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# Nutrient provisioning facilitates homeostasis between tsetse fly (Diptera: Glossinidae) symbionts

**Anna K. Snyder[1], Jason W. Deberry[1,†], Laura Runyen-Janecky[2] and Rita V. M. Rio[1,*]**

[1]*Department of Biology, West Virginia University, 53 Campus Drive 5106 LSB, Morgantown, WV 26506, USA*
[2]*Department of Biology, University of Richmond, Richmond, VA 23173, USA*

Host-associated microbial interactions may involve genome complementation, driving-enhanced communal efficiency and stability. The tsetse fly (Diptera: Glossinidae), the obligate vector of African trypanosomes (*Trypanosoma brucei* subspp.), harbours two enteric Gammaproteobacteria symbionts: *Wigglesworthia glossinidia* and *Sodalis glossinidius*. Host coevolution has streamlined the *Wigglesworthia* genome to complement the exclusively sanguivorous tsetse lifestyle. Comparative genomics reveal that the *Sodalis* genome contains the majority of *Wigglesworthia* genes. This significant genomic overlap calls into question why tsetse maintains the coresidence of both symbionts and, furthermore, how symbiont homeostasis is maintained. One of the few distinctions between the *Wigglesworthia* and *Sodalis* genomes lies in thiamine biosynthesis. While *Wigglesworthia* can synthesize thiamine, *Sodalis* lacks this capability but retains a thiamine ABC transporter (*tbp*A*thi*PQ) believed to salvage thiamine. This genetic complementation may represent the early convergence of metabolic pathways that may act to retain *Wigglesworthia* and evade species antagonism. We show that thiamine monophosphate, the specific thiamine derivative putatively synthesized by *Wigglesworthia*, impacts *Sodalis* thiamine transporter expression, proliferation and intracellular localization. A greater understanding of tsetse symbiont interactions may generate alternative control strategies for this significant medical and agricultural pest, while also providing insight into the evolution of microbial associations within hosts.

**Keywords:** symbiosis; tsetse fly; homeostasis; comparative genomics

## 1. INTRODUCTION

Microbial associations are significant drivers of evolution (Margulis & Fester 1991). Since most microbes are localized within a complex consortium, little is known regarding how species interact, and even less is known about mechanisms that prevent species antagonism, which can ultimately compromise the integrity of the biological system. Elucidating these complex microbe–microbe interactions can be enabled through the use of host model systems that harbour naturally simple microbial communities.

The haematophagous tsetse fly (Diptera: Glossinidae) is the sole vector of African trypanosomes (*Trypanosoma brucei* subspp.), the causative agents of the fatal African trypanosomiasis (commonly known as sleeping sickness) in humans and nagana in other animals. In addition to serving as a vector for African trypanosomes, the tsetse fly also harbours two enteric gamma-proteobacterial symbionts: the obligate mutualist *Wigglesworthia glossinidia* (Aksoy 1995) and a secondary symbiont, *Sodalis glossinidius* (Dale & Maudlin 1999). These symbionts are necessary for tsetse's survival, as they are believed to supplement nutrients that the host is incapable of producing or obtaining from its restricted blood diet. The loss of *Wigglesworthia* and *Sodalis* associations results in significant detriment to tsetse, including reduction in reproductive output and shortened lifespan, respectively (Nogge 1976, 1981; Dale & Welburn 2001; Pais *et al.* 2008). Tsetse may also harbour *Wolbachia*, which is typically found in reproductive tissues (O'Neill *et al.* 1993) and to date has an unknown functional role. Although examples of fecundity enhancement and greater competitive efficiency have been described (Wade & Chang 1995; Dedeine *et al.* 2001; Hosokawa *et al.* 2010), *Wolbachia* associations are generally construed as a form of facultative parasitism within insects (reviewed in Dobson 2003).

In contrast to the majority of insects, tsetse flies have a unique reproductive strategy known as adenotrophic viviparity (i.e. live birth). Progeny develop through three larval instars *in utero* where they are provided with protein- and lipid-rich nutrients, and also inoculated with *Wigglesworthia* and *Sodalis* via maternal milk gland secretions (Ma & Denlinger 1974; Attardo *et al.* 2008). The maternal transmission of tsetse symbionts is associated with significant population bottlenecks at each generation (Rio *et al.* 2006). Consequently, stability between the different microbial symbiont species is especially critical towards maintaining the cohesiveness and evolutionary success of the biological system.

* Author for correspondence (rita.rio@mail.wvu.edu).
† Present address: West Virginia University School of Medicine, Robert C. Byrd Health Sciences Center, Morgantown, WV 26506, USA.

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Molecular phylogenetic analysis of the association between *Wigglesworthia* and tsetse supports an ancient establishment, dating back 50–80 Myr with a high degree of concordance (Chen *et al.* 1999). Extensive host coevolution has streamlined *Wigglesworthia*'s genome to complement the exclusively sanguivorous tsetse lifestyle (Akman *et al.* 2002). In comparison to the *Wigglesworthia* symbiosis, molecular phylogenetic analyses date the *Sodalis*–tsetse association to be of recent origin (Aksoy *et al.* 1997; Weiss *et al.* 2006). Also supporting its recent transition into symbiosis, *Sodalis* remains one of the few insect symbionts that can still be maintained in culture outside of its host (Welburn *et al.* 1987).

