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A Tale of Three Species: Adaptation of Sodalis glossinidius to Tsetse Biology, Wigglesworthia Metabolism, and Host Diet

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ABSTRACT The tsetse fly is the insect vector for the Trypanosoma brucei parasite, the causative agent of human African trypanosomiasis. The colonization and spread of the trypanosome correlate positively with the presence of a secondary symbiotic bacterium, Sodalis glossinidius. The metabolic requirements and interactions of the bacterium with its host are poorly understood, and herein we describe a metabolic model of S. glossinidius metabolism. The model enabled the design and experimental verification of a defined medium that supports S. glossinidius growth ex vivo. This has been used subsequently to analyze in vitro aspects of S. glossinidius metabolism, revealing multiple unique adaptations of the symbiont to its environment. Continued dependence on a sugar, and the importance of the chitin monomer N-acetyl-D-glucosamine as a carbon and energy source, suggests adaptation to host-derived molecules. Adaptation to the amino acid-rich blood diet is revealed by a strong dependence on l-glutamate as a source of carbon and nitrogen and by the ability to rescue a predicted l-arginine auxotrophy. Finally, the selective loss of thiamine biosynthesis, a vitamin provided to the host by the primary symbiont Wigglesworthia glossinidia, reveals an intersymbiont dependence. The reductive evolution of S. glossinidius to exploit environmentally derived metabolites has resulted in multiple weaknesses in the metabolic network. These weaknesses may become targets for reagents that inhibit S. glossinidius growth and aid the reduction of trypanosomal transmission.

IMPORTANCE Human African trypanosomiasis is caused by the Trypanosoma brucei parasite. The tsetse fly vector is of interest for its potential to prevent disease spread, as it is essential for T. brucei life cycle progression and transmission. The tsetse’s mutualistic endosymbiont Sodalis glossinidius has a link to trypanosome establishment, providing a disease control target. Here, we describe a new, experimentally verified model of S. glossinidius metabolism. This model has enabled the development of a defined growth medium that was used successfully to test aspects of S. glossinidius metabolism. We present S. glossinidius as uniquely adapted to life in the tsetse, through its reliance on the blood diet and host-derived sugars. Additionally, S. glossinidius has adapted to the tsetse’s obligate symbiont Wigglesworthia glossinidia by scavenging a vitamin it produces for the insect. This work highlights the use of metabolic modeling to design defined growth media for symbiotic bacteria and may provide novel inhibitory targets to block trypanosomal transmission.

KEYWORDS metabolism, microbiome, physiology, symbiosis, vector biology

It has been estimated that only 1% of all microbial life is culturable (1–3). Included in this are a vast array of symbiotic bacteria. Interspecies competition, as well as sensitivity to temperature and pH and availability of oxygen and nutrients, means many...
species cannot be cultured using standard conditions (3–5). The ability to culture medically significant microorganisms is an important tool in disease control. Medically significant microorganisms include pathogens and the key symbionts within the system. Improved culture methods, combining microbiology with genomics, have been used to analyze the microbial flora of a number of disease vectors. Notable examples are members of the family Paenibacillaceae and Serratia marcescens in the Asian malarial vector Anopheles stephensi (6), the more-complex flora of Aedes aegypti (7), and the defined microbiome of the tsetse fly, the insect vector for the Trypanosoma brucei parasites that cause human African trypanosomiasis (HAT) (8).

HAT is endemic in 36 countries in sub-Saharan Africa, with an estimated 65 million people at risk of infection (9–11). The tsetse, genus Glossina, also hosts a limited bacterial microbiome alongside the parasitic T. brucei. The microbiome consists of a primary, obligate symbiont Wigglesworthia glossinidia and typically, a secondary facultative symbiont Sodalis glossinidius (12, 13). S. glossinidius is of medical importance, as its presence correlates positively with the ability of the tsetse to be infected by T. brucei (14–17). Its complement of more than 1,500 pseudogenes and its large genome size of 4.17 Mb are consistent with it making a rapid and recent movement from free-living to a host-restricted niche (18, 19). The high rate of pseudogene accumulation is consistent with the loss of many cellular processes and metabolic pathways that are no longer needed for life in the tsetse. These include genes involved in the transport of carbohydrates not present in the blood meal (18) and of l-arginine biosynthesis (19). The recent discovery of a closely related, free-living species of Sodalis, Sodalis praecaptivus, provides a useful, relevant comparison (20). It enables informed predictions to be made about the presence or absence of key metabolic genes in S. glossinidius. The hypothesis that S. glossinidius has specifically lost metabolic capabilities during its transition to symbiosis can also be tested.

