Drosophila* ovary consists of 15 to 18 discrete tubular ovarioles (Fig. 1A). Increasingly more mature egg chambers extend from the anterior to the posterior of the ovariole. The germ line stem cells are located at the anterior of the ovariole, in a region termed

the germarium. Egg chamber development continues into the vitellarium region, where the oocyte takes up yolk and completes development into a fully formed unfertilized egg (14). Using immunofluorescence microscopy, we observed that *Spiroplasma* bacteria accumulate in the muscular epithelium that surrounds ovarioles. Specifically, *Spiroplasma* bacteria accumulate in the region of this epithelium that is proximal to the posterior end of the egg chamber (Fig. 1B), where endocytic activity is highest (15). We observed that *Spiroplasma* bacteria are not present in the germ line at the germarium stage, early in oogenesis (Fig. 1C1 and C2). *Spiroplasma* bacteria are known to be found occupying the abdominal cavity of females (16, 17). Therefore, to achieve vertical transmission, these bacteria must be able to reach the germ line at later oogenic stages from the hemolymph. We observed that *Spiroplasma* enters the germ line over specific stages of oogenesis (Fig. 1C3 and C4). *Spiroplasma* bacteria distinctly colonize the germ line during the vitellogenic stages (stages 8 to 10) of oogenesis, when yolk is incorporated into the oocyte. We also show that *Spiroplasma* bacteria are present in the extracellular space between follicle cells (Fig. 1D). In the oocyte, *Spiroplasma* bacteria are localized to vesicles like those formed by the endocytosis of yolk, also known as yolk granules (Fig. 1E1). Transmission electron microscopy (TEM) images reveal that *Spiroplasma* bacteria are found in the space between the yolk granule and the surrounding vesicular membrane (Fig. 1E2), which is consistent with a previous electron microscopy study (18). In our TEM images, we also observed *Spiroplasma* bacteria penetrating the vesicular membrane surrounding yolk granules (Fig 1E3); presumably, these cells are exiting yolk granules and gaining access to the oocyte cytoplasm. Altogether, this pattern of infection indicates that the route taken by *Spiroplasma* bacteria to reach the germ line involves invasion of the ovary, followed by passage between follicle cells of vitellogenic egg chambers, translocation across the oocyte membrane into the vesicles that become yolk granules, and finally traversal of the yolk vesicular membrane to access the oocyte cytoplasm.

The Yolkless receptor is required for efficient *Spiroplasma* transmission. In *Drosophila*, the nutritional demands of embryonic development are fulfilled mainly by yolk proteins, which are synthetized primarily in the fat body (an organ functionally similar to the mammalian liver) and secreted into the hemolymph. The yolk proteins then travel through the hemolymph to enter the ovaries, traversing the peritoneal sheath and muscular epithelium before passing between follicle cells, and ultimately are taken up into the oocyte by a process very similar to general receptor-mediated endocytosis (19). The endocytosis of yolk into the oocyte requires the Yolkless receptor, which belongs to the low-density lipoprotein receptor superfamily and is localized to the surface of the oocyte (20). The pattern of *Spiroplasma* germ line colonization appears very similar to yolk uptake, lending support to the hypothesis that *Spiroplasma* could use the vitellogenic machinery to ensure access to the germ line.