Large-scale sequencing and annotation has begun to shed light on the functional capabilities of host-associated microbes and their potential roles towards host biology and development (Moran *et al.* 2008). The comparative analyses of the annotated *Sodalis* (Toh *et al.* 2006) and *Wigglesworthia* (Akman *et al.* 2002) genomes enable the identification of complementary pathways of potential metabolic integration. Additionally, during intensive periods of host development, the growth dynamics of *Wigglesworthia* and *Sodalis* mirror one another's, suggestive of intertwined metabolic pathways (Rio *et al.* 2006). Interestingly, the *Sodalis* proteome contains most of the putative *Wigglesworthia* products (i.e. greater than 90% of *Wigglesworthia* coding sequences are orthologues within the *Sodalis* genome). This significant genomic overlap calls into question why tsetse maintains the energetically expensive coresidence of both symbiont species and how symbiont homeostasis is maintained.

The deficiency of B vitamins in blood (Edwards *et al.* 1957) coupled with the inability of insects to synthesize these essential nutrients (Sweetman & Palmer 1928; Craig & Hoskins 1940) suggest their provisioning to tsetse through microbial interactions. One of the few distinctions between the *Sodalis* and *Wigglesworthia* genomes lies in thiamine (vitamin B1) biosynthesis and transport. Although *Wigglesworthia* retains de novo thiamine biosynthetic capabilities, *Sodalis* is incapable of its production. To complement its thiamine biosynthetic deficiency, the *Sodalis* genome contains a putative thiamine ABC transport system (TbpAThiPQ), which in other closely related prokaryotes is used to salvage exogenous thiamine (Webb *et al.* 1998). We believe that this complementation of genetic inventory between *Wigglesworthia* and *Sodalis* may represent the early convergence of metabolic pathways that may act to ensure the maintenance of the *Wigglesworthia* association while also evading antagonism between the symbiont species.

Here, we examine one aspect of possible interplay between tsetse symbionts: the dependence of *Sodalis* on the provisioning of thiamine by *Wigglesworthia*. We investigate the effect of thiamine and its derivatives towards *Sodalis* proliferation and intracellular localization, a lifestyle feature that is associated with enhanced replication for this microbial symbiont (Dale *et al.* 2001). Functional assays characterizing the expression and regulatory patterns of the *Sodalis* thiamine ABC transporter were performed. We present evidence for the necessity of exogenous thiamine towards *Sodalis* fitness, both *in vitro* and within the tsetse fly. The biosynthesis and utilization of thiamine by *Wigglesworthia* and *Sodalis*, respectively, may be pivotal not only towards the retention of the tsetse–*Wigglesworthia* association, but also to preserve homeostasis of the microbial community within the host. Understanding the metabolic interactions of tsetse symbionts can lead to the identification of novel control strategies towards combating trypanosomiasis prevalence, while also providing insight towards the evolution of microbial associations within hosts.

## 2. MATERIAL AND METHODS

### (a) *Insects*

Tsetse flies, *Glossina morsitans morsitans*, were maintained at West Virginia University within the Department of Biology insectary at $24 \pm 1^{\circ}$C with 50 to 55 per cent relative humidity on a 12/12 h light/dark schedule. Tsetse flies received defibrinated bovine blood (Haemostat, Dixon, CA, USA) every 48 h through an artificial membrane feeding system (Moloo 1971).

### (b) *Cell cultures*

*Sodalis* were isolated from surface-sterilized *G. m. morsitans* pupae and cultured on *Aedes albopictus* C6/36 cells as described previously (Dale & Maudlin 1999). *Sodalis* were subsequently maintained cell-free *in vitro* at 28°C in Mitsuhashi–Maramorosch (MM) medium (Weiss *et al.* 2006) supplemented with 5 per cent heat-inactivated foetal bovine serum (FBS). C6/36 cells were maintained in MM medium supplemented with 15 per cent FBS at 28°C.

### (c) *Growth assays*

*Sodalis* growth was compared upon inoculation into six different M9 minimal glucose media types (Sambrook & Russell 2001; plus additional supplements as indicated in figure 1*a*). Log-phase *Sodalis* was diluted to an initial $OD_{600}$ of 0.01. Subsequently, 1 ml of diluted culture was inoculated into 4 ml of each of the various media types and grown at 28°C without shaking. $OD_{600}$ readings were taken every 24 h for 5 days, with three independent trials performed.

### (d) *Impact of thiamine monophosphate on* Sodalis *fitness*

Log-phase *Sodalis* was inoculated at an $OD_{600}$ of 0.01 into Media 1 (M9 minimal glucose media $+50\ \mu g\ ml^{-1}$ Bacto Vitamin Assay Casamino Acids; BD, Franklin Lakes, NJ, USA) with the addition of 0, 50 or 500 μM thiamine monophosphate (TMP; Sigma-Aldrich, St Louis, MO, USA). Every 24 h for 7 days, $OD_{600}$ readings were obtained to measure growth. Three independent trials were performed.

### (e) *Analysis of symbiont gene expression* in vitro

To examine the transcription of the *Sodalis* thiamine ABC transporter relative to TMP concentration, we chose to analyse the expression of the *tbp*A gene that encodes the thiamine transporter substrate-binding subunit. RNA was isolated during *in vitro* growth in Media $1 \pm 50$ μM TMP using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The absence of DNA contamination was verified through PCR using an RNA template lacking a reverse-transcription step. First-strand cDNA synthesis was performed with Superscript III Reverse Transcriptase (Invitrogen), 25 ng random hexamer primers and 200 ng RNA. Real-time quantitative PCR (qPCR) was performed in an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using Bio-Rad iQ SYBR Green Supermix, 10 mM of primers (tbpAQTfor and tbpAQTrev; electronic supplementary material, table S1) and 2 μl cDNA template.