Symbiotic bacteria often present with small, degraded genomes (21). As a result of gene loss and inactivation, symbionts often cannot be grown outside their host. S. glossinidius can be cultured, but it requires undefined rich media (13) and a longer incubation time than that for the free-living S. praecaptivus (20). This increases the risk of contamination by faster-growing organisms and limits in vitro study of metabolic essentiality. A rationally designed growth medium was achieved in a landmark paper for the causative agent of Whipple’s disease Tropheryma whippelii (22), but this medium still contained undefined components. An entirely defined medium will improve the culturing of S. glossinidius and the study of its physiology dramatically. This may then enable genetic manipulation of this organism to express antiparasitic molecules toward the elimination of T. brucei (23–25). This process is already a consideration for the control of other vector-borne diseases (26, 27).

To define an S. glossinidius-specific growth medium, with the eventual aim of understanding more about the symbiont’s biology and metabolic dependencies, an experimental approach was combined with whole-genome metabolic modeling (GEM) and flux balance analysis (FBA) to model S. glossinidius in silico. This is a powerful method when based on a well-annotated genome and the ability to test in silico hypotheses experimentally (28). Analysis of the metabolic network of S. glossinidius was first undertaken by Belda et al. (29), who described a network of 458 gene products and 560 reactions, iEB458. A key finding was the pseudogenization of the phosphoenolpyruvate (PEP) carboxylase gene (ppc), preventing the conversion of PEP to oxaloacetate for the tricarboxylic acid (TCA) cycle. The pseudogenization of components of the l-arginine biosynthesis pathway indicate the requirement of an external source of l-arginine to supplement growth in silico. They concluded that exogenous l-arginine is required both as a biomass component and to form succinate via putrescine in order to supplement the TCA cycle in the absence of ppc. The common hexose sugar D-glucose is given as the sole carbon source. Importantly, this construction of iEB458 was limited by the lack of a well-annotated relative from the same genus, which is no longer an issue since the discovery of S. praecaptivus.

We present here a significantly advanced and improved model, ILF517, and describe
how it has enabled the development of an entirely defined medium that supports *S. glossinidius* growth *in vitro* (SGM11). Our data indicate the use of a carbon source lacking in the blood meal, namely, N-acetyl-D-glucosamine (GlcNAc). This suggests a complex nutritional interaction of *S. glossinidius* with the tsetse chitinous peritrophic matrix. Degradation of this by a microbe-derived chitinase might explain the increased persistence of the trypanosome when *S. glossinidius* is present (14, 15). Using SGM11, we demonstrate that *S. glossinidius* is not, as thought previously, a true auxotroph for L-arginine. Rather, it has a unique vitamin auxotrophy for thiamine, likely provided by the primary symbiont *W. glossinidia* (30, 31), through an interaction currently undefined.

**RESULTS**

The genome of *S. praecaptivus* enables an improved analysis of the *S. glossinidius* metabolic network. *S. praecaptivus* is the only free-living member of the *Sodalis* genus to have been characterized. It has a 5.16-Mb genome with a 57.5% GC content (20). Using this discovery, *S. praecaptivus* was compared to *S. glossinidius* to reassess the existing metabolic model of the symbiont. This additional information verified many of the important findings in *iEB458*, while others relating to carbon and nitrogen usage were not supported.

One central hypothesis derived from *iEB458* comes as a consequence of the inactivation of the PEP carboxylase reaction encoded by *ppc* (29). This loss in *S. glossinidius* should pose a problem for its metabolism, as it loses a route to replenish oxaloacetate from PEP. This represents an important anapleurotic reaction to maintain high flux through the TCA cycle in the related bacterium *Escherichia coli*. To compensate for this loss, Belda et al. (29) hypothesized a threefold function for exogenous L-arginine for *S. glossinidius*: as a biomass component, as a biosynthetic precursor to putrescine and spermidine, and as an anapleurotic substrate via succinate (Fig. 1). A functional *ppc* gene is present in *S. praecaptivus* (*Sant_3959*), whereas the gene in *S. glossinidius* contains multiple frameshifts and premature stop codons (see Fig. S1 in the supplemental material), suggesting loss as a result of selection pressures or genetic drift.

