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Interspecific Transfer of Bacterial Endosymbionts between Tsetse Fly Species: Infection Establishment and Effect on Host Fitness▽

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Tsetse flies (*Glossina* spp.) can harbor up to three distinct species of endosymbiotic bacteria that exhibit unique modes of transmission and evolutionary histories with their host. Two mutualist enterics, *Wigglesworthia* and *Sodalis*, are transmitted maternally to tsetse flies' intrauterine larvae. The third symbiont, from the genus *Wolbachia*, parasitizes developing oocytes. In this study, we determined that *Sodalis* isolates from several tsetse fly species are virtually identical based on a phylogenetic analysis of their *ftsZ* gene sequences. Furthermore, restriction fragment-length polymorphism analysis revealed little variation in the genomes of *Sodalis* isolates from tsetse fly species within different subgenera (*Glossina fuscipes fuscipes* and *Glossina morsitans morsitans*). We also examined the impact on host fitness of transinfecting *G. fuscipes fuscipes* and *G. morsitans morsitans* flies with reciprocal *Sodalis* strains. Tsetse flies cleared of their native *Sodalis* symbionts were successfully repopulated with the *Sodalis* species isolated from a different tsetse fly species. These transinfected flies effectively transmitted the novel symbionts to their offspring and experienced no detrimental fitness effects compared to their wild-type counterparts, as measured by longevity and fecundity. Quantitative PCR analysis revealed that transinfected flies maintained their *Sodalis* populations at densities comparable to those in flies harboring native symbionts. Our ability to transinfect tsetse flies is indicative of *Sodalis* ' recent evolutionary history with its tsetse fly host and demonstrates that this procedure may be used as a means of streamlining future paratransgenesis experiments.

Trypanosomiasis in humans and nagana in other animals are highly debilitating diseases that are fatal if left untreated. According to the World Health Organization, at least 100,000 new cases of trypanosomiasis were reported each year over the past decade, and 60 million more people in 36 African nations are at risk of acquiring the disease.

Trypanosome transmission requires interaction between several organisms, including the pathogen, the insect vector, wild reservoirs, and the human host. While this interdependence can be complicated, it provides numerous opportunities for interfering with disease transmission. However, current solutions, which involve treating infected hosts chemotherapeutically and/or inhibiting further transmission by attempting to reduce the insect vector population with traps or insecticides, are impractical, are harmful to the environment and public health, and have had limited sustainability in the field (4, 12, 13). Recent advances in molecular biology provide opportunities to use transgenic technologies for the purpose of diminishing vectorial capacity via genetic manipulation (5).

Tsetse flies (Diptera: Glossinidae), which feed exclusively on vertebrate blood, harbor three distinct species of endosymbiotic bacteria that presumably play different roles in the flies. The obligate mutualist *Wigglesworthia glossinidia* is a member of the family *Enterobacteriaceae*. This bacterium resides mainly within the cells (bacteriocytes) of a specialized organ in the tsetse fly's anterior midgut called the "bacteriome" and has a highly streamlined 700-kb genome. A large proportion of its proteome encodes vitamin biosynthesis pathways that may help supplement the tsetse fly's vertebrate-blood-specific diet (2). This hypothesis is supported by the fact that tsetse flies become sterile in the absence of *Wigglesworthia*, but this reaction can be reversed by feeding the flies vitamin-supplemented blood (27, 28).

Tsetse flies harbor a second mutualistic symbiont, of the genus *Sodalis*, which is also a member of the family *Enterobacteriaceae*. This secondary symbiont has broad tissue tropism and can be found both inter- and intracellularly in the midgut, muscle, fat body, milk gland, and salivary glands of certain species (8). Analysis of the *Sodalis* genome sequence reveals that this organism is closely related, both in terms of genome size and chromosomal synteny, to several free-living pathogens, including *Salmonella* and *Yersinia* (35). However, the large number of pseudogenes (972) present on the *Sodalis* chromosome, in conjunction with an unusually low protein coding density (49.2%), may represent early genome erosion events during the course of symbiosis as the organism adapted to its host's physiological environment (1, 35). Using *Escherichia coli* macroarrays, Rio et al. (30) utilized a heterologous array hybridization approach to determine that significant differences exist between the genomes of *Sodalis* and another, closely related symbiotic bacterium (*Sitophilus oryzae* primary

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endosymbiont [SOPE]) from the rice weevil (18). These differences may account for the fact that each organism has adapted to the unique nutritional requirements of its host. These data suggest a specific, anchored relationship between symbiont and host and, furthermore, indicate an inability to readily undergo horizontal transmission and establishment in distinct insect taxa (30).

The tsetse fly's third symbiont (9), a member of the genus *Wolbachia*, is a rickettsia-like parasitic bacterium found only in certain populations of tsetse flies, as well as some other insects, mites, crustaceans, and filarial nematodes (29, 41). The role of *Wolbachia* in tsetse flies is unknown, although in other organisms it causes a variety of reproductive abnormalities, the most common of which is called "cytoplasmic incompatibility" (CI) (35). Recently, several groups have performed *Wolbachia* "transinfection" experiments, during which this bacterium was transferred between different hosts with the intention of determining the impact each organism exerts on the other. For example, McGraw et al. (24) transinfected *Drosophila simulans* with the overreplicating *Wolbachia* sp. strain popcorn, originally isolated from *Drosophila melanogaster*. Initially, high *Wolbachia* ovary densities likely caused a reduction in the reproductive fitness of the recipient flies. However, both conditions (high density and detrimental fitness costs) were rapidly reversed in subsequent generations, leading the authors to speculate that the host was intimately involved in regulating *Wolbachia* replication rates. In a different study, a *Wolbachia* strain (*w*Kue) that induces CI in its native host (*Ephestia kuehniella*) was transferred to another moth host (*Ostrinia scapulalis*) that normally expresses *Wolbachia*-induced male killing (*O. scapulalis* was cleared of its native *Wolbachia* by tetracycline treatment). Thereafter, the recipient host expressed CI similar to that of the donor, thus demonstrating the crucial role of the *Wolbachia* genotype in determining the type of reproductive abnormality (34).