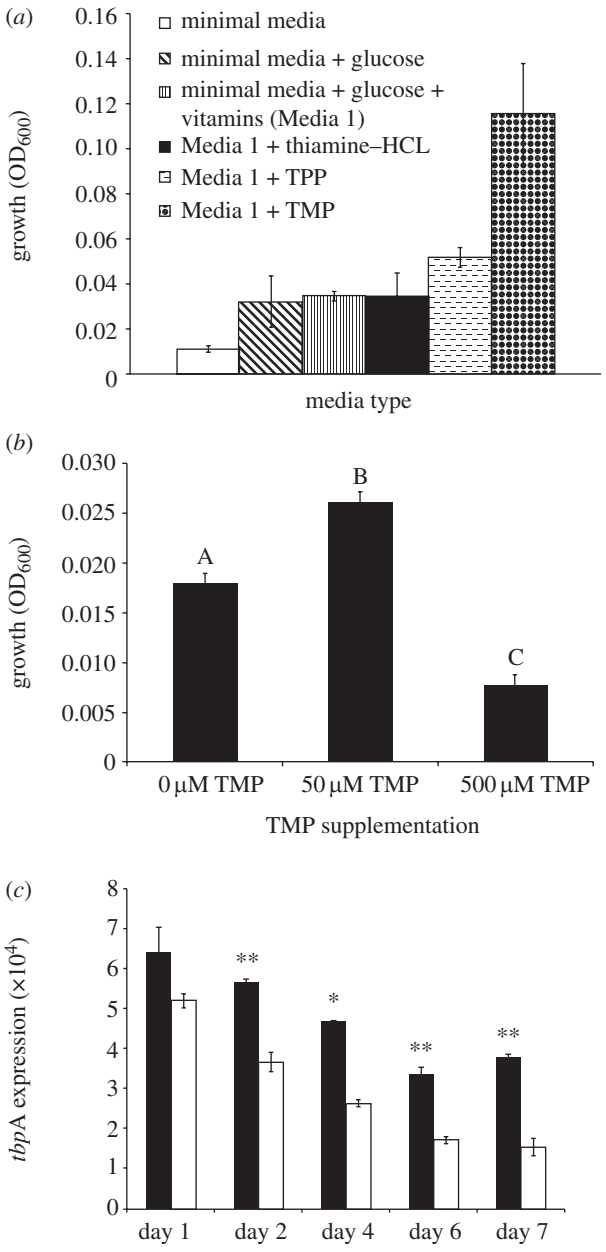


Figure 1. *Sodalis* growth and thiamine ABC transporter expression *in vitro* within TMP supplemented minimal media. (*a*) *Sodalis* growth in M9 minimal media ± glucose ± vitamins ± various thiamine derivatives (100 μM) at 120 h post-inoculation (vitamins contain a negligible amount of thiamine). (*b*) Mean *Sodalis* growth through 168 h in Media 1 and 0, 50 or 500 μM TMP. Letters designate treatments that are significantly different from others (ANOVA, $p < 0.0001$). (*c*) qRT–PCR analysis of *tbp*A expression from *Sodalis* grown in Media 1 ± 50 μM TMP. * and ** denote statistically significant differences, ANOVA $p < 0.05$ and $p < 0.01$, respectively, within each time point. Error bars signify ±1 standard error of the mean (s.e.m.). ($n \geq 6$ samples at each time point). Black bars, 0 μM TMP; white bars, 50 μM TMP.

The amplification settings were an initial 3 min denaturation step at 95.0°C, followed by 40 cycles of 10 s at 95.0°C and 30 s at 54.1°C. Internal standard curves were developed by cloning *tbp*A into the pGEM-T vector (Promega, Madison, WI, USA) using tbpAlongF and tbpAlongR primers (electronic supplementary material, table S1). Quantification of the amplicons relative to the standard curves was performed using Bio-Rad iCycler iQ multi-colour real-time PCR optical system software v. 2.0. The respective $OD_{600}$ readings of each time point were used for the normalization of *tbp*A expression. All assays were performed in triplicate and replicates were averaged for each sample.

### (f) *Regulation of* Sodalis *thiamine transporter*

The control of the *Sodalis* thiamine ABC transporter by a *thi* box regulatory region was examined using the plasmid-borne *tbp*A–*gfp* fusions pRJ12, pRJ13 and pRJ14 (figure 3*b*) in wild-type *Escherichia coli* MG1655. To construct the *tbp*A–*gfp* reporter fusions, primers (UR281 and UR282, UR283 or UR284) were used to amplify promoter DNA from three different *tbp*A regions of the *Sodalis* chromosome (figure 3*b*). The PCR products were digested with *Bam*HI and *Xba*I and cloned into the promoterless *gfp* vector pLR29 (Runyen-Janecky & Payne 2002) to generate pRJ12, pRJ13 and pRJ14, respectively. Overnight cultures of MG1655 containing each respective plasmid were started from freezer stocks inoculated into M9 minimal glucose media and 125 μg $ml^{-1}$ carbenicillin. Cultures were grown overnight at 37°C with shaking. Following overnight growth, each culture was pelleted and resuspended in the original volume of M9 media and carbenicillin. Resuspended cultures (20 μl) were inoculated into 2 ml of M9 media and 125 μg $ml^{-1}$ of carbenicillin ± 50 μM TMP. Cultures were grown at 37°C with shaking. At 24 h, 500 μl of each sample was fixed in 2 per cent paraformaldehyde and green fluorescence was quantified using an FACSCaliber (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) fluorescence-activated cell sorter with an excitation at 488 nm to measure single-cell fluorescence. FACSCaliber settings were forward scatter = E01, side scatter = 505 and relative fluorescence between 515 and 545 nm = 798. Three independent trials were performed, with 10 000 cells analysed per sample.