The proposed anapleurotic link from L-arginine to succinate is not supported in our analysis, with little or no evidence for these genes being present in *S. glossinidius* (Fig. 1; see also Data Set S1 in the supplemental material). L-Arginine must link to the TCA cycle to serve as an anapleurotic substrate. In *iEB458*, this linkage was proposed to occur via putrescine transaminase (PTRCTA), aminobutyraldehyde dehydrogenase (ABUTD) and 4-aminobutyrate transaminase (ABTA) (29) (Data Set S1). PatA is required for this PTRCTA reaction, but BLASTp searches find no evidence of an orthologue in *S. glossinidius* (Fig. 1). There is, however, a functional *patA* gene in *S. praecaptivus* (*Sant_1573*). Similarly, there is no functional orthologue of *patD* for ABUTD, nor of *puuE* or *gabT* for ABTA. L-Arginine can still be converted to agmatine and then to putrescine via arginine decarboxylase (ARGDC, SG2018) and agmatinase (AGMT, SG2017). This is the only route to the synthesis of this biomass component, meaning a source of L-arginine in the cell is still predicted to be essential. However, it is not supporting the proposed additional anapleurotic function.

An alternative organic compound must therefore serve the role of supplying TCA cycle intermediates in the absence of *ppc*. Removal of the ABTA reaction from *iEB458*, breaking the link of putrescine to succinate, results in zero biomass production. This can be rescued by the in silico addition of metabolites that can be introduced easily into the TCA cycle: the amino acid L-aspartate or L-glutamate or the organic acid succinate, fumarate, or α-ketoglutarate. The loss of *ppc* therefore requires the addition of a second organic substrate in addition to glucose for growth, suggesting an adaptation to an amino acid-rich environment that results from the tsetse blood diet. Important components, including L-aspartate and L-glutamate, are predicted to be present at high concentrations.
A revised metabolic model, iLF517, for S. glossinidius. A systematic reanalysis of the S. glossinidius genome enabled the construction of an independent metabolic model, iLF517. This model has significant differences to iEB458 (29). Growth was not supported in iLF517 using the uptake of oxygen, α-glucose, and L-arginine given in iEB458, indicating that alternative carbon and nitrogen sources are used. S. praecaptivus was used as a comparator to assess the presence of important metabolic genes in S. glossinidius, a resource not available to Belda et al. (29). Full details of all reactions removed from iEB458 and those added to iLF517 are highlighted in Data Set S1. iLF517 contains 517 genes, 703 metabolites, and 638 reactions (excluding pseudoreactions). This model can be viewed and analyzed through a web-based FBA browser on DETOXbase (www.detoxbase.org/publications/iLF517).

iLF517 was analyzed via FBA to investigate metabolite essentiality and the presence of predicted auxotrophies. Eighty reactions are included in iLF517 that were absent in iEB458, and 32 have been removed (Data Set S1). iEB458 simulates high oxygen transfer rates, using an uptake value of 20 mmol g DW⁻¹ h⁻¹ (DW stands for dry weight). This value was selected originally for E. coli and indicates highly aerated growth conditions.
in a chemostat (32–34). However, S. glossinidius is sensitive to high levels of oxygen (13). Cultures used here were grown in conditions of reduced aeration in comparison to E. coli or S. praecaptivus. The oxygen uptake rates given in iEB458 are therefore unrealistic for the simulations. Decreasing the oxygen supplied to iEB458 in silico results in a decrease in biomass output (Fig. S2), demonstrating that using unrealistic oxygen uptake rates exaggerate possible growth. The oxygen uptake rate in iLF517 was subsequently reduced to 12 mmol g DW⁻¹ h⁻¹, guided by the value given in a model of another microaerophile, Helicobacter pylori (35).

**A defined medium, SGM11, supports S. glossinidius growth.** The use of complex media is insightful for examining certain aspects of bacterial physiology. However, it does limit the ability to investigate all metabolic functions. A defined medium with components of known concentrations is therefore desirable. iLF517 was used to design a defined medium, SGM11, containing metabolites that the model predicts may enhance S. glossinidius growth or become limiting.