These symbioses are successful in large part because the above-mentioned bacteria have adapted to the tsetse fly's unique viviparous reproductive physiology. Adult females produce a single egg per gonotrophic cycle, which hatches in utero. The offspring completes its development through three larval instars in utero, during which time it acquires nutrients and both gut symbionts via milk gland secretions from the mother. *Wolbachia* bacteria, which reside primarily in reproductive tissue, are transmitted transovarially (29). Given the sterile nature of its vertebrate-blood-specific diet, the tsetse fly's resident microbial community is highly restricted in comparison to those of insects that feed on multiple diets throughout their development.

Sodalis is of great interest from the perspective of controlling trypanosomiasis. A procedure previously developed, called "paratransgenesis," involves expressing a foreign protein in *Sodalis* (6). These recombinant *Sodalis* bacteria are then microinjected into the hemocoels of fertile female flies. The tsetse fly's unique viviparous reproductive biology (females use milk gland secretions to nourish three larval instars) ensures that recombinant symbionts are vertically transmitted to the progeny, where they inhabit the same body compartments as they do in their native host (8). *Sodalis* is ideally suited for use in paratransgenesis because it resides in the tsetse fly's midgut in close proximity to pathogenic trypanosomes, it can be cultured and genetically modified, and recombinant clones can be introduced into females, where they are vertically transmitted to the offspring (31). Furthermore, *Sodalis*' annotated genome indicates that the bacterium has reduced metabolic capabilities that would severely hinder (or most likely completely eliminate) survival outside of its normal host (36). In fact, attempts to colonize flies by including recombinant *Sodalis* in their blood meals have not been successful, in support of the maternal-transmission mode (unpublished data). Several antimicrobial peptides, which have little or no effect on *Sodalis* but are toxic to trypanosomes, have been identified for use in this system as transgene products (16, 21).

We conducted experiments to determine the level of biological integration shared by *Sodalis* and its tsetse fly host species, with the goal of streamlining the paratransgenesis approach for future field studies. Tsetse flies were chosen from distinct species groups (*Glossina fuscipes fuscipes* from the *palpalis* group and *Glossina morsitans morsitans* from the *morsitans* group) (7) with the intention of using individuals that were as distantly related as possible. Phylogenetic reconstruction analysis and restriction fragment length polymorphism (RFLP) analysis were performed to determine the relatedness of *Sodalis* strains from the aforementioned tsetse fly species. A transinfection procedure novel to this model system was developed to ascertain whether *Sodalis* from one tsetse fly species can successfully colonize another tsetse fly species and be vertically transmitted to the offspring without altering maternal fecundity. Finally, quantitative PCR (QT-PCR) analysis was initiated to investigate *Sodalis* symbiont densities in the progeny of wild-type (WT) and transinfected tsetse flies. We discuss the significance of our findings in the context of improving the application of tsetse fly paratransgenesis.

MATERIALS AND METHODS

Insects and bacterial cultures. Phylogenetic studies were conducted on field-collected tsetse flies (*Glossina austeni*, Shimba Hills, Kenya; *Glossina pallidipes*, Arba Minsch, Ethiopia; *Glossina brevipalpis*, Shimba Hills, Kenya; *Glossina morsitans submorsitans*, Tanzania; and *Glossina tachinoides* and *Glossina pallidipes gambiensis*, Burkino Faso) and individuals colony-reared at Yale University. Rice weevils (*Sitophilus oryzae*) were reared in Villeurbanne (BF2I), France. Transinfection experiments were performed using *G. fuscipes fuscipes* and *G. morsitans morsitans* tsetse flies maintained in Yale's insectary at 24°C with 40 to 50% relative humidity. These flies received defibrinated bovine blood every 48 h through an artificial-membrane feeding system (26).

Sodalis glossinidius morsitans and *Sodalis glossinidius fuscipes* were isolated from surface-sterilized *G. morsitans* and *G. fuscipes* pupae and cultured on *Aedes albopictus* C6/36 cells as described previously (10). The cultures were subsequently maintained in vitro (in the absence of C6/36 cells) at 25°C in Mitsuhashi-Maramorosch (M-M) medium [1 mM $CaCl_2$, 0.2 mM $MgCl_2$, 2.7 mM KCl, 120 mM NaCl, 1.4 mM $NaHCO_3$, 1.3 mM NaH_2PO_4, 22 mM D-(+) glucose, 6.5 g/liter lactalbumin hydrolysate, and 5.0 g/liter yeast extract] supplemented with 5% heat-inactivated fetal bovine serum (6).

Phylogenetics. The gene sequence encoding the cell division protein FtsZ was analyzed to phylogenetically compare different species of *Sodalis* and other closely related enterics. The gene fragment was PCR amplified using primers (F, 5′-GACGCGGTGATTAAAGTC-3′; R, 5′-CGATATCTCCAGCAACG-3′) designed on the basis of conserved regions (730 bp) of the *ftsZ* gene among eubacteria. All 50-μl PCR amplification reactions were performed in an MJ Research thermal cycler using the following procedure: 95°C for 5 min, followed by 35 cycles at 95°C, 55°C, and 72°C, each for 1 min, and a final 7-min elongation/extension. All PCR products were cloned into the pGEM-T vector (Promega) and sequenced using the T7 and SP6 primers. A minimum of three clones were sequenced from each amplification reaction. Symbiont genes were analyzed from a minimum of three flies for each *Sodalis* and *Sitophilus* species.

Comparative sequence analyses for FtsZ genes were conducted using the

DNAStar software package (Lasergene, Madison, WI), and tree generation was performed using the algorithms in PAUP 4.0 (Swofford).

RFLP analysis. WT *S. glossinidius morsitans* and *S. glossinidius fuscipes* were embedded in 0.5% agarose plugs (~10^9 cells/plug) and subsequently treated with the following incubations: (i) 50°C and 37°C, each for 12 h, in 20 volumes of lysis solution (100 mM EDTA [pH 8.0], 10 mM Tris-HCl [pH 8.0], 1% *N*-lauroylsarcosine, 100 μg/ml proteinse K); (ii) three times for 1 h each time at room temperature in 10 volumes of storage buffer (100 mM EDTA [pH 8.0], 10 mM Tris-HCl [pH 8.0], 1 mM phenylmethylsulfonyl fluoride); and (iii) three times for 30 min each time at room temperature in 10 volumes of 10 mM Tris-HCl (pH 8.0). To remove plasmid and phage DNA, the plugs were subjected to contour-clamped homogeneous electric field (CHEF) gel electrophoresis at 14°C, with a ramping interval of 150 to 200 s, for 20 h at 200 V (1). The plugs were subsequently removed from the gel (1% agarose) and treated with PacI restriction endonuclease (TTAAT↓TAA [the arrow indicates the cut site]; New England Biolabs, Boston, MA) for 24 h at 37°C. The digested samples were again subjected to CHEF electrophoresis, this time using a 9.1- to 13.1-s ramping interval. Finally, the gel was stained with ethidium bromide, destained in double-distilled H_2O, and viewed using a Kodak S2000 imaging system.

