Paternal Transmission of a Secondary Symbiont during Mating in the Viviparous Tsetse Fly

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Abstract

***Sodalis glossinidius*, a maternally inherited secondary symbiont of the tsetse fly, is a bacterium in the early/intermediate state of the transition toward symbiosis, representing an important model for investigating establishment and evolution of insect–bacteria symbiosis. The absence of phylogenetic congruence in tsetse-*Sodalis* coevolution and the existence of *Sodalis* genotypic diversity in field flies are suggestive for a horizontal transmission route. However, to date no natural mechanism for the horizontal transfer of this symbiont has been identified. Using novel methodologies for the stable fluorescent-labeling and introduction of modified *Sodalis* in tsetse flies, we unambiguously show that male-borne *Sodalis* is 1) horizontally transferred to females during mating and 2) subsequently vertically transmitted to the progeny, that is, paternal transmission. This mixed mode of transmission has major consequences regarding *Sodalis*' genome evolution as it can lead to coinfections creating opportunities for lateral gene transfer which in turn could affect the interaction with the tsetse host.**

Key words: **symbiont, paternal transmission, genetic diversity, *Sodalis glossinidius*, *Glossina*.**

Main Text

To ensure successful symbiont inheritance and dissemination within insect populations, mechanisms have evolved within different insect-symbiont associations that optimize the transmission of bacterial symbionts from one generation to another (Bright and Bulgheresi 2010). The mode of transmission plays a key role in the evolutionary trajectory of these associations where strict vertical symbiont transmission results in congruent host–symbiont phylogenies whereas horizontal transfer tends to eliminate congruence with partner phylogenetic trees (reviewed in Ebert [2013]). The obligate blood-feeding tsetse fly, the sole vector of human pathogenic African trypanosomes, harbors several bacterial symbionts that influence important aspects of its physiology and ecology. Tsetse flies display a unique viviparous mode of reproduction where the offspring develops in utero where it is nourished by maternal milk gland secretions containing tsetse's two main symbiotic bacteria, that is, an anciently associated obligate mutualist *Wigglesworthia glossinidia* and a more recently established commensal *Sodalis glossinidius*. Tsetse's third maternally inherited endosymbiont, *Wolbachia*, has a transovarial transmission route. It is assumed that these three symbionts are strictly vertically transferred from the mother fly to the intrauterine progeny. However, the absence of phylogenetic congruence in the tsetse-*Sodalis* coevolution (Toh et al. 2006) and the ability to experimentally establish a stable heritable symbiosis in tsetse flies cleared of their native *Sodalis* symbionts with a *Sodalis* species isolated from a different tsetse fly species (Weiss et al. 2016) are indicators that horizontal transmission of *Sodalis* symbionts could occur in tsetse populations. To date, no natural mechanism for the horizontal transfer of this symbiont has been identified. The presence of *Sodalis* in male testes (Balmand et al. 2013) and female spermathecae (Cheng and Aksoy 1999) has led us to hypothesize that transfer of *Sodalis* might occur during copulation. Such transfer would be biologically relevant in terms of host–symbiont coevolution if the symbionts are subsequently transmitted maternally to the progeny, that is, paternal transmission.

Experimental evidence for the latter is currently lacking due to the absence of an appropriate methodology to reliably trace this alternative transmission route. To date, only a few studies have utilized genetic techniques to explore the nature of host–symbiont interactions due to the fastidious nature of these bacteria.

In this study, we adapted and optimized the Tn7 transposition technology for the genetic manipulation of *Sodalis*. Furthermore, we developed novel techniques for introducing genetically modified *Sodalis* in tsetse allowing us to explore sexual transmission and ensuing vertical transmission of sexually acquired *Sodalis* to the offspring, that is, paternal transmission. Although paternal transmission has been shown to have important implications regarding the coevolution of hosts and symbionts, it has been reported in only a few

Letter

insect-symbiont associations to date (Moran and Dunbar 2006; Damiani et al. 2008). In this study, we show unambiguously that male-borne green fluorescent protein (GFP)-tagged *Sodalis* is horizontally transferred to females during mating and subsequently vertically transmitted to the progeny.