To test this hypothesis, we used quantitative PCR (qPCR) and fluorescence microscopy to quantify *Spiroplasma* transmission in flies lacking the yolk receptor, Yolkless. yl^{13} is a strong loss-of-function mutation in *yolkless* that causes a marked decrease in yolk uptake by oocytes and an increase in the amount of yolk in the hemolymph (21). Without sufficient yolk, the eggs laid by yl^{13} mutant flies do not complete early embryonic development (21). We first quantified *Spiroplasma* titers by qPCR of the *Spiroplasma dnaA* gene in control Oregon-R (OR^R) and yl^{13} homozygous female flies, as well as in their embryos. Although we initially quantified the *Spiroplasma dnaA* copy number relative to that of a host nuclear gene, *RPS17*, we decided to remove this from all of our analyses because the host nuclear gene copy number was, unsurprisingly, much lower in nonviable eggs. We observed that yl^{13} homozygous flies transmit four times fewer *Spiroplasma* bacteria to eggs than their wild-type counterparts do, while *Spiroplasma* levels in whole flies are not significantly different (Fig. 2A). Consistent with these observations, immunofluorescence microscopy revealed that oocytes of *yolkless* homozygous infected females contained much lower *Spiroplasma* levels than did wild-type oocytes of the same stage (compare Fig. 2B1 and B2; see Fig. S1A in the supplemental material for image quantification). We observed that, in some cases, *Spiroplasma* bacteria tended to accumulate between follicle cells and on the outer surface of the yl^{13} oocyte, suggesting that the blockage occurs at the point of oocyte entry. We observed a similar phenotype when using another independently derived mutant allele of *yolkless*, yl^{15}.

To rule out the possibility that the low *Spiroplasma* titers observed by qPCR in the embryos laid by yl^{13} mutants were somehow linked to their being nonviable, we also quantified *Spiroplasma* transmission by $dec\text{-}1^{VA28}$ females, which have a mutation in the *defective chorion 1* (*dec-1*) gene affecting chorion formation and causing nonviability of eggs (22). We did not observe decreased levels of *Spiroplasma* transmission in $dec\text{-}1^{VA28}$ flies (see Fig. S2 in the supplemental material). As an additional control, we also examined *Spiroplasma* transmission in *Df*(3r) *LpR2* flies, which are mutants that lack another member of the low-density lipoprotein receptor superfamily, lipophorin receptor 2 (LpR2). This receptor is involved in the uptake of lipids and lipoproteins into the developing oocyte (23). We found that the lack of LpR2 receptors did not decrease *Spiroplasma* transmission (see Fig. S2 in the supplemental material), suggesting that, for transmission, *Spiroplasma* interacts specifically with certain receptors in the low-density lipoprotein superfamily. We also show in Fig. S2 that the white-eyed mutant background (w^{1118}) does not itself affect *Spiroplasma* transmission.

To establish whether *Spiroplasma* bacteria are translocated into the oocyte by endocytosis, we also examined *Spiroplasma* colonization of $Rab5^2$ mutant oocytes (Fig. 2B3), which exhibit a general blockage of endocytosis and are therefore also unable to accumulate yolk proteins (24). Since the $Rab5^2$ mutation used causes early embryonic lethality (25), we used the FLP-FRT mosaic system to generate female flies with $Rab5^2$-deficient oocytes (24). Our analysis was complicated by the fact that $Rab5^2$-deficient oocytes were severely deformed, with invaginations of the peripheral oocyte cortex. Nevertheless, we clearly observed lower *Spiroplasma* levels in the oocytes of these flies (Fig. 2B3; for image quantification, see Fig. S1A in the supplemental material). Of note, most of the few internalized *Spiroplasma* bacteria were associated with these invaginations, raising the possibility that they enter through disrupted regions of the oocyte cortex. Altogether, these observations lead us to conclude that Yolkless receptor-mediated endocytosis is important for *Spiroplasma* uptake by host oocytes.

The presence of yolk protein in hemolymph is required for *Spiroplasma* transmission. Having shown that efficient *Spiroplasma* uptake requires the Yolkless receptor, we investigated whether *Spiroplasma* also needs yolk as a vehicle to enter the germ line. *D. melanogaster* synthesizes three major yolk proteins, Yp1, Yp2, and Yp3 (26), mainly in the fat body but also to a lesser extent