***Sodalis* transformation.** WT *S. glossinidius morsitans* and *S. glossinidius fuscipes* were genetically transformed using a modified version of a previously described protocol (11). In brief, 100 ml of log-phase cells (optical density at 600 nm, ~0.5; SmartSpec Plus spectrophotometer; Bio-Rad, Hercules, CA) were washed consecutively in 50 ml, 10 ml, and 2 ml of sterile 10% glycerol. The cell pellets were resuspended in 200 μl of sterile 10% glycerol, and 50-μl aliquots were mixed with ~50 ng of eGFPuv plasmid (BD Bioscience, Palo Alto, CA) and subjected to electroporation (voltage, 1.9 kV; capacitance, 25 μF; resistance, 200 Ω). Recombinant *Sodalis* (rec*Sodalis*) cells were immediately placed into 950 ml of M-M medium and allowed to recover for 24 h. Following this recovery, the *Sodalis* cells were placed onto a feeder layer of C6/36 cells, and transformants were selected with ampicillin (10 μg/ml) for another 24-h period. Finally, the cells were plated onto M-M agar (supplemented with antibiotics), and a single rec*Sodalis* colony was placed into liquid culture (37).

The growth rates of rec*Sodalis* cultures were established by measuring the optical densities at 600 nm of two clonal populations of each species over two 1-week periods.

Symbiont reintroduction into tsetse flies. Newly eclosed adult female *G. morsitans morsitans* and *G. fuscipes fuscipes* flies were given a blood meal supplemented with 30 μg/ml ampicillin prior to injection. Each fly species was then divided into two groups. One group from each fly species received an injection of ~1.5×10^4 rec*Sodalis* bacteria originally isolated from its own species, and the other group received an injection of rec*Sodalis* bacteria originally isolated from the other species. Hereafter, the treated fly groups will be referred to as follows: tsetse species/*Sodalis* species (e.g., *G. morsitans morsitans*/*S. glossinidius morsitans*).

All injections were performed on flies anesthetized with CO_2, using glass needles and a Narashige IM300 microinjector. Following injection, all groups of flies (n = 20 for each group) were mated (n = 10 males) and subsequently maintained on their designated diets. Two other groups, both of which received no injection, were fed either normal blood (*G. fuscipes fuscipes*^{REG} and *G. morsitans morsitans*^{REG}) or blood supplemented with ampicillin (*G. fuscipes fuscipes*^{AMP} and *G. morsitans morsitans*^{AMP}). All groups were subsequently maintained in separate cages and received the appropriate diet (as indicated in Table 1) every 48 h throughout the course of the experiment.

Tsetse fly fitness and rec*Sodalis* reestablishment. The number of pupae and the number of deceased mothers from each group were recorded following each of four 15-day larval-deposition cycles. Total DNA was extracted from mothers and offspring at designated times using the method of Holmes and Bonner (19). The presence of rec*Sodalis* in treated females was determined by using a PCR amplification assay. The primers used (F, 5′-AGTGGAGAGGGTGAAGGTGA-3′; R, 5′-ATCCCAGCAGCAGTTACAAA-3′) targeted a 600-bp fragment of the gene encoding the enhanced green fluorescent protein (eGFP) from the eGFPuv transformation plasmid (BD Biosciences, Palo Alto, CA). To confirm vertical transmission of rec*Sodalis*, fly DNA from 14-day-old adult progeny from each group of treated mothers was PCR amplified (F, 5′-ACCGACTGGGGACAGTACGATG-3′; R, 5′-CAAAGAAGTCATAGGTCATAAC-3′) using primers specific for the *Sodalis* chromosomal exochitinase gene (849-bp amplicon; GenBank accession no. BSPY11391). PCR amplification, using an MJ Research (PTC-200) thermal cycler, consisted of a 2-min hot start at 94°C, followed by 30 cycles at 94°C for 30 s, 54°C for 40 s, and 72°C for 1 min and a final elongation/extension at 72°C for 7 min. The amplified products were verified by agarose gel electrophoresis.

TABLE 1. Fly groups, *Sodalis* reconstitution each group received, and their subsequent blood meals

Experimental group	Fly species	*Sodalis* sp. used for reconstitution[a]	Blood meal supplement
1	*G. morsitans morsitans*	None	None
2	*G. fuscipes fuscipes*	None	None
3	*G. morsitans morsitans*	None	Amp[b]
4	*G. fuscipes fuscipes*	None	Amp
5	*G. morsitans morsitans*	*S. glossinidius morsitans*	Amp
6	*G. morsitans morsitans*	*S. glossinidius fuscipes*	Amp
7	*G. fuscipes fuscipes*	*S. glossinidius fuscipes*	Amp
8	*G. fuscipes fuscipes*	*S. glossinidius morsitans*	Amp

[a] All injections were performed with recombinant *Sodalis* harboring the eGPFuv plasmid. Each fly injected received ~1.5×10^4 rec*Sodalis* bacteria.
[b] Amp, ampicillin (30 μg Amp/ml blood).

Measuring symbiont genome numbers and densities. Total DNA was extracted from (i) in vitro cultures of *S. glossinidius fuscipes* and *S. glossinidius morsitans*, (ii) 2-week-old adult offspring of WT mothers, (iii) 2- and 60-day-old mothers treated with rec*Sodalis* harboring the eGFPuv plasmid, and (iv) 2-week-old adult offspring of aposymbiotic WT flies (these individuals were fed an antibiotic-supplemented diet and served as a background control). The Holmes and Bonner (19) method of DNA extraction was used in all cases.