Glossina morsitans morsitans (Westwood) from the colony at the Institute of Tropical Medicine (Antwerp, Belgium) were used in all experiments. Flies, maintained at 26 °C and 65% relative humidity, were fed 3 days per week with defibrinated bovine blood using an artificial membrane system. A chromosomally GFP-tagged *Sodalis* strain (*Sod*:GFPi) was constructed by Tn7-mediated transposition. Briefly, the *lacZ* promoter_*gfp* fusion cassette was cloned into the multiple cloning site of the pGRG25 plasmid (McKenzie and Craig 2006) encoding the *Tn7* transposition machinery which allows for the site-specific transposition at the *attTn7* locus of *Sodalis* (fig. 1*A*; additional details are provided as supplementary material, Supplementary Material online). We found that the *lacZ* promoter drives strong expression of GFP (fig. 1*B*) in a constitutive manner due to the absence of a *Sodalis lac* repressor.

Subsequently, a novel technique for the introduction of the GFP-labeled endosymbionts into tsetse flies was developed. Third-instar larvae, collected immediately after larviposition, were microinjected with 10^6 CFU of *Sod*:GFPi using a 5 µl Hamilton 75RN microsyringe with gauge 34 removable electrotapered needles and allowed to pupate resulting in efficiently colonized male flies upon emergence 30 days later (fig. 2). Microscopic observations of the male reproductive organs of *Sod*:GFPi colonized flies revealed that *Sod*:GFPi was found predominantly in the testes (fig. 3*A*), although not apparent within the sperm heads themselves. Interestingly, *Sod*:GFPi could not be detected in the accessory glands.

Prior to being mated with wild-type (WT) virgin female flies from the colony, *Sod*:GFPi containing males flies were given three blood meals. During mating, male tsetse deposit a "spermatophore" (i.e., a capsule consisting of male accessory gland secretions and sperm) in the uterus of female flies which can be isolated from the uterus of the female tsetse by dissection immediately after the mating event (fig. 3*B*). Using confocal microscopy *Sod*:GFPi was found to be present in low densities (up to 20 *Sod*:GFPi cells/spermatophore) in