No growth was observed after 72-h incubation in M9 medium (36) supplemented with GlcNAC as a carbon source (Fig. 2A). The addition of trehalose, L-serine, L-arginine, L-proline, L-glutamate, L-aspartate, nicotinamide, α-ketoglutarate, fumarate, and thiamine monophosphate (TMP), to create SGM11, resulted in higher yields than with LB alone, although this difference was not statistically significant (Fig. 2A). Cell concen-
Approximately two times greater endpoint growth is exhibited with an equimolar amount of GlcNAc compared to D-glucose (P < 0.05 by one-way ANOVA) (Fig. 2B). Normalizing the carbon added from D-glucose with regard to GlcNAc has no significant effect on the optical density reached (data not shown). This demonstrates that the difference in growth between D-glucose and GlcNAc is not a result of the additional carbon. S. glossinidius grows significantly better on GlcNAc than mannitol (P < 0.05 by one-way ANOVA), reflecting the in silico results qualitatively.
**S. glossinidius** has adapted to thiamine produced by the primary tsetse symbiont. The addition of L-arginine, L-glutamate, and a carbon source, namely, GlcNAc, does not produce a positive biomass output in iLF517. It is likely that **S. glossinidius** requires supplementation from certain vitamins that it cannot synthesize. During the transition to symbiosis, **S. glossinidius** may lose genes that encode components of the vitamin and cofactor biosynthetic pathways in favor of retaining transporters. There is also thought to be a connection between aspects of the tsetse microbiome in terms of vitamin biosynthesis (19, 39).

**S. glossinidius** appears to have retained the components of pantothenate, biotin, riboflavin, protoheme, NAD, PLP, and tetrahydrofolate biosynthesis pathways found in **S. praecaptivus** (Table 1 and Table S1). Neither **S. praecaptivus** nor **S. glossinidius** can synthesize cobalamin. The key difference between the two species is in the pathway for thiamine biosynthesis. The loss of this pathway in **S. glossinidius** has been noted previously (39), but here the complete pathway in **S. praecaptivus** (Sant_3916-21) is used as a comparison. The loss of this pathway in **S. glossinidius** may be a specific adaptation to the tsetse. To investigate this further, three other **Sodalis**-allied symbionts were examined: "**Candidatus** Sodalis pierantonius" strain SOPE from the rice weevil *Sitophilus oryzae* (40, 41), and **Sodalis**-like symbionts from the meadow spittlebug *Philaenus spumarius* (42) and the seed bug *Henestaris halophilus* (43). The *P. spumarius* symbiont appears most similar to **S. praecaptivus**, with the fewest number of genes predicted to be absent or pseudogenized, whereas the *H. halophilus* symbiont has lost the ability to encode the components of the entire biotin and protoheme biosynthetic pathways (Table 1). “**Candidatus** Sodalis pierontonius” differs from **S. glossinidius** in that it cannot synthesize biotin (Table 1). However, this organism, along with the *H. halophilus* symbiont, does share similarities with **S. glossinidius** in the pseudogenization of genes encoding components of the thiamine pathway, including *thiF*, *thiG*, and *thiH*. This suggests an adaptation to symbiosis with certain insects. It is important to note that **S. glossinidius** is unusual in that it can function as either a primary or secondary symbiont depending on the insect host, and therefore, interspecies comparisons should be treated with caution (44). Thiamine is a cofactor for many enzymes, including pyruvate dehydrogenase (45), that are essential in *iLF517*. The potential thiamine auxotrophy in **S. glossinidius** was assessed *in silico*, and supplementation of thiamine or TMP was required to produce a positive biomass output in *iLF517* (Data Set S1).

The reliance of **S. glossinidius** on an external source of thiamine in order to produce TMP was then investigated experimentally. TMP was removed from SGM11, and the ability of the symbiont to grow was measured. Removal of TMP from SGM11 resulted in a significant reduction in **S. glossinidius** growth ($P < 0.05$ by one-way ANOVA) (Fig. 2C). A thiamine ABC transporter (SG0431-3) that likely transports TMP is also present (46). This adds weight to the *in silico* evidence from *iLF517* and the elegant

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### TABLE 1 Completeness of vitamin biosynthesis pathways in **Sodalis** species

| Pathway       | Sp | Sg | CSp | S-Hh | S-Ps |
|---------------|----|----|-----|------|------|
| Pantothenate  | +  | +  | +   | +    | +    |
| Biotin        | +  | +  | +   | +    | +    |
| Riboflavin    | +  | +  | +   | +    | +    |
| PLP           | +  | +  | +   | +    | +    |
| Protoheme     | +  | +  | +   | +    | +    |
| Thiamine      | +  | +  | +   | +    | +    |
| Tetrahydrofolate | + | + | + | + | + |
| Nicotinamide  | +  | +  | +   | +    | +    |

*The presence (pink) or absence (white) of vitamin biosynthesis genes, found using tBLASTn and BLASTp, in **S. praecaptivus** (Sp), **S. glossinidius** (Sg), "**Candidatus** Sodalis pierontonius" (CSp), **Sodalis**-like symbiont of *Henestaris halophilus* (S-Hh), and **Sodalis** endosymbiont of *Philaenus spumarius* (S-Ps).*
empirical work of Snyder et al. (86) that *S. glossinidius* relies on TMP from its environment. The only source of TMP in the tsetse microenvironment is that excreted by *W. glossinidia*, which is thought to supply it to the tsetse. *S. glossinidius* may therefore have adapted to not only its host but also to the metabolism of the primary symbiont in order to scavenge the available TMP.