### (g) *Examining the effect of thiamine on* Sodalis *intracellular replication*

Intracellular localization and proliferation, followed by host cell lysis, is a process associated with *Sodalis* replication both in culture and within the tsetse host (Welburn *et al.* 1987; Dale & Maudlin 1999; Dale *et al.* 2001). To examine the influence of TMP towards intracellular infection and replication by *Sodalis*, C6/36 cells were split into six-well culture plates with MM media + 15 per cent FBS. Log-phase *Sodalis* grown in various media types (Media 1 ± 50 μM TMP or MM media + 5% FBS) were inoculated into a confluent lawn of C6/36 cells at an $OD_{600}$ of 0.01. Prior to inoculation, the supernatant from the wells was replaced with the media used to grow the respective *Sodalis*. To account for any potential effects of the various media types towards C6/36 viability, a replicate of the experiment was performed that lacked *Sodalis* inoculation. At 24 and 168 h post-inoculation, the total well contents (including any adhered C6/36 cells) were aspirated and total DNA isolation was performed using the Holmes–Bonner method (Holmes & Bonner 1973). The quantification of C6/36 cells was determined through qPCR using the rpL8QTfor and rpL8QTrev primers (electronic supplementary material, table S1), which amplify the *A. albopictus* ribosomal protein (rpL8) gene (GenBank accession no. M99055). The quantification of *Sodalis* density was also determined through qPCR, with corresponding SgexochiQTfor and

SgexochiQTrev oligonucleotides (electronic supplementary material, table S1), which amplify the single-copy exochitinase gene (*chi*; GenBank accession no. BSPY11391; Rio *et al.* 2006). Internal standard curves were developed by cloning amplicons for *A. albopictus* rpl8, using rpl8for and rpl8rev primers (electronic supplementary material, table S1), and *Sodalis chi* was produced with Sg exochifor and Sgexochirev (electronic supplementary material, table S1) into the pGEM-T vector (Promega, Madison, WI, USA), as described previously (Rio *et al.* 2006). Quantification of the amplicons relative to the standard curves was performed using SYBR Green I Dye (Bio-Rad) and Bio-Rad iCycler iQ multi-colour real-time PCR optical system software v. 2.0. The experiment was performed twice with multiple replicates within each trial.

### (h) *Expression of* Sodalis *thiamine ABC transporter through tsetse fly development*

Tsetse flies, *G. m. morsitans*, were sacrificed at distinct developmental stages (i.e. late larval, dissected approx. 6–9 days *in utero*; early pupal, less than 48 h post-maternal deposition; late pupal, approx. 28–30 days post-maternal deposition; teneral, newly eclosed adults prior to first blood meal; and two-week-old adults. Whole-fly RNA was isolated from single tsetse fly individuals using TRIzol (Invitrogen, Carlsbad, CA, USA) and treated with RNase free–DNase I (Invitrogen). The absence of DNA contamination was verified using PCR. First-strand cDNA synthesis was performed with 200 ng RNA, a 2 μM primer cocktail of tbpArev and gapDHrev (table S1), and Invitrogen Superscript II Reverse Transcriptase. Second-strand synthesis was performed with the addition of complementary 5′ end gene primers (electronic supplementary material, table S1) at 55°C for 35 cycles. The amplification products were analysed by agarose gel electrophoresis and visualized with Kodak one-dimensional image analysis software. The expression level of endogenous *Sodalis* glyceraldehyde-3-phosphate dehydrogenase (*gap*DH) within respective time points was used as a loading control.

### (i) *The effect of TMP-supplemented blood meals towards* Sodalis *thiamine ABC transporter expression within tsetse*

Teneral tsetse were maintained on blood meals supplemented with 50 or 500 μM TMP for two weeks. Whole-fly RNA was isolated from single tsetse individuals using TRIzol, and *tbp*A and *gap*DH reverse-transcriptional analyses were performed as described above.

### (j) *The impact of TMP-supplemented blood meals towards tsetse biology*

Teneral tsetse were maintained on blood meals supplemented with TMP as described previously. Tsetse flies were sacrificed at two weeks of age and DNA isolation performed using the Holmes–Bonner protocol (Holmes & Bonner 1973). DNA from each experimental sample was analysed to quantify the density of *Wigglesworthia*, *Sodalis* and *Wolbachia* symbionts as described previously (Rio *et al.* 2006).

### (k) *Statistical analysis*

The data were analysed using JMP 7.0 software (SAS Institute, Cary, NC, USA). A one-way analysis of variance (ANOVA) and Tukey–Kramer *post hoc* pairwise comparison of the mean were performed where appropriate to determine whether symbiont density, thiamine ABC transporter expression or C6/36 density differed between the various treatments. Student's *t*-tests were employed to assess the differences in gfp fluorescence of the plasmid constructs. *F*-tests were applied to assess the homogeneity of variances. The normality of density distributions was determined with a goodness-of-fit test. *Wolbachia* densities were square-root-transformed to satisfy normality. Significant differences ($p \leq 0.05$) are reported.

## 3. RESULTS

### (a) *The role of thiamine in* Sodalis *fitness*

The growth of *Sodalis* in media supplemented with glucose, vitamins and various thiamine derivatives including thiamine–HCl, thiamine pyrophosphate (TPP) or TMP was observed over 120 h. An increase in the *Sodalis* growth yield was observed with the incremental supplementation of various nutrients, such as glucose and vitamins, to an M9 minimal media base (Media 1). *Sodalis* proliferation increased significantly in cultures supplemented with TMP (figure 1*a*); however, a similar enhancement was not observed upon the addition of other thiamine derivatives (i.e. TPP or thiamine–HCl) to Media 1. These results indicate that *Sodalis* requires an exogenous thiamine source, preferably in the form of TMP, for optimal growth. Furthermore, *Sodalis* growth is impacted through time, not only by the presence of TMP, but also by different concentrations of this nutrient. A significantly higher mean *Sodalis* density was realized in Media 1 supplemented with 50 μM TMP (ANOVA, $p < 0.0001$; figure 1*b*) in comparison to 500 μM TMP. Moreover, a detrimental growth effect was observed when *Sodalis* was inoculated into Media 1 containing 500 μM TMP. These results demonstrate that *Sodalis* requires exogenous nutrients including TMP for its cultivation outside of the tsetse host.