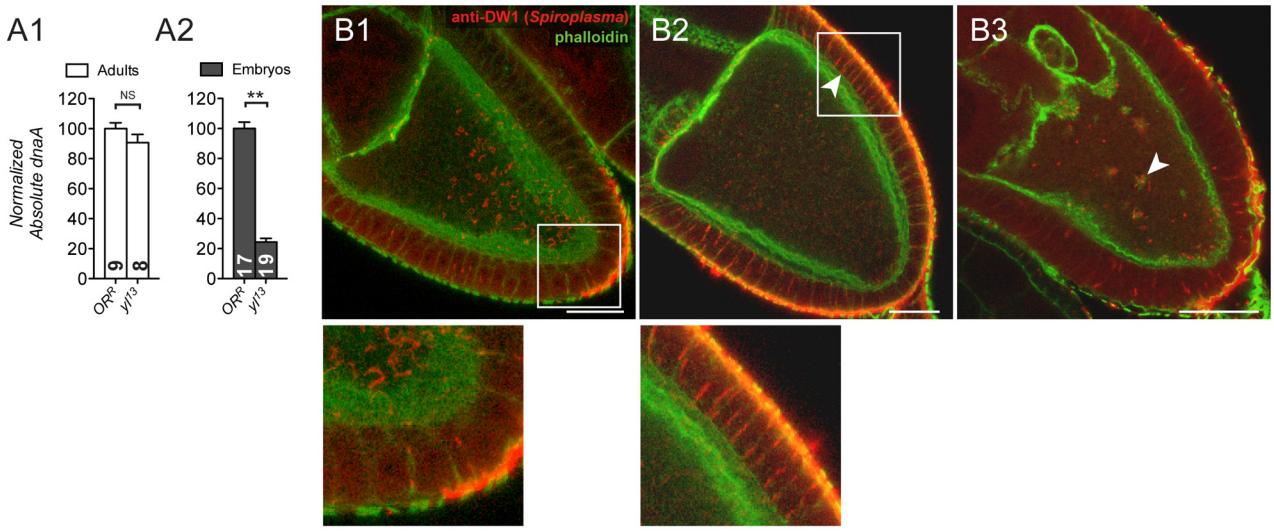

FIG 2 Involvement of Yolkless receptor-mediated endocytosis in *Spiroplasma* transmission. (A) *Spiroplasma* levels in flies and embryos are shown for the control (OR^R) and *yolkless* (yl^{13}) mutants. *Spiroplasma* levels were monitored by qPCR with a *Spiroplasma*-specific gene (*dnaA*). Each value was normalized to the average of the control values for that experiment (OR^R flies or embryos), which was set at 100%. All of the repeats from all of the experiments were then pooled. The number of samples collected independently for DNA extraction is shown by the value in each bar. Error bars represent the standard error of the mean. NS and *** denote levels of statistical significance in a Mann-Whitney U test of difference when yl^{13} mutants are compared to the control (OR^R) for flies ($P = 0.6298$) and for embryos laid by these flies ($P < 0.0001$). (B) Stage 10 oocytes from control (OR^R) (B1), yl^{13} (B2), and $Rab5^2$-deficient germ line clone (B3) flies. Note that $Rab5^2$-deficient clones exhibit structural deformations. The arrowhead in B2 denotes an accumulation of *Spiroplasma* bacteria between the follicle cells surrounding yl^{13} mutant oocytes. The arrowhead in B3 denotes actin-rich cortical invagination that is associated with the presence of *Spiroplasma* (see image analysis in Fig. S1 in the supplemental material). The images at the bottom are higher magnifications of the insets.