*S. glossinidius* is dependent on external sources of L-glutamate or L-aspartate. Culturing *S. glossinidius* in SGM11 enables the thorough testing of amino acid usage in *ilF517*. Metabolites can be removed individually, and the effect on biomass production can be measured. *ilF517* requires either L-glutamate or L-aspartate to produce a positive biomass output. Both L-glutamate and L-aspartate likely enter the cell through the GltP transporter SG2121 (Fig. 1). *S. glossinidius* is predicted to have transporters for 14 amino acids (Table S2), so this reliance on L-glutamate or L-aspartate is not merely due to the ability to transport only these amino acids.

Removing L-aspartate or L-glutamate from SGM11 individually resulted in a significant decrease in the growth yield achieved by *S. glossinidius* after incubating for 72 h (P < 0.05 by one-way ANOVA) (Fig. 2D). Removing both L-aspartate and L-glutamate abolished growth completely (P < 0.001 by one-way ANOVA). This confirms that an exogenous source of one of these amino acids is essential for *S. glossinidius* growth. Examination of *ilF517* reveals that L-glutamate feeds directly into the TCA cycle through deamination to α-ketoglutarate. The direct route to feed L-aspartate into the TCA cycle via fumarate (L-aspartase) is however missing in *S. glossinidius* (Fig. 1). When *ilF517* is supplied with L-aspartate instead of L-glutamate, 69% of the available L-aspartate is channelled into the aspartate transaminase reaction (ASPTA), producing L-glutamate and oxaloacetate. The resulting biomass output was reduced from 0.35 to 0.31 g DW (mmol glucose)\(^{-1}\) h\(^{-1}\), demonstrating that *S. glossinidius* can use L-aspartate if L-glutamate is not available, although the latter may be preferred. L-Aspartate is therefore likely an important energy source in *ilF517* both to form L-aspartate and to replenish the TCA cycle at α-ketoglutarate (Fig. 1).

*S. glossinidius* is not an L-arginine auxotroph. Initial analysis of amino acid biosynthesis in *S. glossinidius* appeared to confirm existing opinion (29) that the only amino acid with an incomplete biosynthetic pathway is L-arginine (Table S2). A functional uptake system is also present, suggesting that *S. glossinidius* is indeed an L-arginine auxotroph. To assess this experimentally, *S. glossinidius* was grown in SGM11 with L-arginine removed. The growth yield decreased (Fig. 2E), but surprisingly, it was not totally abolished as expected for a true auxotroph. Excess L-glutamate was added to SGM11 to determine whether it could rescue this reduction in growth, as L-glutamate appears to be a key metabolite to *S. glossinidius*. A fivefold increase in L-glutamate concentration to 85 mM increased the bacterial yield (Fig. 2E), likely due to the extra carbon and nitrogen available. Remarkably, the excess L-glutamate rescued the growth defect caused by the removal of L-arginine completely (Fig. 2E and Fig. S4).

The *S. glossinidius* pathway for L-arginine biosynthesis was subsequently reanalyzed using the latest genome annotation (GenBank accession no. LN854557) to assess its completeness in comparison to *S. praecaptivus*. This revealed that argB, argE, argF/argI, and argH are full length and therefore likely functional (Table 2). It has been noted previously that argC is pseudogenized (29), but the new annotation indicates that this may not be the case. ArgC is also detected by proteomics, suggesting that this gene is indeed likely functional (47).

The new annotation suggests that the *S. glossinidius* argG gene (SGGMB4_03590) has a fragment (argG\_2) that is almost full length in comparison to its *S. praecaptivus* orthologue. This indicates that argG is also likely functional in spite of its description as a pseudogene in the previous annotation.