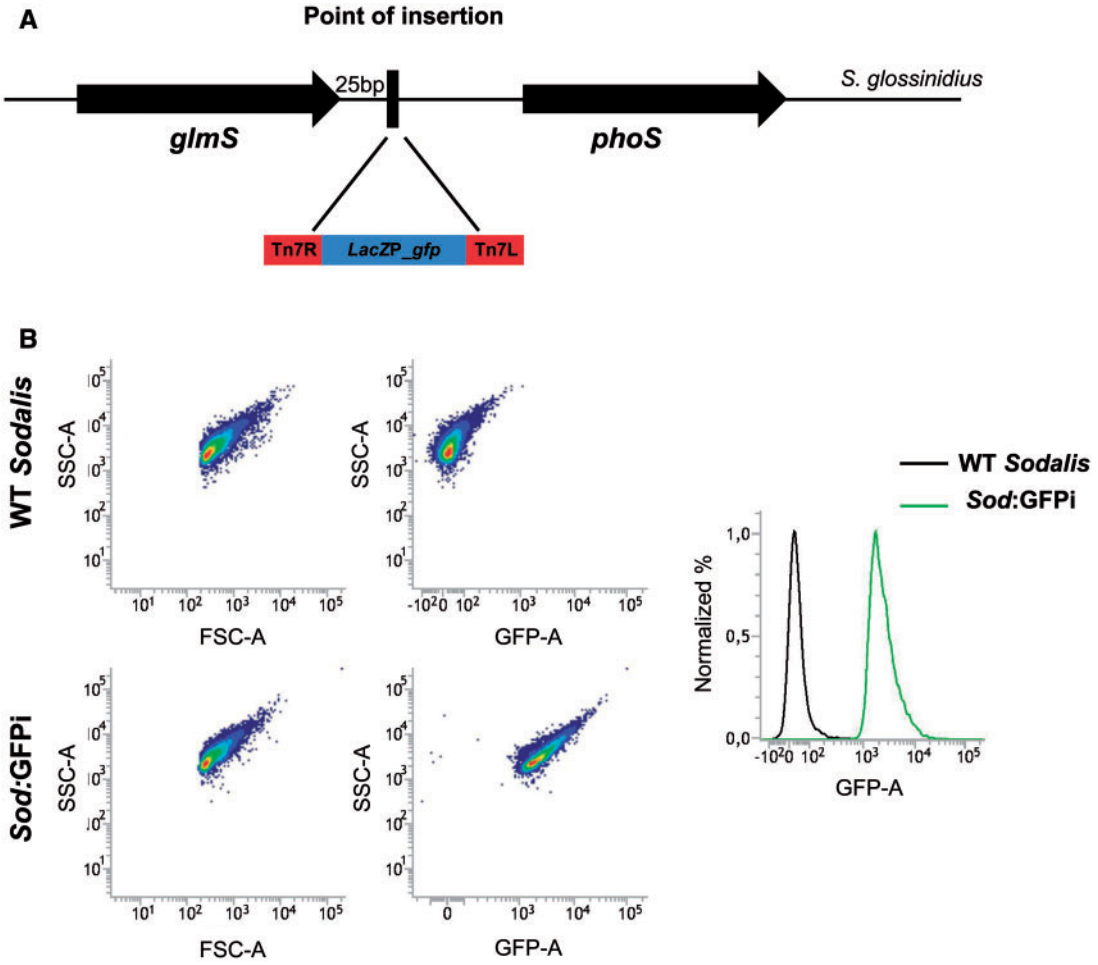

FIG. 1. Characteristics of *Sod*:GFPi. (*A*) The preferred site for Tn7 insertion in the *Sodalis glossinidius* chromosome, that is, 25 nucleotides downstream of the *glmS* gene. Tn7 insertions at this site are orientation-specific with the right end of Tn7 (Tn7R) adjacent to the 3′-end of the *glmS* gene, as shown in the figure. (*B*) Flow cytometric analysis of WT *Sodalis* (upper panel) and *Sod*:GFPi (lower panel) showing forward (FSC) and side scatter profiles (SSC) and GFP fluorescence.

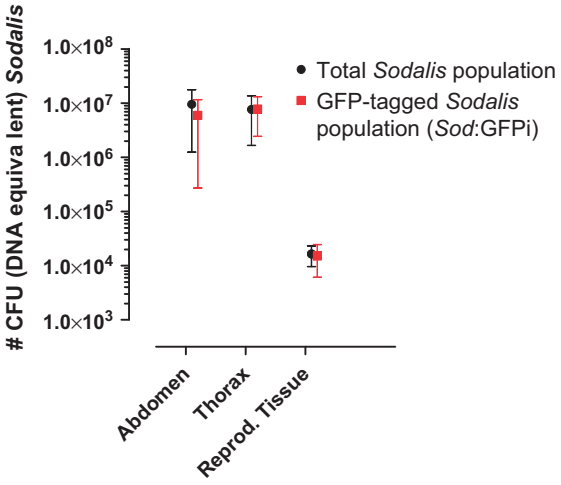

Fig. 2. Characteristics of *Sod*:GFPi colonized male flies. Number of GFP-tagged *Sodalis* (*Sod*:GFPi) present in abdomen, thorax, and reproductive tissues of 4-week-old male flies emerged from larvae injected with 10^6 *Sod*:GFPi CFU versus the total number of *Sodalis* (WT + *Sod*:GFPi). In these tissues, *Sod*:GFP constituted 63%, 100%, and 93% of the total *Sodalis* population, respectively. The number of WT and GFP-tagged *Sodalis* CFU was estimated using an already validated quantitative real time-PCR protocol (De Vooght et al. 2014 and supplementary material, Supplementary Material online). The bars represent the mean total *Sodalis* and *Sod*:GFP CFU (plus SD) present in abdomen, thorax, and reproductive tissues of at least five individual flies. The number of CFU is represented in log scale on the *y*-axis.