in the follicle cells surrounding the developing oocyte (27). We analyzed *Spiroplasma* transmission in mutant flies with reduced yolk production. $Yp1^{TS1}$ is a temperature-sensitive dominant female sterile mutation that causes a drastic decrease in circulating Yp1, as well as a reduction of circulating Yp2 and Yp3 (28, 29). $Yp1^{TS1}$/+ flies raised at the restrictive temperature of 29°C secrete Yp1, as well as Yp2 and Yp3, into the subbasement membrane space of their fat bodies, but the secreted oligomers condense and cannot cross the basement membrane to be released into the hemolymph (29). The qPCR *Spiroplasma* titer measurement shown in Fig. 3A2 reveals that the *Spiroplasma* transmission to embryos laid by $Yp1^{TS1}$/+ flies at the restrictive temperature is decreased about 4-fold. Importantly, Fig. 3A1 shows that $Yp1^{TS1}$/+ does not decrease the overall *Spiroplasma* level in whole female flies, indicating that the lack of *Spiroplasma* bacteria in embryos was due to impaired transmission and not to a growth defect in the hemolymph. In fact, Fig. 3A1 reveals that *Spiroplasma* levels were around 30% higher in $Yp1^{TS1}$/+ flies. *Spiroplasma* levels in flies have been positively correlated with the efficiency of *Spiroplasma* transmission to eggs (30), and therefore our data might underestimate the actual transmission blockage caused by $Yp1^{TS1}$/+. Using fluorescence microscopy, we were able to observe significantly fewer *Spiroplasma* bacteria inside the vitellogenic oocytes of $Yp1^{TS1}$/+ flies than in wild-type fly oocytes at the restrictive temperature (compare Fig. 3B1 and B2; for image quantification, see Fig. S1B in the supplemental material). Together, these findings indicate that, in addition to Yolkless, yolk is also involved in *Spiroplasma* transmission into the germ line. In contrast to the situation observed with the *yolkless* mutant and wild-type flies, we noted that *Spiroplasma* bacteria often appeared to be less abundant in the vicinity of the oocyte and between follicle cells of $Yp1^{TS1}$/+ flies. This suggests that an association between *Spiroplasma* bacteria and yolk could be required for *Spiroplasma* bacteria to reach the oocyte surface prior to Yolkless-mediated translocation into the oocyte.

DISCUSSION

While reproductive manipulation and other strategies that directly increase host fitness can contribute to the persistence of facultative endosymbionts in insect populations, these bacteria will not be maintained without highly efficient vertical transmission (31). Therefore, facultative endosymbionts must have developed reliable and effective strategies to colonize the germ line of their hosts. To date, little is known about these mechanisms and no mutation blocking endosymbiont vertical transmission has been previously described.

In this study, we analyzed the mechanism underlying the vertical transmission of *S. poulsonii* MSRO in *D. melanogaster*. By using a *Spiroplasma*-specific antibody and immunofluorescence microscopy, we characterized the route used by *Spiroplasma* to colonize the germ line. Our results reveal that *Spiroplasma* colonizes host oocytes at specific stages, coinciding with vitellogenesis. Immunofluorescence images show that *Spiroplasma* cells accumulate at the posterior pole of the oocyte, pass between follicle cells, and are ultimately internalized within large yolk granules in the oocyte. Consistent with our observations, an electron microscopy study also demonstrated the presence of *Spiroplasma* cells within granules in the oocyte (18). Using a genetic approach, we further demonstrate that *Spiroplasma* requires the yolk transport and uptake machinery to achieve efficient vertical transmission. First, we observed that *Spiroplasma* bacteria are blocked and tend to accumulate in the region surrounding the oocytes of females