### (b) *Impact of exogenous TMP towards* Sodalis *thiamine ABC transporter expression* in vitro

In free-living bacterial species, exogenous thiamine and its derivatives can be imported into the cell through an ATP-driven thiamine ABC transporter localized to the cell wall (Webb *et al.* 1998; Rodionov *et al.* 2002). At sufficient levels, thiamine and its derivatives can transcriptionally repress further TMP uptake by binding to a riboswitch localized upstream of the thiamine ABC transporter operon known as the *thi* box (Winkler *et al.* 2002).

To determine whether a similar expression pattern occurs with the *Sodalis* thiamine ABC transporter, we analysed the expression of *tbp*A in media containing or lacking TMP using qPCR. *Sodalis* grown in media lacking TMP exhibited significantly higher *tbp*A expression than cultures grown in the presence of TMP (ANOVA, $p < 0.001$; figure 1*c*). The significantly higher expression of *tbp*A in cells lacking exogenous TMP through time suggests that these *Sodalis* are attempting to import a vital nutrient for growth via its transporter, and that its functional regulation is intact and similar to that exhibited by free-living bacteria. Interestingly, a similar relationship of decreased *tbp*A expression through time was observed for *Sodalis* in both media types, suggesting the significance of TMP early in growth.

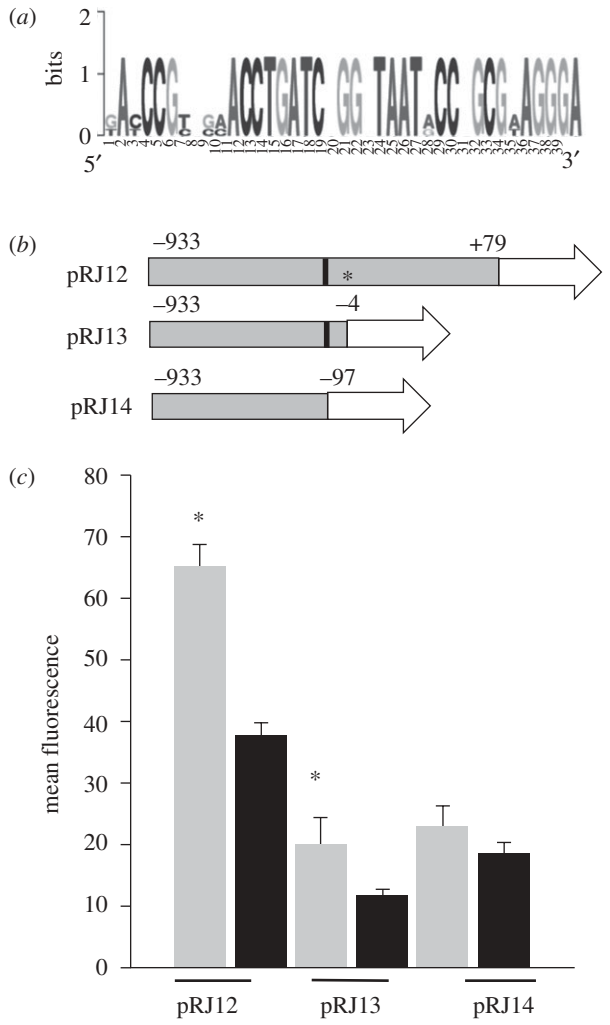


Figure 2. Conservation of *thi* box and regulatory regions of *Sodalis* thiamine ABC transporter. (*a*) Graphical representation of *thi* box (Miranda-Ríos *et al.* 1997) nucleotide sequence alignment of *Sodalis*, *E. coli* and *Salmonella typhimurium*. Image generated through the WebLogo website (http://weblogo.berkeley.edu/). (*b*) *tbp*A–*gfp* fusions are depicted. The *thi* box and *tbp*A start codon are represented by a black box and an asterisk, respectively. The *gfp* sequences are represented by arrows. (*c*) *Escherichia coli* MG1655 carrying the *tbp*A–*gfp* fusions were grown for 24 h in Media 1 and carbenicillin in the absence (grey bars) or presence (black bars) of 50 μM TMP, and the fluorescence quantified with FACS. Asterisks denote significant differences, Student's *t*-test, $p < 0.05$, within each construct. The data represent the mean fluorescence of at least three independent trials, with 10 000 cells analysed per sample. Standard deviations are indicated.

### (c) *Regulation of* Sodalis *thiamine ABC transporter*

The *Sodalis tbp*A promoter has a putative *thi* box (Miranda-Ríos *et al.* 1997) at nucleotides 58–97 5′ of the transcriptional start site. Thus, based on the high conservation of the *thi* box region upstream of *tbp*A (figure 2*a*), we hypothesized that the *thi* box still mediates thiamine repression of *Sodalis*'s thiamine ABC transporter. To test this hypothesis, we constructed *tbp*A–*gfp* fusions (± *thi* box, figure 2*b*) and examined GFP expression in *E. coli* containing these fusions grown with and without TMP. *Escherichia coli* containing the two *tbp*A–*gfp* fusions containing the *thi* box (pRJ12 and pRJ13) showed significant reductions (Student's *t*-test, $p<0.05$)–specifically, decreases of 42 and 37 per cent, respectively, in GFP levels when grown in media containing TMP when compared with media lacking TMP (figure 2*c*). In contrast, there was no statistically significant change in the GFP level with *E. coli* containing the pRJ14 fusion (Student's *t*-test, $p > 0.05$), which lacks the *thi* box, in either media type. These data suggest that the *thi* box remains functionally relevant for TMP regulation of *tpbA* expression by *Sodalis*.