The argA gene appears in two fragments: SGGMB4_04654 (argA\_1) and SGGMB4_04655 (argA\_2). It has been demonstrated in *Pseudomonas aeruginosa* that the two separate ArgA protein domains can be expressed individually (48, 49). The C-terminal acetyltransferase domain can also function as a stand-alone protein when a
A high concentration of L-glutamate is provided (48). The SGGMBB4_04655 (argA_2) fragment of this gene has a GTG start codon and may therefore be functional under the conditions shown in Fig. 2E. The S. glossinidius argD orthologue also appears in two pieces: SGGMBB4_05438 (argD_1) and SGGMBB4_05439 (argD_2). Lal et al. (50) showed that an argD mutant of E. coli can still exhibit some N-acetylornithine aminotransferase activity, demonstrating that other proteins can compensate for a loss of this gene. The hypothesis that the loss of certain genes in the L-arginine biosynthesis pathway is not lethal was then assessed in vivo. E. coli argA, argD, and argG knockouts from the Keio collection (51) were grown in M9 minimal medium (36) alone or with the addition of L-arginine. The argD knockout mutant can grow in the absence of L-arginine (Fig. 4), confirming that the loss of this gene can be compensated for by alternative proteins in E. coli. Indeed, candidate aminotransferase genes exist in S. glossinidius, including bioA (SG0902) or hemL (SG0500), that may provide functional alternatives.

The data here suggest that under certain conditions, S. glossinidius can synthesize L-arginine, surviving when it is not supplied exogenously. Therefore, it is not a true auxotroph and could instead be described as a relic of a prototroph transitioning to auxotrophy.

**DISCUSSION**

Symbiotic bacteria are important components of medically significant microbiomes. However, studying their physiology and metabolism is limited frequently by culturabil-
ity issues. Tools to improve this are therefore desirable. This work describes a new refined FBA model of the *S. glossinidius* metabolic network and demonstrates its application in designing defined growth media for the symbiont. The carbon source for *i*LF517 is GlcNAc, as opposed to *D*-glucose in *i*EB458. It has been verified empirically that *S. glossinidius* achieves a significantly better growth yield with GlcNAc than with *D*-glucose (Fig. 2B). This is an important progression in the development of a metabolic model of *S. glossinidius*. The use of GlcNAc may be a result of both the pseudogenization of the glucose-specific PTS transporter (Fig. 3) and the availability in vivo of this host-derived sugar. This inclusion of GlcNAc may also support the theory that *S. glossinidius* is connected to the persistence of the trypanosomes within the tsetse. GlcNAc can inhibit *D*-glucose uptake by procyclic trypanosomes, resulting in a metabolic switch to the more-efficient oxidative phosphorylation with *L*-proline and a higher growth rate (38, 52). The free GlcNAc may derive from a breakdown of the tsetse peritrophic membrane by a chitinase secreted by *S. glossinidius* (15). The data here indicate that there may indeed be a link between the symbiont, the parasite, and the availability of GlcNAc within the tsetse.

The experimental evidence demonstrates that *S. glossinidius* still requires a sugar for growth, even though it clearly relies on amino acids such as *L*-glutamate (Fig. 2D). This is important, as other bacterial species have been shown to reduce their metabolic networks to grow on amino acids alone (53–57). The fact that this is not the case suggests an adaptation to use an abundant host-derived sugar, namely, GlcNAc. Furthermore, this may have then allowed the loss of *ppc* to occur during the transition to a symbiotic lifestyle (Fig. 1). The result is a more-constrained metabolic network that makes the organism less metabolically flexible than its free-living relative.

*L*-Glutamate has been shown in silico and empirically to be an essential nutrient for *S. glossinidius* (Fig. 2D; see also Data Set S1 in the supplemental material). It supplements the TCA cycle at α-ketoglutarate and forms *L*-arginine via ornithine (Fig. 1). An excess of this amino acid rescued the growth defect caused by the removal of *L*-arginine, previously thought to be an essential metabolite (Fig. 2E). The *argD* gene has become fragmented, but all other genes thought previously to be pseudogenized appear functional in the new genome annotation (Table 2). *S. glossinidius* is therefore an *L*-arginine prototroph, not an auxotroph as thought previously, capable of growth in the absence of *L*-arginine when sufficient *L*-glutamate is available. Unusual amino acid biosynthesis pathways are not uncommon in symbiotic bacteria. Indeed, components of the *L*-arginine biosynthesis pathway can function differently in symbionts. One
example is a potential fusion of ArgA and ArgG in Sulcia muelleri, symbiont of the sap-feeding sharpshooter Homalodisca vitripennis (58). The experimental conditions used here aim to reflect the tsetse microenvironment; metabolite concentrations vary according to the stage of the hunger cycle or the tissue sampled, but internal L-glutamate has been measured at 34 mM in the tsetse tissue (59, 60). SGM11 could be considered “low” L-glutamate at 18 mM, and therefore, the $5 \times$ (85 mM) medium subsequently removes any limitations caused by insufficient L-glutamate.