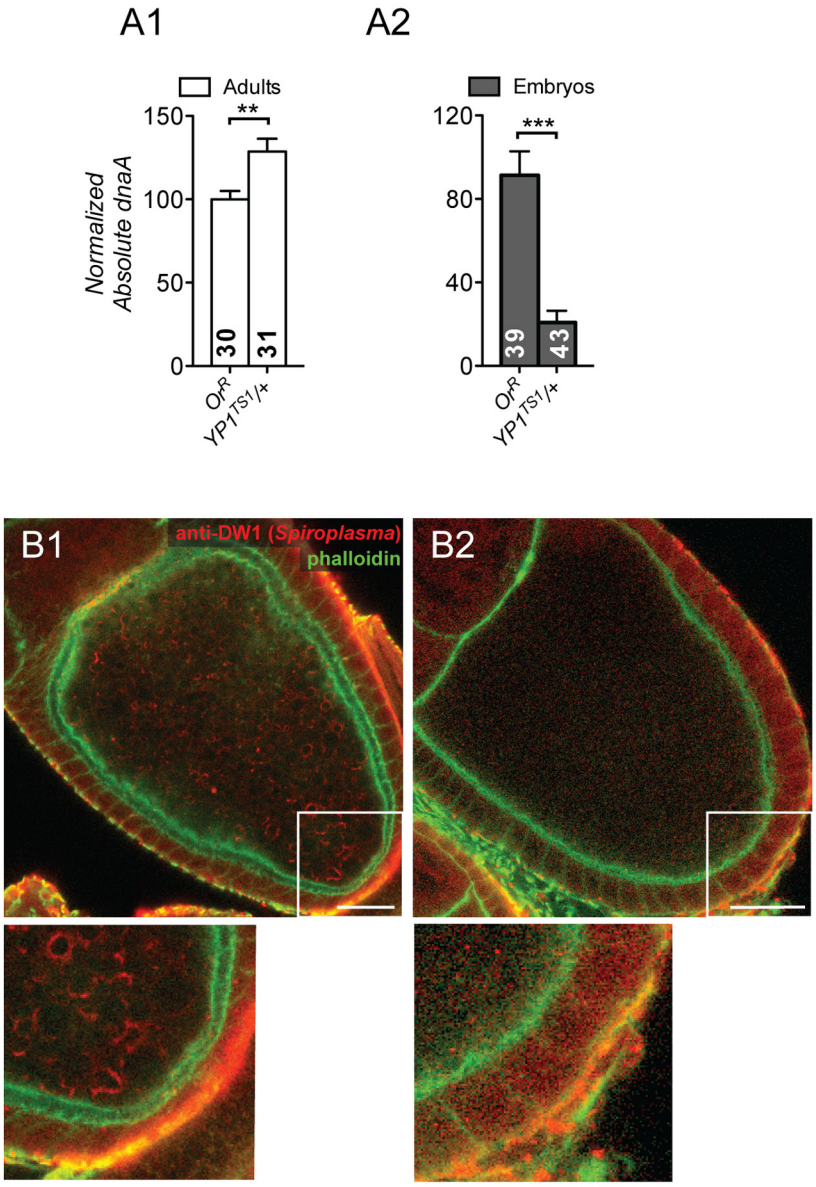

FIG 3 Yolk protein involvement in *Spiroplasma* transmission. (A1) *Spiroplasma* levels in flies are shown for the control (OR^R) and Yolk protein 1 ($YP1^{TS1}/+$). The qPCR data were normalized in a manner identical to that used for Fig. 2A. $YP1^{TS1}/+$ flies had significantly more *Spiroplasma* bacteria than their OR^R counterparts ($P = 0.0017$) (A2). Mean *Spiroplasma* levels in embryos are shown for the control (OR^R) and Yolk protein 1 ($YP1^{TS1}/+$). Embryos collected from $YP1^{TS1}/+$ flies had significantly fewer *Spiroplasma* bacteria than control embryos did ($P < 0.0001$). Error bars represent the standard error of the mean; the number of samples collected independently for DNA extraction is reflected by the value in each bar. ** and *** denote the levels of statistical significance in a Mann-Whitney U test. (B) Stage 10 oocytes from OR^R control (B1) and $YP1^{TS1}/+$ mutant (B2) flies. Note the lack of *Spiroplasma* bacteria in the follicle cell layer and the lower levels associated with the muscular epithelium in B2 (see image analysis in Fig. S1 in the supplemental material). The images at the bottom are higher magnifications of the insets.