spermatophores collected from WT females immediately after mating (fig. 3*B*). Polymerase chain reaction (PCR)-analysis revealed a transmission efficiency of 27.5% (8 of 29 spermatophores). Furthermore, in 18.7% of WT females (3 of 16) mated with *Sod*:GFPi colonized males, *Sod*:GFPi was found to remain present within the reproductive organs for up to 4 weeks postmating, indicating that *Sod*:GFPi bacteria were able to persist within the host upon sexual acquisition. Finally, a proportion of females ($n = 23$) mated with *Sod*:GFPi colonized males were allowed to give birth to fully developed third-instar larva to evaluate the presence of *Sod*:GFPi in the F_1 progeny. The percentage of F_1 individuals carrying the recombinant bacteria was found to be 17.4% using PCR analysis (4 of 23 individuals). These results indicate that transmission of only a few *Sodalis* cells, residing within the spermathopore, is sufficient for establishment in the female "receiving" fly and subsequent transfer to the offspring.

In this study we have demonstrated unequivocally that paternal transmission through sexual reproduction is an additional route for *S. glossinidius* symbiont transmission to the progeny, besides maternal vertical transmission. This mixed mode of transmission could strongly impact *Sodalis*' genome evolution as transfer between tsetse fly individuals infected with different symbiont strains can lead to coinfections creating opportunities for lateral gene transfer or exchanging phage or phage genes increasing symbiont genetic diversity

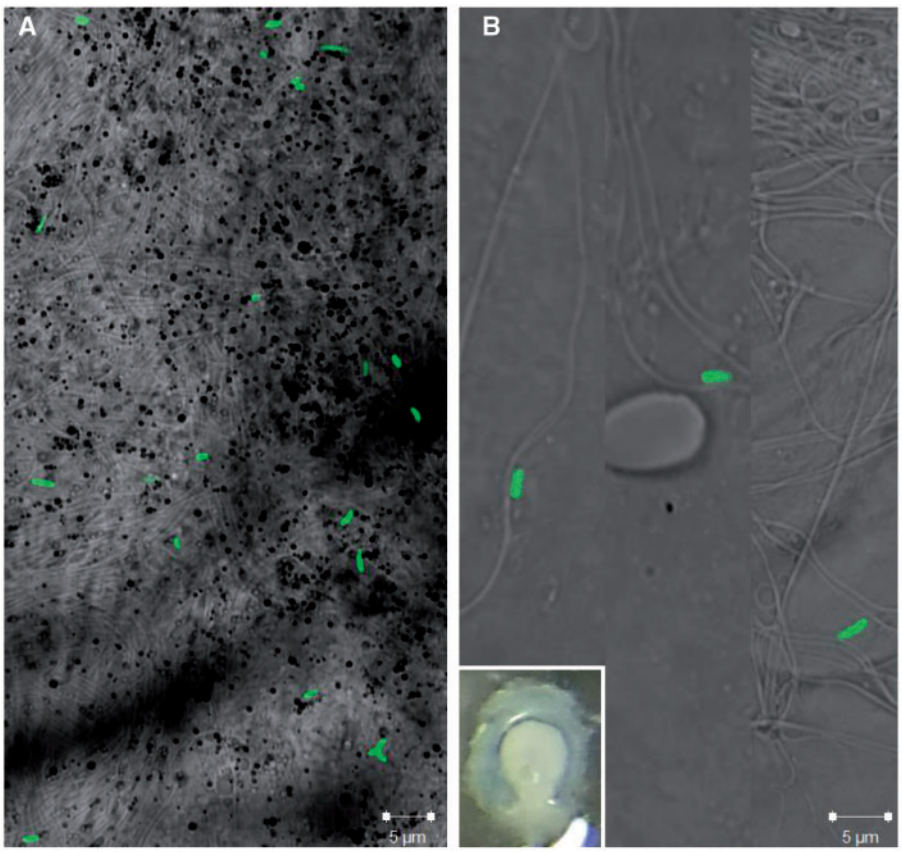

Fig. 3. (*A*) Localization of *Sod*:GFPi within the testes. (*B*) *Sod*:GFPi in spermatophores collected from the female uterus after mating with *Sod*:GFPi infected males (compilation of three separate locations in the same spermatophore). Inset: Macro-photograph of a spermatophore dissected from the female uterus after mating.