It may be that the L-arginine biosynthesis pathway is undergoing the process of inactivation and will become entirely pseudogenized over evolutionary time. This is supported by the complete pathway for L-arginine biosynthesis in S. praecaptivus, suggesting that these genes may have been lost within the tsetse environment as a result of selection pressure or drift. It implies that L-glutamate is not limiting inside the tsetse, allowing relaxed selection pressure on the L-arginine biosynthesis genes. It also emphasizes strongly the importance of using in vitro experiments to test in silico assertions. This is particularly relevant in symbiotic bacteria where the functionality of broken or fragmented genes is not certain. Indeed, a recent report implies that some S. glossinidius genes thought to be pseudogenized are in fact under transcriptional control (47).

TMP has been described here as an essential external metabolite. S. glossinidius is reliant on an external source of thiamine, both in silico in iLF517 and experimentally in the form of TMP in SGM11 (Fig. 2C). S. glossinidius may use its intact transporter to obtain TMP in vivo from W. glossinidia, which has retained the ability to synthesize this vitamin (18, 39). The results presented here provide the first clear experimental evidence of a potential metabolic linkage between the two important symbionts of the tsetse, S. glossinidius and W. glossinidia, and suggest that the TMP released by W. glossinidia is transported around the tsetse for use by both host cells and other symbionts.

iLF517 and SGM11 can now be used as a tool to predict with accuracy how S. glossinidius might respond to genetic manipulation. Using genomics to investigate and implement custom growth conditions is an area of research that is progressing rapidly, aided by advancements in gene sequencing and analysis. This includes the design of defined microbiological growth media (61–63), enabling metabolic and physiological investigations that would not be possible with complex or standard media (64–67). This has implications in disease control, both for HAT and for other diseases where the insect vectors have characterized bacterial microbiomes (68, 69). Wolbachia, for example, has been introduced successfully into the mosquito Aedes aegypti, with a notable reduction in infection by the pathogenic dengue and chikungunya viruses and the malaria parasite Plasmodium (70, 71). Wolbachia is found naturally in a range of medically significant insects, including Phlebotomus chinensis (visceral leishmaniasis) (72) and Aedes albopictus (dengue, yellow fever, West Nile, and chikungunya) (73–75). While some tsetse populations do present with Wolbachia infection (76–79), the persistence of S. glossinidius and its colocalization with the parasitic T. brucei make the latter an ideal candidate for novel disease control methods. It is hoped that the techniques described here may also translate to the microbiomes of other medically significant insects, including Rhodococcus rhodnii from the Chagas disease vector Rhodnius prolixus (80), and Acetobacteraceae spp. and Pseudomonadaceae spp. in Leishmania infantum-infected sand flies (Lutzomyia longipalpis) (81).

Use of iLF517 and the S. glossinidius metabolic network has enabled the design of a defined growth medium that supports growth of the symbiont. While several FBA models for insect symbionts have been published (82–85), this study is the first example of using FBA to improve the in vitro culture of these organisms. SGM11 facilitated the discovery that S. glossinidius is not a true L-arginine auxotroph and demonstrates its reliance on exogenous sources of thiamine and L-glutamate. SGM11 will greatly improve the ability to test other aspects of S. glossinidius metabolism and growth kinetics that have until now been limited by the restrictions of rich media. The continued transition of S. glossinidius to a symbiotic lifestyle can
now be predicted using this model. By comparing its dispensable, redundant genes to those in both free-living and symbiotic bacteria, it is possible to assess the trajectory of this symbiosis.

MATERIALS AND METHODS

Refinement of the S. glossinidius metabolic network. The previously published whole-genome metabolic model (GEM) iEB458 (29) was assessed for missing or potentially incorrect gene assignments. A reaction was removed if there could be no functional gene identified, either through absence or through pseudogenization based on the size of the gene in comparison to its E. coli or S. praecaptivus orthologues. Those reactions for which a gene assignment had been uncovered were added to the new model. Reactions were maintained if removing them resulted in a lethal phenotype, observed when the biomass output returned a value of zero. BiGG Models, KEGG, and EcoCyc databases were used to identify E. coli genes encoding the reactions for which an S. glossinidius gene assignment had not been found. Translated nucleotide and protein BLAST searches were used to look for known S. glossinidius proteins, and confirmation of genes and pseudogenes was performed using the Artemis genome visualization tool. Candidate pseudogenes were aligned with functional orthologues using ClustalX2.