lacking the Yolkless receptor, indicating that this endocytic receptor is specifically required for the translocation of *Spiroplasma* cells across the surface of the oocyte. Blocking endocytosis by removing Rab5 had a similar effect on *Spiroplasma* levels in the oocyte, although the results were less clear since the absence of Rab5 also caused major deformations of the oocyte. Collectively, our results suggest that endocytosis through the Yolkless receptor is the main route of germ line colonization and is crucial for ensuring vertical *Spiroplasma* transmission. In addition, we observed that germ line colonization was also impaired in $Yp1^{TS1}/+$ flies, which are known to have a reduced amount of yolk in their hemolymph. In contrast to yl^{13} flies, fewer *Spiroplasma* bacteria appeared to reach the follicle cell layer and the oocyte vicinity of $Yp1^{TS1}/+$ females. Comparison of the observed *Spiroplasma* fates due to these two mutations allows us to envisage a model in which yolk is involved in *Spiroplasma* bacteria gaining access to the exterior of the oocyte and then the yolk receptor Yolkless becomes important for endocytosis of *Spiroplasma* cells in yolk granules into the germ line. It is notable that despite their lowered levels, *Spiroplasma* bacteria were still detected in oocytes and eggs of yl^{13}

and $Yp1^{TS1}$/+ females. This might be due to the residual yolk endocytic activity of yl^{13} mutant oocytes and the fact that the $Yp1^{TS1}$/+ mutants do not experience a complete blockage of yolk secretion. However, the existence of a less efficient, yolk-independent entry route also cannot be excluded.

Spiroplasma citri is a plant pathogen that is vectored by leafhoppers and whose infection cycle involves crossing the insect gut, moving through the hemolymph, and colonizing the salivary glands (32). At several stages during the process of infection, *S. citri* has been observed undergoing endocytosis (33). In conjunction with our present study on *S. poulsonii* MSRO, these findings suggest that *Spiroplasma* might have a general capacity to interact with the host endocytic machinery to ensure its transmission. Future studies should identify *Spiroplasma* factors, presumably cell membrane based, that mediate an interaction with host endocytic machinery. Endocytosis is likely to play an important role in the vertical transmission of other endosymbionts, as in the aphid obligate symbiont *Buchnera*, which is vertically transmitted via a process that involves highly specific exocytosis from the bacteriocyte, followed by endocytosis into the blastula (34).

Interactions between *Spiroplasma* and insect hosts usually exhibit a high degree of specificity. For example, inherited *Spiroplasma* strains introduced into novel *Drosophila* species are often poorly transmitted from mother to offspring (35). *S. citri*, which normally infects leafhoppers, grows well in *D. melanogaster* hemolymph but cannot gain access to the oocyte and as a consequence is not vertically transmitted (36). Our results suggest that *Spiroplasma* host specialization could be linked to its capacity to interact with the yolk transport and uptake machinery of its native host. *Drosophila* yolk proteins are not homologous to vitellogenin, which is the principal component of yolk in many other insect species (37). It is therefore interesting that, while there are a numerous examples of interspecific transfers of *Spiroplasma* between *Drosophila* species (38), there are no documented cases of *Spiroplasma* having been transferred from *Drosophila* to, and maintained by vertical transmission in, more distantly related insect taxa. It is also notable that most heritable spiroplasmas in *Drosophila* are members of the *S. citri*-*S. poulsonii* clade, whereas most heritable spiroplasmas in other insect taxa (*Coleoptera*, *Lepidoptera*) belong to the *S. ixodetis* clade (10). It would be interesting to determine whether differences in the nature of yolk can explain the global patterns of *Spiroplasma* host distribution.

Our results indicate that yolk is required for efficient vertical transmission but does not affect the growth of *Spiroplasma*. Indeed, we observed normal and higher *Spiroplasma* titers in the hemolymph of yl^{13} homozygous or $Yp1^{TS1}$ heterozygous flies, respectively, despite the fact that these, respectively, have higher or lower levels of hemolymph yolk than wild-type flies do (21, 29). In addition, *S. poulsonii* MSRO also grows when injected into the body cavity of a male host (data not shown). Since males do not produce yolk, this indicates that the host factors exploited for the transmission and growth of *Spiroplasma* bacteria are distinct. This might have evolutionary significance, since the use of yolk as a nutrient source by *Spiroplasma* could have a direct negative impact on host fecundity and as a consequence would be detrimental to the fitness of a strictly maternally transmitted symbiont, such as *Spiroplasma*. In agreement with this, *Spiroplasma* does not detrimentally affect the fecundity of its *Drosophila* host under laboratory conditions (39). It is also worth noting that a recent microarray study of *D. melanogaster* harboring different strains of *Spiroplasma* revealed that these had the capacity to perturb the expression of host yolk genes (40). This suggests that that the interaction between *Spiroplasma* and yolk might be multifaceted, but the implications of these gene expression changes for *Spiroplasma* transmission are not clear.