QT-PCR was performed with an icycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA). A 117-bp region of the single-copy *Sodalis* exochitinase gene was amplified with the following primers: F, 5′-TTATACCGACTGGGGACAGT-3′, and R, 5′-ATAGGCGGTCGGGGATAATT-3′. A specific fluorescent beacon designed for the central portion of the amplicon (Sg*chi* probe, 5′-FAM-CCGCCGCGTTGATGTCGGGAGTCGTGGCCGGCGG-DABCYL-3′) was utilized for chitinase quantification. PCR reagents included PLATINUM Quantitative PCR SuperMIX-UDG (Invitrogen, Carlsbad, CA; catalog no. 11730-017) and the addition of 1.7 μl of template, specific primers, and beacon (1.5 U PLATINUM *Taq* DNA polymerase, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 200 μM dATP, 200 μM dGTP, 200 μM dCTP, 400 μM dUTP, 1 U UDG, 0.2 μM of each primer, and 0.2 μM beacon) in a final reaction volume of 50 μl. The amount of fluorescence generated was measured during each amplification cycle (8 min and 30 seconds at 95°C, followed by 40 cycles at 95°C for 15 seconds and 1 min at 56°C). Optical-density readings were used to normalize the amount of individual DNA (μg) analyzed so that *Sodalis* density would be represented as the *chitinase* copy number per tsetse fly). Symbiont density estimates were obtained by comparison to a standard curve using Bio-Rad icycler iQ Multi-Color Real Time PCR data analysis software. All assays were carried out in 96-well format plates in duplicate and were averaged for each sample. Negative controls were included in all amplification reactions.

Statistical analysis. Fitness and QT-PCR data are all presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using SAS system v9.1 for Windows. The specific statistical test used to analyze each data set is indicated where appropriate in Results. Where densities appeared skewed, the data were log transformed to satisfy normality. Differences were considered significant at a P value of ≤0.05.

RESULTS

Molecular phylogenetics. A phylogenetic tree was generated based on the FtsZ gene sequences of different *Sodalis* species. This gene has been used successfully for the same purpose with different *Wolbachia* strains (41). Our results indicated that all mutations observed in the *ftsZ* locus were synonymous and that the insect symbionts *Sodalis* and SOPE form a clade related to the other enterics examined. However, the FtsZ gene sequence did not demonstrate enough variability to determine the ancestral relationships among the *Sodalis* species, although the *Sodalis* and SOPE could be described as sister taxa (Fig. 1A).

RFLP analysis was used as a means of identifying large-scale divergence between the *S. glossinidius fuscipes* and *S. glossinidius morsitans* genomes. Prior to enzymatic treatment of aga-

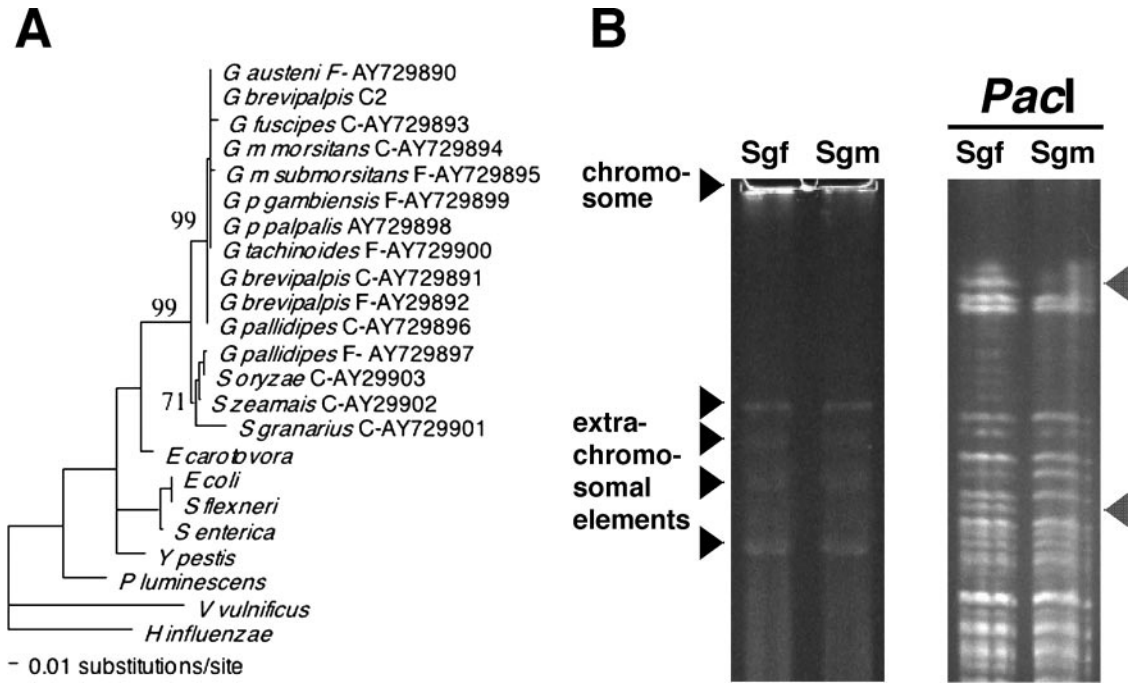

FIG. 1. Molecular phylogenetic analysis of *S. glossinidius fuscipes* and *S. glossinidius morsitans*. (A) Phylogenetic tree, based on FtsZ gene sequence information, showing the relationship between *Sodalis* strains from different *Glossina* species and related *Enterobacteriaceae* (*E. carotovora*, *Erwinia carotovora*; *S. flexneri*, *Shigella flexneri*; *S. enterica*, *Salmonella enterica*; *Y. pestis*, *Yersinia pestis*; *P. luminescens*, *Photorhabdus luminescens*; *V. vulnificus*, *Vibrio vulnificus*; *H. influenzae*, *Haemophilus influenzae*). The tree was generated by heuristic search using parsimony analysis with a bootstrap value of 500. C, laboratory culture; F, field collected. GenBank accession numbers follow each collection location. (B) RFLP analysis was used to identify variations in the chromosomes of the two *Sodalis* species examined. Extrachromosomal and genomic DNAs were separated by subjecting agarose plugs to CHEF electrophoresis at 14°C using ramping intervals of 150 to 200 s for 20 h at 200 V. The plugs containing only genomic DNA were subsequently removed from the gel, treated with PacI restriction endonuclease, and again subjected to CHEF electrophoresis (9.1- to 13.1-s ramping interval; 24 h; 200 V). The gray arrowheads indicate chromosomal polymorphisms. Sgf, *S. glossinidius fuscipes*; Sgm, *S. glossinidius morsitans*.