within the host population. The importance of genetic diversity in *Sodalis* is emphasized by the fact that different genotypes of *Sodalis* harbored by *G. palpalis gambiensis* have been shown to correlate with the tsetse fly ability to transmit trypanosomes including the human-pathogenic *Trypanosoma brucei gambiense* (Geiger et al. 2007). Although the paternal transfer of *Sodalis* in tsetse is a low to moderately frequent type of transmission, it has been demonstrated for other symbionts that even very low rates of paternal transmission can be consequential especially when particular combinations of host and symbiont genotypes have a direct effect on host fitness (Oliver et al. 2005). We propose that paternal gene transfer could be an important evolutionary factor that should be taken into account to explain the mechanisms and consequences of genetic diversity of *S. glossinidius*. However, assessing the importance of paternal transmission for the dynamics of *Sodalis* symbionts with a particular trait will require an understanding of the relative importance of the different natural transmission routes and transmission rates through these routes in natural tsetse populations.

Finally, in the context of a *Sodalis*-based paratransgenic approach (i.e., genetic manipulation of symbiotic microorganisms to interfere with pathogen development in the host) to control African trypanosomiasis (De Vooght et al. 2014), paternal transfer could provide an additional route for introducing genetically modified symbionts into populations as a single infected male can transfer symbionts to the progeny of several females.

Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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References

Balmand S, Lohs C, Aksoy S, Heddi A. 2013. Tissue distribution and transmission routes for the tsetse fly endosymbionts. *J Invertebr Pathol.* 112:S116–S122.

Bright M, Bulgheresi SA. 2010. A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol.* 8:218–230.

Cheng Q, Aksoy S. 1999. Tissue tropism, transmission and expression of foreign genes *in vivo* in midgut symbionts of tsetse flies. *Insect Mol Biol.* 8:125–132.

Damiani C, Ricci I, Crotti E, Rossi P, Rizzi A, Scuppa P, Esposito F, Bandi C, Daffonchio D, Favia G. 2008. Paternal transmission of symbiotic bacteria in malaria vectors. *Curr Biol.* 18:1087–1088.

De Vooght L, Caljon G, De Ridder K, Van Den Abbeele J. 2014. Delivery of a functional anti-trypanosome nanobody in different tsetse fly tissues via a bacterial symbiont, *Sodalis glossinidius*. *Microb Cell Fact.* 13(1):156.

Ebert D. 2013. The epidemiology and evolution of symbionts with mixed-mode transmission. *Annu Rev Ecol Evol Syst.* 44:623–643.

Geiger A, Ravel S, Mateille T, Janelle J, Patrel D, Cuny G, Frutos R. 2007. Vector competence of *Glossina palpalis gambiensis* for *Trypanosoma brucei* s.l. and genetic diversity of the symbiont *Sodalis glossinidius*. *Mol Biol Evol.* 24:102–109.

McKenzie GJ, Craig NL. 2006. Fast, easy and efficient: site-specific insertion of transgenes into enterobacterial chromosomes using Tn7 without need for selection of the insertion event. *BMC Microbiol.* 6:39.

Moran NA, Dunbar HE. 2006. Sexual acquisition of beneficial symbionts in aphids. *Proc Natl Acad Sci U S A.* 103:12803–12806.

Oliver KM, Moran NA, Hunter MS. 2005. Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proc Natl Acad Sci U S A.* 102:12795–12800.

Toh H, Weiss BL, Perkin SA, Yamashita A, Oshima K, Hattori M, Aksoy S. 2006. Massive genome erosion and functional adaptations provide insights into the symbiotic lifestyle of *Sodalis glossinidius* in the tsetse host. *Genome Res.* 16:149–156.

Weiss BL, Mouchotte R, Rio RV, Wu YN, Wu Z, Heddi A, Aksoy S. 2006. Interspecific transfer of bacterial endosymbionts between tsetse fly species: infection establishment and effect on host fitness. *Appl Environ Microbiol.* 72:7013–7021.