Recent studies have elucidated aspects of endosymbiont transmission in two insect species whose mode of reproduction is quite different from that of *Drosophila*. In the viviparous tsetse fly, vertical transmission of the endosymbionts *Sodalis* and *Wigglesworthia* appears to be linked to the transfer of milk from mother to offspring (41). Endosymbiont transmission has also been studied in aphids, where the endosymbiont *Buchnera* is transferred from maternal bacteriocytes to ovary blastulae by a series of coordinated exocytosis-endocytosis events (34).

In *Drosophila*, aspects of maternal transmission of *Wolbachia* have been elucidated. *Wolbachia* is the only other bacterial endosymbiont group harbored by *D. melanogaster* (42). In contrast to *Spiroplasma*, *Wolbachia* has a predominantly intracellular lifestyle, being found in many cell types throughout the body of the host. High-fidelity vertical transmission of *Wolbachia* appears to be linked to embryonic colonization and maintenance of the bacteria in the germ line (43–45), as well as tropism for the ovariole's somatic stem cell niche, which could enable recolonization of the germ line in adults (46). The situation observed with *Wolbachia* is rather different from that which we describe, as *Spiroplasma* reaches the germ line only in adults and at later stages of oogenesis, using the yolk uptake machinery. This difference may result from the fact that, in contrast to *Wolbachia*, *Spiroplasma* bacteria are primarily extracellular. Cooption of the yolk transport and uptake machinery appears to be a powerful route of germ line entry for endosymbiotic bacteria with an extracellular niche. Intriguingly, this strategy of gaining access to the germ line could have relevance beyond bacterial endosymbionts. A study has shown that the endogenous retrovirus of *D. melanogaster*, ZAM, is transmitted from follicle cells to the oocyte by a mechanism that is linked to endocytic yolk uptake (47). It has also been reported that the vertically transmitted protozoan parasite *Babesia* exhibits transmission efficiencies that are affected by the rate of tick-host yolk uptake (48). Recruitment and subversion of the yolk machinery could therefore be a widely used strategy to specifically target the germ line. As a crucial point of interaction between the host and a vertically transmitted endosymbiont, this interface could be central for determining endosymbiont-host specificity. A better understanding of the mechanistic basis of routes to germ line colonization is important because it might facilitate the generation of novel endosymbiont-insect vector combinations that do not transmit pathogens that cause disease in humans.

MATERIALS AND METHODS

Fly stocks and handling. We used a wild-type OR^R fly stock that harbors *S. poulsonii* MSRO (36, 49) but not *Wolbachia*. Fly stocks with mutant alleles were derived by crosses with *Spiroplasma*-harboring OR^R females and maintained as previously described (36). Embryos were collected from 2- to 4-day-old flies by using embryo collection cages and yeasted grape juice plates. All embryos were less than 4 h old when collected for DNA extraction. The alleles yl^{13} and yl^{15} originate from different mutagenesis screenings (50, 51). To generate $rab5^2$ homozygous germ line clones, *Spiroplasma*-infected females of the genotype *y w hs-flp*; $Rab5^2$ *FRT40A*/*P*[*ovoD1*] *FRT40A* were heat shocked for 1.5 h at 37°C several times during late larval stages to induce FLP (flippase)-mediated recombination at the FRT site. The female-sterile dominant mutation *P*[*ov*-

odD1] was used to eliminate Rab5$^+$ germ line cells as previously described (25, 52). The *Rab5*^{2} *FRT40A*/*CyO* stock was obtained from Antoine Guichet. *Yp1*^{TS1}/+ larvae and controls were shifted to the restrictive temperature of 29°C as L1 larvae and maintained at this temperature for the duration of the experiments.