rose plugs containing total bacterial DNA, the *Sodalis* multicopy extrachromosomal elements were run out of the samples via CHEF electrophoresis (Fig. 1B). The remaining intact chromosomal DNA was then treated with PacI restriction endonuclease, which was chosen because of its low cutting frequency (76 cut sites within the 4.2-Mb chromosome). Analysis of the banding patterns of the digested chromosomal DNAs from the two species revealed few polymorphisms: only two over the range of DNA fragments visible on the gel (Fig. 1B). Furthermore, RFLP analysis using the PmeI restriction endonuclease (which cuts the chromosome 52 times) revealed no visible polymorphisms (data not shown). These results, in conjunction with the phylogenetic analysis of *ftsZ*, provided preliminary evidence leading us to conclude that *S. glossinidius fuscipes* and *S. glossinidius morsitans* represent closely related organisms symbolic of a recent symbiotic association with their host taxa.

rec*Sodalis* growth, reestablishment, and vertical transmission. Prior to transinfecting female flies, the in vitro growth rates of rec*Sodalis glossinidius fuscipes* and rec*Sodalis glossinidius morsitans* were established. As indicated in Fig. 2, clonal populations of both bacterial strains grew at virtually identical rates following their initial passage.

We next set out to determine whether rec*Sodalis* was able to successfully colonize transinfected females and be subsequently transmitted to their offspring. To achieve this goal, a PCR amplification approach was used to detect the *Sodalis* chromosomal exochitinase gene in female flies and offspring harboring WT *Sodalis*, and rec*Sodalis*. PCR was also used to detect the eGFPuv plasmid in mothers treated with rec*Sodalis*. All groups of treated mothers and their offspring retained the introduced rec*Sodalis* throughout the course of these experiments (Fig. 3A).

Of note is our inability to detect the GFP gene in the off-

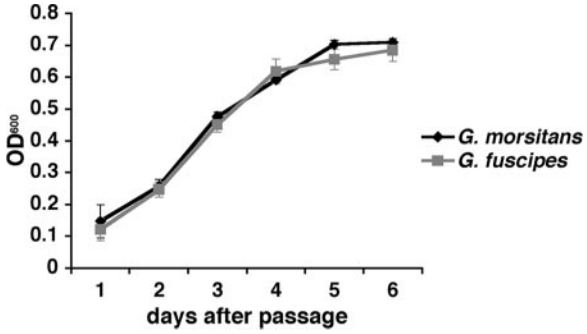

FIG. 2. Growth rates of recombinant *Sodalis glossinidius morsitans* (*G. morsitans*) and *Sodalis glossinidius fuscipes* (*G. fuscipes*) in vitro. Measurements (represented as the daily average of each population ± SEM) were recorded from two individual clonal populations over two 1-week periods following passage. OD_{600}, optical density at 600 nm.

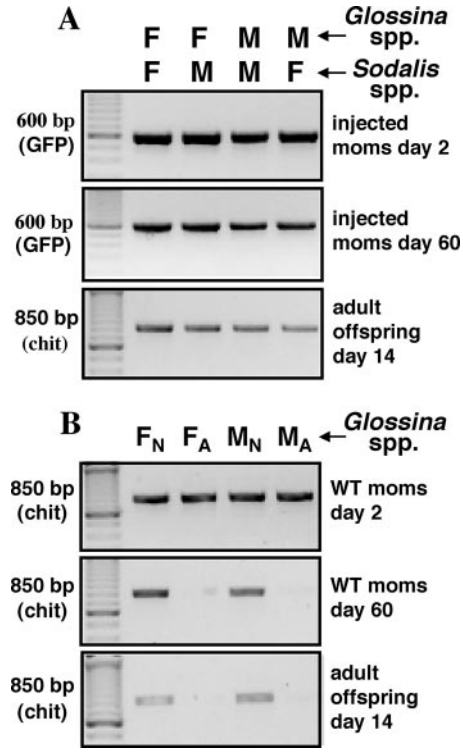

FIG. 3. Tsetse fly reconstitution with rec*Sodalis* and the effect of antibiotic treatment on symbiont flora. (A) Female tsetse flies transinfected with rec*Sodalis* and subsequently fed antibiotics. PCR revealed the presence of the eGFP gene in all mothers, thus indicating that the *Sodalis* bacteria were able to establish and maintain infections regardless of which tsetse fly species they were originally isolated from. Offspring from these mothers contained *Sodalis*, as evidenced by the presence of the chitinase gene (however, the eGFP gene was undetectable in these samples). (B) Control female flies receiving no rec-*Sodalis*. In this population, WT *Sodalis* bacteria were eliminated by day 60 from females fed antibiotics (A). Furthermore, no *Sodalis* bacteria were present in the offspring of these flies. In contrast, all females (and their offspring) that were fed regular blood (N) retained *Sodalis*. The data were generated from one individual of each group. However, initially three individuals were randomly selected for PCR analysis, and all tested positive. F, *fuscipes*; M, *morsitans*. GFP, eGFP gene; chit, *Sodalis* exochitinase gene.

spring of transinfected mothers. This phenomenon was unlikely to have resulted from loss of the eGFPuv plasmid during the 30-day pupal stage (on the contrary, unpublished data from our laboratory indicated that rec*Sodalis* bacteria grown in vitro retained their plasmids in the absence of antibiotic selection over a 3-month test period). Oxygen deprivation in the sealed environment of the tsetse fly's pupal case more likely accounted for our inability to consistently detect this gene (although very faint bands were present in some samples), as the replication and stability of plasmids carrying ColE1 replication origins (i.e., pGFPuv) can be severely impeded under such conditions (15, 20). We are currently developing a method of inserting exogenous DNA directly into the *Sodalis* chromosome via homologous recombination.

In the case of untreated mothers, all groups contained WT *Sodalis* flora at day 2. However, by day 60, WT *Sodalis* had been eliminated from those mothers fed an ampicillin-supplemented diet, and it was not present in any subsequent offspring. In contrast, when the flies were fed normal blood, WT *Sodalis* bacteria were present in all mothers and their offspring (Fig. 3B). These results demonstrate that female flies are able to reproduce when maintained on ampicillin (which does not affect the intracellular primary symbiont, *Wigglesworthia*) and that experimentally introduced *Sodalis* bacteria are efficiently transmitted to offspring.