Flux balance analysis. The flux balance analysis (FBA) solutions were generated using the GNA linear programming kit (GLPK) integrated with custom software in Java. Oxygen uptake was constrained to 12 mmol g DW⁻¹ h⁻¹ in order to simulate a reduced oxygen environment. The uptake of ammonia, water, phosphate, sulfate, potassium, sodium, calcium, carbon dioxide, protons, and essential transition metals was unconstrained. Uptake of all other metabolites was set at zero, with the exception of those used in the analyses which have been set at 2 mmol g DW⁻¹ h⁻¹. Uptake of all other metabolites was set at zero, with the exception of those used in the analyses which have been set at 2 mmol g DW⁻¹ h⁻¹. Cofactor constraints were implemented by introducing these metabolites to the biomass functions at small fluxes (0.00001 mmol g DW⁻¹ h⁻¹) (82). The phenotype was considered viable if the biomass production rate was greater than 1 × 10⁻³ g DW (mmol glucose)⁻¹ h⁻¹. Furtile cycles, identified as reactions carrying biochemically unsustainable flux, were altered to the correct reaction stoichiometry where possible with guidance from EcoCyc and BiGG. The full description of the model is provided in Data Set S1 in the supplemental material.

Bacterial strains, growth conditions, and reagents. S. glossinidius strain GMM4 was obtained from the University of Liverpool. Working stocks were established by growing starter cultures on brain heart infusion (BHI) (Sigma-Aldrich) plates under microaerophilic conditions generated by Oxoid CampyGen sachets (Thermo Fisher Scientific) until growth was visible. Colonies were then transferred to liquid BHI sachets (Thermo Fisher Scientific) until growth was visible. Colonies were then transferred to liquid BHI medium and incubated for 4 to 7 days in cell culture flasks at room temperature until growth was visible.

S. glossinidius from the working stock was then transferred to 5 ml fresh Luria-Bertani (LB) medium (Sigma-Aldrich) and supplemented with either D-glucose or GlcNAc (Sigma-Aldrich).

iLF517 and the S. glossinidius metabolic network were used to design in silico an entirely defined medium, SGM11, in which to grow the bacterium. S. glossinidius from the working stock was transferred to 5 ml of M9 minimal medium (36) containing the following supplements; 17 mM GlcNAc, 17 mM trehalose, 17 mM L-serine, 17 mM L-arginine, 4 mM L-proline, 17 mM L-glutamic acid monosodium salt hydrate, 17 mM L-aspartate, 4 mM nicotinamide, 9 mM α-ketoglutarate, 9 mM fumaric acid, and 0.4 mM thiamine monophosphate (Sigma-Aldrich). Metabolites were added to SGM11 individually and in combination to test the model predictions.

The culture flasks were incubated for 48 h (LB) or 72 h (SGM11) at 25°C in a temperature-controlled water bath. Gentle agitation was achieved using magnetic stir bars to achieve a suitable balance between oxygenation, settling, and disturbance, and a stirring speed of 500 rpm was used. Intermediate time points were found to compromise the sterility of the cultures, and therefore, destructive sampling was the only reliable method of investigation, negating the possibility of higher-resolution temporal data. Endpoint increase in S. glossinidius growth was measured at an optical density of 650 nm. Preliminary experiments using variable sampling times indicated that this was the most appropriate sampling time for S. glossinidius to reproducibly capture the final steady state but still retain a proxy for growth rate to guide the modeling results.

E. coli gene deletion mutants were obtained from the Keio collection (51). Cells were cultured in M9 minimal medium (34) with 0.4% D-glucose, and either 20 or 100 mM L-glutamic acid monosodium salt hydrate or 20 mM L-arginine in a microplate reader for 24 h at 37°C.

Flow cytometry. Cell count was generated using S. glossinidius taken from a starter culture in BHI and diluted in M9 salts. Cells were stained with DAPI at 2 μl/ml for 10 min at room temperature and measured on the CytoFLEX S flow cytometer (Fig. S3). The flow cytometer was calibrated using counting beads from Beckman Coulter (Miami, FL, USA).

Statistical analysis. All statistical analyses were performed using SciPy in Python (version 2.7.10, www.python.org). Error bars show standard errors of the means, and statistical significance was assessed using one-way analysis of variance (ANOVA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.02106-18.

FIG S1, TIF file, 4.4 MB.
FIG S2, TIF file, 5.5 MB.
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FIG S3, TIF file, 3.8 MB.
FIG S4, TIF file, 3.2 MB.
TABLE S1, DOCX file, 0.01 MB.
TABLE S2, DOCX file, 0.01 MB.
DATA SET S1, XLSX file, 0.1 MB.

Metabolic Adaptations in the Tsetse Microbiome
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