Fluorescence microscopy. Ovaries were processed and stained by standard immunofluorescence techniques (53). Stages of oogenesis were based on the published descriptions (14). All flies were 2- to 4-day-old virgins at the time of dissection. The following stains and antisera were used at the indicated dilutions: rabbit polyclonal anti-DW1 antibody against *Spiroplasma poulsonii* strain DW-1T (54) (1:500), anti-MSRO antibody generated against *S. poulsonii* MSRO isolated from *Drosophila* hemolymph (1:500), 488-conjugated phalloidin (Molecular Probes) (1:200), and 594 goat anti-rabbit IgG secondary antibody (Molecular Probes) (1:200). Ovaries were observed on a Zeiss LSM 700 confocal microscope. Images were analyzed with the ImageJ software package (55). We projected the maxima of all of the confocal sections corresponding to the interior of stage 10 oocytes. After gamma correction (value = 0.45), all maximum projected images were thresholded to create binary images; we then calculated the percentage of the area inside the oocyte that has a signal in the 594-nm (red) channel. To reduce noise, we did not count any signal from particles of fewer than 3 pixels. Percent coverage was normalized to the average of *OR*^{R} controls for each experiment (average of *OR*^{R} controls reflecting 100% for that experiment) so that data from different experiments could be pooled. We noted that oocytes lacking yolk (*yl*^{13}, *YP1*^{TS1}, and *Rab5*^{2}) were more transparent, since a signal could be observed from stacks that correspond to positions deeper in the tissue, and as a result, we expect that these quantifications are a conservative estimate of reduced transmission to oocytes of these genotypes. Microscope settings were kept constant within each experiment.

Electron microscopy. Ovaries were dissected in phosphate-buffered saline and then transferred to heptane that had been equilibrated with a fresh solution of cacodylate buffer containing 2% acrolein and 2.5% glutaraldehyde. They were left at room temperature for 10 min and then rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) before being postfixed for 40 min in 1% osmium tetroxide in the same buffer. They were then dehydrated in a graded series of ethanol and embedded in Epon epoxy resin and cured overnight at 60°C. Fifty-nanometer-thick sections were cut with a diamond knife by using a Leica ultramicrotome (UC7; Leica Microsystems), stained with uranyl acetate, and lead citrate, and then imaged in a Phillips TECNAI Spirit electron microscope operating at 80 kV.

DNA extraction and qPCR. We extracted DNA from between 25 and 50 embryos per sample. In all cases, the quantity of embryos collected was the same for all treatments within a single experiment. For extractions from flies, five flies were used per sample in all cases. DNA extraction, qPCR protocols, and standard curves have been previously described (36). *Spiroplasma* absolute *dnaA* gene quantification values indicate the amounts of *Spiroplasma* bacteria in embryos or flies and were not normalized to a host gene because of variability in host gene levels between embryo genotypes (nonviable genotypes having lower host gene levels). For adults, we found that normalization to a host gene, *RPS17*, did not alter the results and these data are therefore not shown.

Data treatment and statistical analysis. Each experiment was repeated independently a minimum of three times. The data shown in Fig. 2A and 3A were treated as follows. Each repeat was normalized to the average of the control values (*OR*^{R} embryos or flies) for the experiment in question, and then the normalized values of all repeats from all experiments were pooled. Image quantification data were also normalized and pooled between experiments. Statistical significance was calculated by using a two-tailed Mann-Whitney test, and differences were considered significant if the *P* values were lower than 0.05. Asterisks indicate the levels of significance as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, nonsignificant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00532-12/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.

Figure S2, PDF file, 0.1 MB.

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