Tsetse fly fitness following rec*Sodalis* reestablishment. The fitness effects of transferring rec*Sodalis* between tsetse fly species are shown in Fig. 4A to D. Over the course of the 60-day observation period, no significant difference in mortality was observed within the *G. fuscipes fuscipes* ($P = 0.9629$; Wilcoxon test) and the *G. morsitans morsitans* ($P = 0.7326$; log rank test) groups (Fig. 4A and B).

Fecundity, as measured by the total number of larvae deposited per group over the course of four deposition cycles, was the second parameter measured as a means of determining the fitness cost associated with interspecific symbiont-host interactions. No significant difference in numbers of larvae produced was observed within any of the *G. fuscipes fuscipes* and *G. morsitans morsitans* groups (Fig. 4C and D) ($P = 0.2492$ and 0.3269, respectively; nested two-factor analysis of variance [ANOVA]).

When comparisons were made between all of the *G. fuscipes fuscipes* groups combined and all of the *G. morsitans morsitans* groups combined, some significant differences were observed. For example, significantly fewer ($P < 0.0001$; log rank test) *G. fuscipes fuscipes* mothers survived (51% ± 10.2%) the 60-day observation period than *G. morsitans morsitans* mothers (90% ± 3%) (Fig. 4A and B). Also, a significant difference ($P < 0.05$; nested two-factor ANOVA) in the cumulative number of larvae (the total over 60 days) was observed between the *G. fuscipes fuscipes* ($n = 143$ pupae) and *G. morsitans morsitans* ($n = 226$ pupae) groups.

Measuring symbiont density. QT-PCR was used to determine the number of genomes per *Sodalis* cell grown in vitro. Both strains maintained multiple chromosomes, with *S. glossinidius fuscipes* ranging from 19 (±6.2) to 28 (±4.3) and *S. glossinidius morsitans* ranging from 23 (±3.1) to 31 (±2.4) (Table 2). This range is not significantly different between *Sodalis* species (experiment 1, $P = 0.11$; experiment 2, $P = 0.16$; Students' t test). Because both strains harbor several chromosomes, all ensuing mention of *Sodalis* density is a reflection of the genome copy number rather than absolute *Sodalis* cell numbers.

A similar approach was utilized to determine if WT *G. fuscipes fuscipes* (*G. fuscipes fuscipes*^{WT}) and WT *G. morsitans morsitans* (*G. morsitans morsitans*^{WT}) maintained similar *Sodalis* densities and if transinfected flies maintained their novel symbionts at titers similar to those of their WT counterparts. With these goals in mind, we used genomic DNA (extracted from treated and control flies) as a template to perform QT-PCR with primers that amplify a region of the *Sodalis* single-copy chitinase gene. The results, which are shown in Fig. 5, are presented as the number of *Sodalis* exochitinase gene copies per tsetse fly (and thus represent the number of *Sodalis* genomes per fly).

Two-week-old adult female progeny of flies from both WT species maintained their symbionts at virtually identical levels (*G. fuscipes fuscipes*^{WT}, $1.33 \times 10^7 \pm 4.3 \times 10^5$; *G. morsitans*

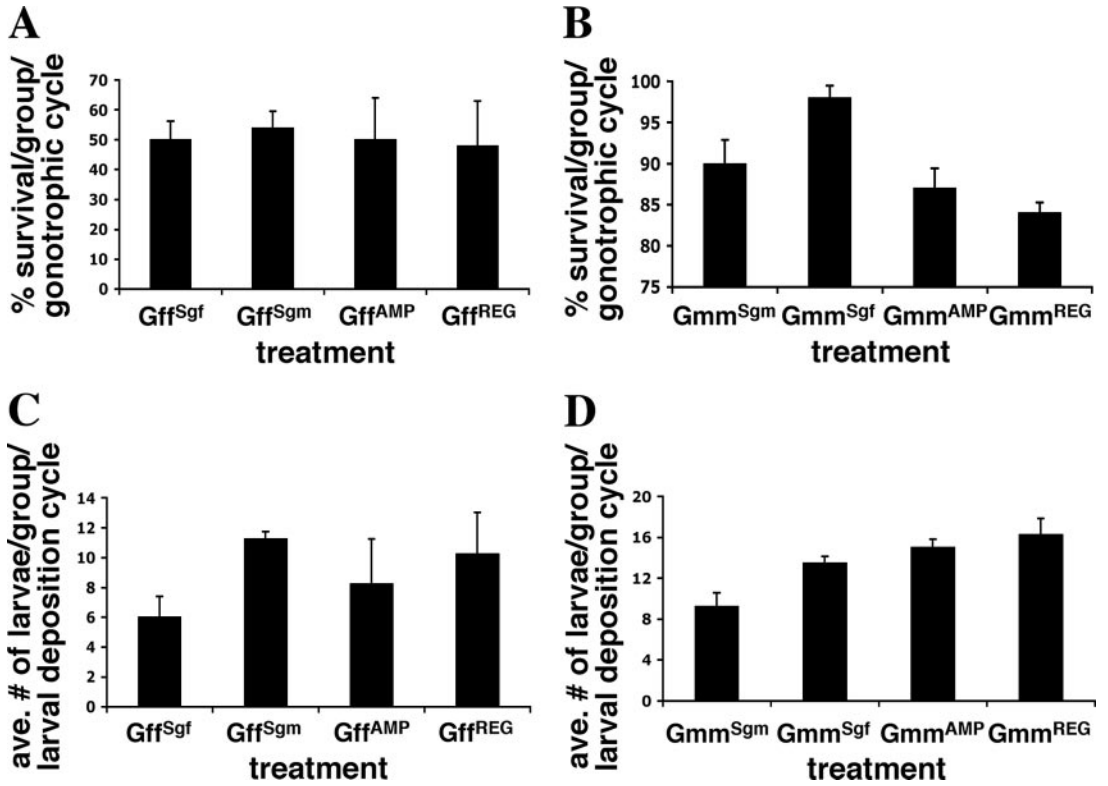

FIG. 4. Host-associated fitness costs when *Sodalis* bacteria are transferred between different tsetse fly species. (A and B) Mortality (as determined by percent survival) of *G. fuscipes fuscipes* (Gff) and *G. morsitans morsitans* (Gmm). The superscript indicates the *Sodalis* treatment received by the given fly group (Sgm, *S. glossinidius morsitans*; Sgf, *S. glossinidius fuscipes*; AMP, no injection/ampicillin-treated blood; REG, no injection/normal blood). (C and D) Fecundity (as determined by the number of larvae produced) of *G. fuscipes fuscipes* and *G. morsitans morsitans*. The error bars represent the SEM.