### (d) *The effect of TMP towards* Sodalis *intracellular localization and replication*

To determine whether *Sodalis* intracellular replication is compromised when grown in the absence of TMP, a monolayer of *A. albopictus* C6/36 cells was inoculated with *Sodalis* grown in the presence or absence of TMP. This particular cell line has previously been demonstrated to support intracellular localization and subsequent increases in *Sodalis* density (Welburn *et al.* 1987; Dale & Maudlin 1999; Dale *et al.* 2001). To ensure that any changes in the C6/36 density were due solely to *Sodalis* infection and not respective media types, replicate assays lacking *Sodalis* were performed and no effects on C6/36 density were found (data not shown). At 24 h post-inoculation, no significant differences were observed in either C6/36 or *Sodalis* density between the various treatments (data not shown and figure 3*b*, respectively). As incubation progressed to 168 h, C6/36 density was significantly lower upon inoculation with *Sodalis* grown in TMP-supplemented media and comparable to when the bacteria are cultured in a rich media base (ANOVA, $p < 0.0001$; figure 3*a*). Moreover, at the 168 h time point, *Sodalis* density was significantly higher with TMP supplementation than with cells cultured in media lacking this nutrient, supporting an increase in replication rate (ANOVA, $*p < 0.05$; figure 3*b*). The highest *Sodalis* density was supported with nutrient-rich MM media. This suggests that although TMP is critical for its proliferation, this metabolite is not the sole dietary necessity as additional nutrients further enhance replication (ANOVA, $**p < 0.01$; figure 3*b*). These results demonstrate that the intracellular infection and subsequent replication of *Sodalis*, typical of its lifestyle within the tsetse fly, is compromised when TMP is lacking.

### (e) Sodalis *thiamine ABC transporter expression through tsetse development and upon TMP supplementation of host blood meals*

Semiquantitative reverse-transcriptional analyses of whole tsetse fly RNA reveals that thiamine transport by *Sodalis* is dynamic through host development (figure 4*a*). Expression levels of *Sodalis tbp*A were highest in the late pupal and teneral adult life stages in both males and females and lowest during the larval and early pupal time points.

Expression levels of *Sodalis tbp*A also demonstrated variability between two-week-old female and male flies, with higher transcriptional activity demonstrated within females (figure 4*a*,*b*). We also examined *Sodalis tbp*A expression in tsetse adults maintained on various TMP-supplemented blood meals (figure 4*b*). The expression of *Sodalis tbp*A decreased in females with greater levels

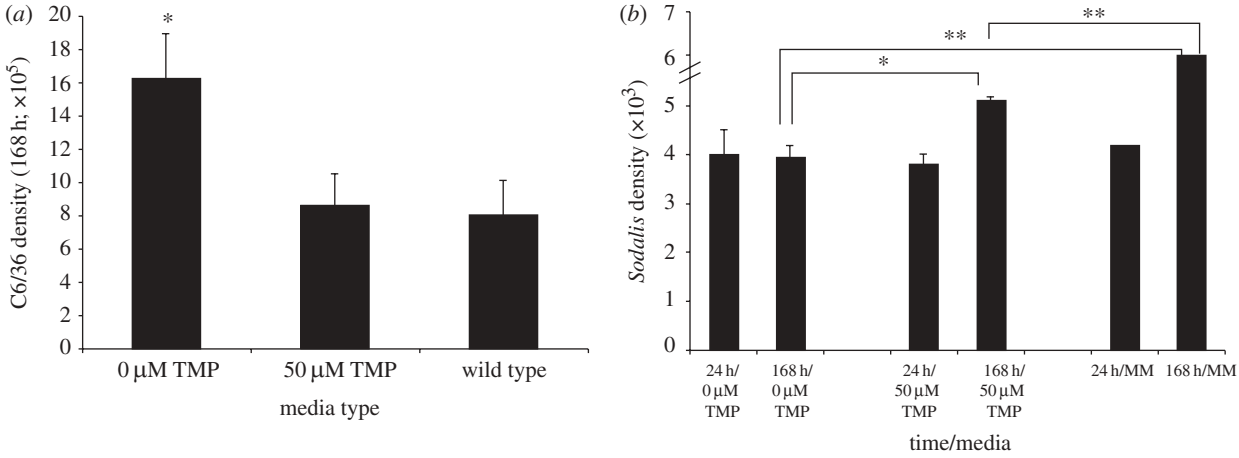


Figure 3. *Sodalis* intracellular invasion and replication is significantly lower in the absence of TMP. (*a*) C6/36 density at 168 h post-inoculation with *Sodalis* grown in Media 1 ($\pm$50 μM TMP) or wild-type media (MM media + 5% FBS). Mean C6/36 density values are represented and errors bars signify 1 s.e.m. Asterisk denotes significant difference (ANOVA, $p < 0.0001$). (*b*) *Sodalis* density at 24 and 168 h post-inoculation of C6/36 cells. Mean *Sodalis* density values are represented and errors bars signify 1 s.e.m. * and ** denote significant difference, ANOVA, $p < 0.05$ and $p < 0.01$, respectively ($n \geq 6$ samples per treatment).