morsitans^{WT}, $1.57 \times 10^7 \pm 5 \times 10^6$; $P = 0.9459$) (Fig. 5) (Nested two-factor weighted ANOVAs were used for all statistical comparisons in this section). Furthermore, no significant difference was observed between *G. fuscipes fuscipes*^{WT} and *G. fuscipes fuscipes*/*S. glossinidius fuscipes* ($P = 0.0731$) and *G. fuscipes fuscipes*^{WT} and *G. fuscipes fuscipes*/*S. glossinidius morsitans* ($P = 0.1096$). However, this was not the case with the *G. morsitans morsitans* groups. Offspring of *G. morsitans morsitans*/*S. glossinidius morsitans* and *G. morsitans morsitans*/*S. glossinidius fuscipes* mothers exhibited bacterial densities significantly lower than those of their *G. morsitans morsitans*^{WT} counterparts (*G. morsitans morsitans*/*S. glossinidius morsitans* versus *G. morsitans*

TABLE 2. Number of genomes per *Sodalis* cell cultured in vitro

Exp no.[a]	*Sodalis* species	*Sodalis* cell no.[b]	*chitinase* copy no.	No. of genomes/ cell[c]
1	*S. glossinidus fuscipes*	2.2×10^8	4.2×10^9	19 ± 6.2
	S. glossinidus morsitans	1.1×10^8	3.4×10^9	31 ± 2.4
2	*S. glossinidus fuscipes*	2.1×10^8	5.9×10^9	28 ± 4.3
	S. glossinidus morsitans	2.3×10^8	5.3×10^9	23 ± 3.1

[a] Each experiment was performed in triplicate for both *Sodalis* species. All data are presented as an average of the triplicate (±SEM for genomes/cell).

[b] To ensure accuracy, *Sodalis* samples were counted twice using a Leavy counting chamber (VWR Scientific Products).

[c] The range of genomes per cell is not significantly different (as determined by Student's *t* test) between *Sodalis* species within each experiment. Experiment 1, $P = 0.11$; experiment 2, $P = 0.16$.

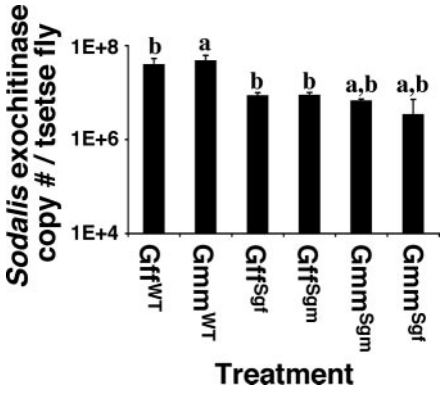

FIG. 5. Quantification of the single-copy *Sodalis* exochitinase gene as a means of determining symbiont density per treated female fly. The bars for each treatment represent the mean (plus SEM) *Sodalis chitinase* copy number for all individual flies in that group (and thus represent the number of *Sodalis* genomes per fly). All treatments have a sample size of 12. The superscript indicates the treatment received by that group of flies. All values were normalized against negative controls (antibiotic-treated aposymbiotic WT flies), which contained less than 10^2 *Sodalis* chitinase gene copies per tsetse fly in all cases. Bars with the letter "a" above them are significantly different ($P < 0.05$) from one another, while those with the letter "b" or "a,b" are not. Gmm, *G. morsitans morsitans*; Gff, *G. fuscipes fuscipes*; Sgm, *S. glossinidius morsitans*; Sgf, *S. glossinidius fuscipes*; WT, wild type.

morsitans^{WT}, $P = 0.0219$; *G. morsitans morsitans*/*S. glossinidius fuscipes* versus *G. morsitans morsitans*^{WT}, $P = 0.0003$) (Fig. 5). When both treated groups within *G. fuscipes fuscipes* (*G. fuscipes fuscipes*/*S. glossinidius fuscipes* plus *G. fuscipes fuscipes*/*S. glossinidius morsitans*) were compared to both treated groups within *G. morsitans morsitans* (*G. morsitans morsitans*/*S. glossinidius morsitans* plus *G. morsitans morsitans*/*S. glossinidius fuscipes*), no significant difference was observed ($P = 0.0911$). Furthermore, no significant difference was observed when the treated groups within each species were compared (*G. fuscipes fuscipes*/*S. glossinidius fuscipes* versus *G. fuscipes fuscipes*/*S. glossinidius morsitans*, $P = 0.8348$; *G. morsitans morsitans*/*S. glossinidius morsitans* versus *G. morsitans morsitans*/*S. glossinidius fuscipes*, $P = 0.1430$). Thus, transinfection does not compromise the tsetse fly's ability to maintain natural *Sodalis* densities.

DISCUSSION

In this study, we examined the efficacy of transinfecting female tsetse flies with nonnative symbiotic flora and whether any fitness costs are associated with this procedure. Because endogenous WT bacteria could have a competitive advantage over recombinant native and nonnative bacteria, effective removal of WT symbionts from experimental flies was imperative for the study. Our results indicate that both *G. morsitans morsitans* and *G. fuscipes fuscipes* female flies were transiently cured (via treatment with ampicillin) of their native *Sodalis* symbionts during the course of these experiments. *Sodalis* bacteria genetically modified in vitro were then able to successfully colonize their antibiotic-treated native hosts. Furthermore, *Sodalis*-free flies were effectively transinfected with rec*Sodalis* originally isolated from a different host species, and the recombinant symbiotic flora was maintained at densities similar to those in WT individuals.

Several studies, which were based on genomics information, have been performed to determine the phylogenetic positioning and species relatedness of tsetse fly symbionts. For example, Aksoy et al. (3) determined that the 16S rRNA gene sequences of *Sodalis* bacteria from five distinct tsetse fly species were practically identical (only 2 out of 1,100 bp were different between *Sodalis* isolates from the most divergent tsetse fly species examined), arguing for a recent association with their tsetse fly hosts. In contrast, a similar analysis of *Wigglesworthia* from the same tsetse fly species revealed significant differences in 16S rRNA genes from this bacterium. Furthermore, analysis of 28S rRNA gene spacer (internal transcribed spacer 2) sequences from corresponding tsetse flies indicated concordant evolution with *Wigglesworthia*, implying that a tsetse fly ancestor had been infected with this bacterium some 50 to 100 million years ago. Then, from this ancestral pair, extant species of tsetse flies and associated *Wigglesworthia* strains radiated without horizontal-transfer events between species (3). This long association is also reflected in the drastically reduced genome size of the *Wigglesworthia* bacterium (2).