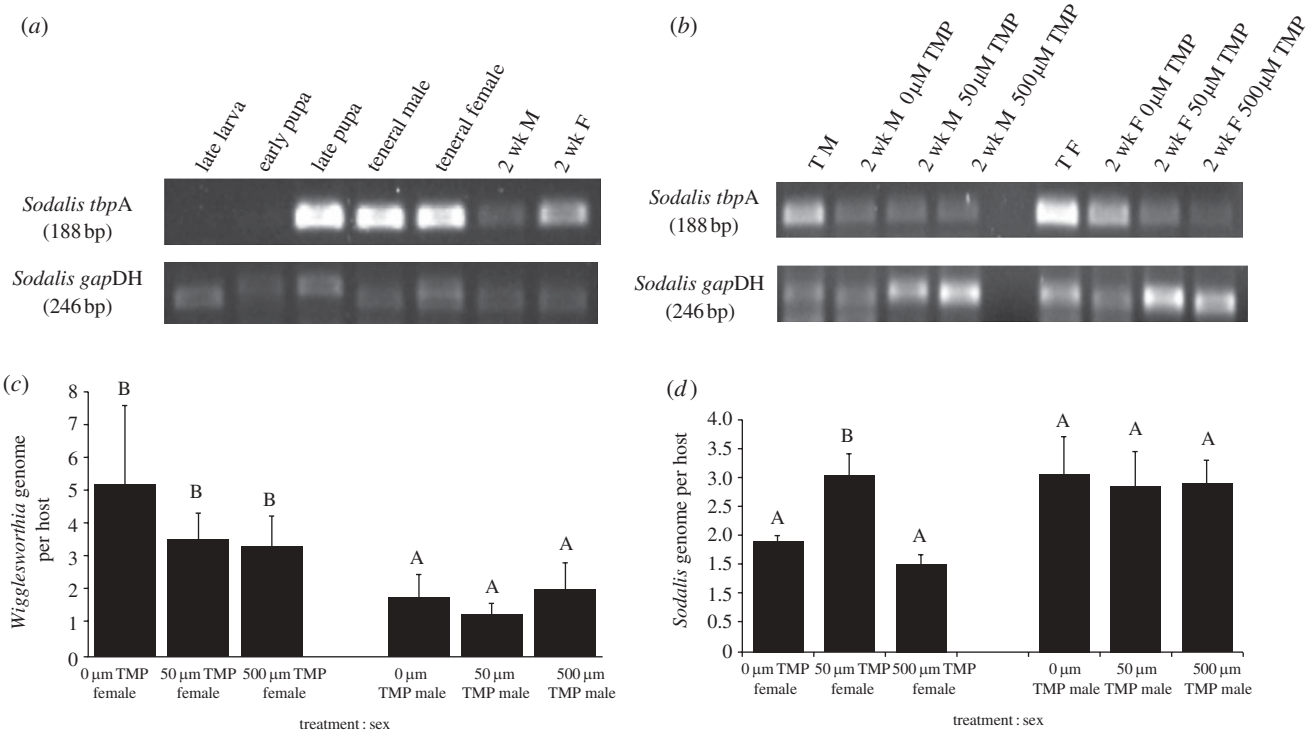


Figure 4. *Sodalis* thiamine ABC transporter expression and tsetse symbiont density through host development and with supplementation of blood meal. Semiquantitative RT–PCR analysis of Sodalis *tbp*A expression (*a*) through host development and (*b*) following two-week TMP supplementation of blood meals. TM, teneral male; 2 wk M, two-week-old male; TF, teneral female; 2 wk F, two-week-old female. *Sodalis gap*DH expression served as a loading control. (*c*) *Wigglesworthia* and (*d*) *Sodalis* density were compared in two-week-old tsetse fed blood-only and TMP-supplemented meals. Mean density values are represented and error bars signify 1 s.e.m. Letters depict significant differences (ANOVA, $p < 0.05$) between treatments ($n \geq 3$ samples at each time point).

of TMP supplementation in blood meals, while this pattern was not observed within males as augmenting TMP had no effect on transcriptional profiles.

### (f) *The impact of TMP-supplemented blood meals towards symbiont density*

Like other obligate insect mutualists, *Wigglesworthia* is unable to be cultured using *in vitro* methods in the laboratory. Consequently, genetic manipulation is not feasible. We bypassed the inability to mutate *Wigglesworthia* to produce increased levels of TMP by supplementing tsetse blood meals with this vitamin derivative and examining the effects on symbiont density. Because symbionts may contain multiple genomes per cell (Komaki & Ishikawa 2000), qPCR was used to determine bacterial genome number by using single copy genes normalized to host single copy genes. In support of

previous descriptions (Rio *et al.* 2006), *Wigglesworthia* abundance was significantly greater within females than males across all treatment groups (ANOVA, $p = 0.01$; figure 4*c*). Within female tsetse, a higher *Wigglesworthia* density was evident within tsetse maintained on blood only in comparison with TMP-supplemented meals, although statistical significance was lacking (ANOVA, $p = 0.71$). Within males, no differences in *Wigglesworthia* density were found between the various treatments (ANOVA, $p = 0.74$). Interestingly, *Sodalis* was more copious within female tsetse fed blood meals supplemented with 50 μM TMP in comparison with those fed blood only or 500 μM TMP-supplemented blood meals (ANOVA, $p = 0.002$; figure 4*d*). A similar reduction in *Sodalis* density was observed when tsetse females were fed a higher TMP concentration (i.e. 500 μM TMP; this finding is similar to what we observed with *Sodalis* in culture). No significant differences in *Sodalis* density were observed among the male treatment groups (ANOVA, $p = 0.96$). In addition, *Wolbachia* density did not significantly differ (ANOVA, $p = 0.6$; data not shown) between the various treatment groups within each sex.

## 4. DISCUSSION

The significance of microbial interactions within hosts is gaining steadfast recognition (Dethlefsen *et al.* 2007). Recent studies have demonstrated that symbionts of ancient origin are associated with genomic complementation, enabling microbial species to reach a synergistic equilibrium that cultivates a highly complex interdependence (Wu *et al.* 2006). In contrast to insect associations where symbionts are of ancient origins (Moran *et al.* 2005; Takiya *et al.* 2006), the tsetse enteric partners have vastly different acquisition times (Aksoy *et al.* 1997; Chen *et al.* 1999), providing a unique opportunity for insight into the adaptation processes associated with early coresidence of microbes within a symbiotic system.