Our phylogenetic analysis of *ftsZ* genes from different *Sodalis* species also supports the concept of a recent symbiotic association between this bacterium and its tsetse fly host. Accordingly, distinct *Sodalis* strains and their respective hosts have evidently undergone little cospeciation to date. Our RFLP analysis, which was performed to determine whether divergence has occurred on a larger, whole-genome scale, also did not reveal extensive genomic polymorphisms. However, the genome sequence of *Sodalis* does show a significantly reduced coding capacity and extreme genome erosion (35), both phenomena indicative of a transitioning process from a free-living to a symbiotic state. Arguing against horizontal transfer between species, O'Neill et al. (29) subjected the extrachromosomal DNAs of *Sodalis* bacteria from five tsetse fly species to RFLP analysis and determined that each one represented a unique isolate. More recently, *Sodalis* cultured from the hemolymph of *G. palpalis gambiensis* and *G. morsitans morsitans* were shown to be genetically distinct when examined by amplified fragment length polymorphism analysis (14). The exact extent of cospeciation between *Sodalis* and tsetse flies thus needs to be further examined, possibly by performing a more comprehensive RFLP analysis of the chromosomes of distinct *Sodalis* species or by utilizing loci that are under more relaxed selection for phylogenetic analysis. Our ability to transinfect tsetse flies with heterologous species of this bacterium, without apparently inflicting detrimental fitness costs, further supports a lack of extensive host-symbiont coevolution that would result in species-specific functional adaptations.

Of crucial importance for a successful paratransgenic strategy is the question of whether transinfected females exhibit a fitness cost compared to their WT counterparts. In the field, such an occurrence would give WT flies an advantage over paratransgenic flies, and the latter group would likely die off. We observed no major inhibitory effects on the fecundity or longevity of transinfected females compared to their WT counterparts. Russell and Moran (33) observed a similar response when performing transinfection experiments in the pea aphid, *Acyrthosiphon pisum*. Clonal strains of *A. pisum* naturally free of secondary symbionts exhibited no apparent reduction in fitness (as determined by measuring the "mean relative growth rate") when transinfected with three different native symbionts from infected donor strains. Interestingly, when the experiment was repeated with a novel symbiont (isolated from the aphid *Aphis craccivora*), donor *A. pisum* exhibited elevated fitness effects. Further experiments are necessary to better determine the functional role of secondary symbionts with regard to host physiology.

Of note is the significant difference in both cumulative mortality and fecundity between the *G. fuscipes fuscipes* and *G. morsitans morsitans* groups examined. Fundamental differences in the habitat preferences of these flies could account for this discrepancy. *G. fuscipes fuscipes* is a member of the *palpalis* group, which inhabits humid riparian/dense-forest environments, while *G. morsitans morsitans* (a member of the *morsitans* group) resides mainly in the drier open-savannah zone (22). All flies used in these studies were reared in an insectary environment favorable to *G. morsitans morsitans*, which may have resulted in a cumulative fitness disadvantage for *G. fuscipes fuscipes*.

Prior studies of symbiont density dynamics in *G. morsitans morsitans* indicated tight regulation of the mutualistic partners through host development and during potentially disruptive events, including host immune challenge, parasite infections, and environmental perturbations (32). Furthermore, different

tsetse fly species can harbor different *Sodalis* loads. Cheng and Aksoy (8) reported high *Sodalis* levels in midgut tissues from *G. morsitans morsitans* and *Glossina palpalis palpalis* and lower levels in *G. austeni* and *G. brevipalpis*. QT-PCR experiments performed in this study indicate similar *Sodalis* densities in *G. morsitans morsitans* and *G. fuscipes fuscipes*. However, symbiont-cured *G. morsitans morsitans* flies repopulated with recombinant native and nonnative *Sodalis* maintained these bacteria at densities significantly lower than those in their WT counterparts. It should be noted, however, that the *P* values for the *G. morsitans morsitans* groups are just below the cutoff delineating significance from nonsignificance. We speculate that had we given the *G. morsitans morsitans* progeny carrying rec*Sodalis* more than 2 weeks prior to harvesting them for assay, their *Sodalis* densities may have increased to levels comparable to those in WT individuals. In contrast, all of the *G. fuscipes fuscipes* groups examined exhibited similar *Sodalis* densities.

Our QT-PCR data are also interesting when considered in the context of tsetse fly-trypanosome interactions. We demonstrated that WT and transinfected individuals of both tsetse fly species maintain similar *Sodalis* densities. Previous experiments indicated that a positive correlation might exist between high *Sodalis* densities and trypanosome infection rates (23, 39). This hypothesis is based on the fact that lectins, which exhibit potent trypanocidal activity (38), are inhibited by sugars (i.e., *N*-acetylglucosamine) produced as by-products of the *Sodalis*-mediated metabolism of tsetse fly chitin (25, 40). Our findings of similar symbiont densities is somewhat surprising considering that *palpalis* group flies (*G. fuscipes fuscipes*) are more refractory to trypanosome infection than flies belonging to the *morsitans* group (17). Contrary to the above-mentioned hypothesis, our results imply that the degree of susceptibility to infection may not correlate solely with the *Sodalis* density. The effect of *Sodalis* transinfection on the tsetse fly's capacity to vector trypanosomes will be the target of future research.

Tsetse flies are the sole vector of African trypanosomes, the causative agent of sleeping sickness in humans and nagana in animals. One potential component of an integrated tsetse fly control strategy is called paratransgenesis, a process whereby flies are made resistant to trypanosome infection by populating them with genetically modified *Sodalis* bacteria that produce a trypanocidal compound (5). For paratransgenesis to be an effective component of a field-based trypanosomiasis control program, all tsetse fly species that vector the causative agent would theoretically need to be manipulated. The results of this study provide preliminary experimental evidence indicating that *Sodalis* strains from two distinct host species are similar enough to interspecifically colonize tsetse flies and have little subsequent impact on host fitness under the conditions tested.

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