Despite a severely reduced genome (Toh *et al.* 2006), *Wigglesworthia* significantly impacts several aspects of tsetse fly biology including reproduction, blood-meal digestion, temperature sensitivity, immunological processing and vector competence (Pais *et al.* 2008; Wang *et al.* 2009). Although *Sodalis* has a relatively large (4.2 Mb) chromosome, a significant degree of genomic decay is apparent, mostly represented in the plethora of pseudogenes. This abundance of pseudogenes results in a diminished coding capacity of only 51 per cent, making the *Sodalis* genome one of the least coding bacterial genomes known to date (Toh *et al.* 2006). The majority of pseudogenes are homologues of proteins that have functions related to immunological defence or transport and metabolism of carbohydrates and inorganic ions in free-living bacteria. These functions are probably no longer necessary, given the fidelity of vertical transmission through successive tsetse generations (Rio *et al.* 2006).

One of the few distinctions between the *Wigglesworthia* and *Sodalis* genomes lies in thiamine biosynthesis. While *Wigglesworthia* is capable of synthesizing thiamine (electronic supplementary material, figure S1), *Sodalis* lacks this capability. While the genes necessary for thiamine biosynthesis have clearly been eroded within the *Sodalis* genome (Toh *et al.* 2006), this biosynthetic inability appears to be circumvented through the retention of genes that encode a thiamine ABC transporter (*tbp*A*thi*PQ). Other *Sodalis* genome tailoring events have occurred following its transition to a host-associated lifestyle. Such events include the alteration of immunogenic components of its cell membrane—notably a truncated lipopolysaccharide, an absent O antigen and modified outer membrane protein A (i.e. *omp*A)—which are believed to protect against a systemic host immune response and enable tsetse establishment (Weiss *et al.* 2008). Additionally, extensive genome divergence between *Sodalis* and closely related *Sitophilus oryzae* primary endosymbiont appears tailored towards acquiring metabolites absent from the restricted diets of their specific hosts (Rio *et al.* 2003). It is tempting to postulate that the evolutionary pressures, resulting in the maintenance of the *Sodalis* thiamine ABC transporter over thiamine biosynthesis capability, may be indicative of selection at the host (Wernegreen & Moran 2000) rather than the individual symbiont level, acting to promote microbial homeostasis and ultimately tsetse fitness. Recognizing mechanisms that drive homeostasis between microbial species provides a basis of understanding fundamental molecular processes associated with the selection, regulation and evolution of symbiotic communities.

Many vitamins must be obtained either through diet or microbial interactions. Thiamine, an important cofactor in carbohydrate and amino acid metabolism, is essential for cellular physiology and growth (Schowen 1998). Within various insect groups, thiamine deficiency results in the degeneration of the fat body, stunted larval growth and reduced fertility (Sweetman & Palmer 1928; Craig & Hoskins 1940). The exclusive blood diet of tsetse, lacking in B-complex vitamins (particularly thiamine; Edwards *et al.* 1957), coupled with the inability of *Sodalis*, *Wolbachia* and tsetse to synthesize thiamine, supports the provisioning of this essential cofactor exclusively by *Wigglesworthia*. With thiamine biosynthesis being a unique *Wigglesworthia* role, provisioning of this vitamin may be essential for both preventing antagonism between tsetse's microbial symbionts and ensuring the maintenance of this obligate mutualist through time.

We demonstrate that *Sodalis* proliferation, both extra- and intracellular, is nutrient-limited, specifically by TMP. In essence, *Sodalis* population dynamics may be regulated not only by presence or absence of TMP but specifically by varying concentrations of this vitamin supplied by *Wigglesworthia*. Nutritional interactions, such as the metabolic interplay of thiamine biosynthesis and transport between the tsetse symbionts, may act to stabilize bacterial cohabitation within a host. The expression of the *Sodalis* thiamine ABC transporter, regulated by TMP through a functionally conserved *thi* box, appears to be reflective of host nutritional status. We observed higher expression of the *Sodalis* thiamine ABC transporter in tsetse's late pupal and teneral life stages. These particular developmental stages, demarcated by only 48 h, culminate a long quiescent developmental period consisting of approximately 30 days in the soil during which nutrient supplies have been vastly reduced (Leak 1999).

Tsetse fly fitness has been shown to influence the susceptibility towards trypanosome infection. Specifically,

starvation periods greatly increase the probability of parasite establishment within tsetse (Kubi *et al.* 2006), with the teneral stage being of highest vector competence (Welburn & Maudlin 1992). The decrease in *Sodalis* thiamine ABC transporter expression in two-week-old adults probably reflects an increase in the *Wigglesworthia* population (Rio *et al.* 2006) and, correspondingly, the ability to synthesize TMP at higher levels. Additionally, *Sodalis* transporter expression was both higher and most affected by TMP supplementation of tsetse blood meals within teneral females in comparison with similarly aged males. This phenomenon is probably due to additional female-specific roles such as reproduction and nourishment of intrauterine progeny, both processes that will result in greater demands and competition for available nutrients.

These studies provide insight into a metabolic factor: the provisioning of TMP by the obligate mutualist *Wigglesworthia*, which may aid the maintenance of microbial homeostasis within tsetse. Future studies will focus on identifying *Sodalis* compensatory roles towards tsetse symbiosis and whether these also act to stabilize the symbiont community. Given the critical role of tsetse symbiosis on host physiology and ecology, these associations provide a weak link in tsetse's biology. A greater understanding of tsetse symbiont interactions may generate alternative biological control methods for use in decreasing the prevalence of African trypanosomiasis.

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