

Chapter 19

Bacterial Symbionts of Tsetse Flies: Relationships and Functional Interactions Between Tsetse Flies and Their Symbionts



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Abstract Tsetse flies (*Glossina* spp.) act as the sole vectors of the African trypanosome species that cause Human African Trypanosomiasis (HAT or African Sleeping Sickness) and Nagana in animals. These flies have undergone a variety of specializations during their evolution including an exclusive diet consisting solely of vertebrate blood for both sexes as well as an obligate viviparous reproductive biology. Alongside these adaptations, *Glossina* species have developed intricate relationships with specific microbes ranging from mutualistic to parasitic. These relationships provide fundamental support required to sustain the specializations associated with tsetse's biology. This chapter provides an overview on the knowledge to date regarding the biology behind these relationships and focuses primarily on four bacterial species that are consistently associated with *Glossina* species. Here their interactions with the host are reviewed at the morphological, biochemical and genetic levels. This includes: the obligate symbiont *Wigglesworthia*, which is found in all tsetse species and is essential for nutritional supplementation to the blood-specific diet, immune system maturation and facilitation of viviparous reproduction; the commensal symbiont *Sodalis*, which is a frequently associated symbiont optimized for survival within the fly via nutritional adaptation, vertical transmission through mating and may alter vectorial capacity of *Glossina* for trypanosomes; the parasitic symbiont *Wolbachia*, which can manipulate *Glossina* via cytoplasmic incompatibility and shows unique interactions at the genetic level via horizontal transmission of its genetic material into the genome in two *Glossina* species; finally, knowledge on recently observed relations between *Spiroplasma* and *Glossina* is

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explored and potential interactions are discussed based on knowledge of interactions between this bacterial Genera and other insect species. These flies have a simple microbiome relative to that of other insects. However, these relationships are deep, well-studied and provide a window into the complexity and function of host/symbiont interactions in an important disease vector.

19.1 Introduction

Human African Trypanosomiasis (HAT, sleeping sickness) and Animal African Trypanosomiasis (AAT, Nagana) are neglected tropical diseases affecting humans and animals throughout countries in sub-Saharan Africa. These diseases are inevitably fatal if left untreated. Recently, coordinated efforts by affected countries have brought case numbers down dramatically (Franco et al. 2018; Meyer et al. 2016). However, these efforts need to be maintained to prevent future resurgences as has occurred in the past when control efforts waned. The animal form of the disease remains a large problem and has tremendous impacts on the economy and people of affected areas (Isaac et al. 2017). The trypanosomes responsible for these diseases are vectored from a reservoir to host or from host to host by tsetse flies (*Glossina spp.*). Flies within the genus *Glossina* are the sole vectors of African trypanosomes in humans and the primary vectors of trypanosomes in animals. Vector control is a primary mechanism for control of trypanosomiasis transmission (Vreysen et al. 2013). This is due to the low natural population numbers of *Glossina* in the wild. This feature differentiates tsetse flies from more prolific vectors such as mosquitoes and is due to their unique biology and unusual adaptations.

Tsetse flies are specialized vectors. They differ from other Dipteran vectors in that both sexes feed on blood alone and derive no nutrition from alternative sources such as floral nectar as mosquitoes do (Buxton 1955; Magnarelli 1978). Tsetse flies also differ in their reproductive biology as females give birth to live fully developed 3rd instar larvae (Mellanby 1937; Tobe 1978). The female fly provides for all of the nutritional requirements of the larvae by secretion of a lactation product (or milk) into the uterus where the developing larvae live (Ma and Denlinger 1974). The evolutionary pressures behind these adaptations are unknown. However, these pressures resulted in extreme adaptations, which required or were facilitated by the presence of symbiotic bacteria. Obligate mutualistic bacterial relationships with insects are well documented and often function to compensate for dietary adaptations to exploit rich but nutrient-deficient dietary sources (Buchner 1965; Douglas 1989). Examples include homopteran insects such as aphids that feed exclusively on plant sap, which lacks essential amino acids. Other insect species with wood-based diets such as carpenter ants (Hymenoptera) (Schroder et al. 1996) and termites (Isoptera) (Jucci 1952) require microbial assistance with the digestion of cellulose. Hematophagous insects subsist entirely on blood, which lacks in B vitamin-associated compounds. In addition to tsetse, examples of other blood feeders with

symbiotic relationships include bed bugs (Cimicidae) (Chang and Musgrave 1973) and lice (Anoplura) (Puchta 1955).

Symbiotic relationships can range from mutualistic where the insect and the bacteria are receiving positive effects from their relationship, to parasitic where the bacteria are exploiting the host resulting in negative impacts on fitness and/or reproduction and some relationships are commensal where the bacteria benefit at no apparent cost to the insect host. To date, research shows that tsetse flies have consistent relationships with a few bacterial species. In general, tsetse flies have a relatively simple microbiome consisting primarily of four characterized species. These include *Wigglesworthia glossinidia*, *Sodalis glossinidius*, *Wolbachia pipientis*, and *Spiroplasma* (Fig. 19.1). The presence or absence of all but one of

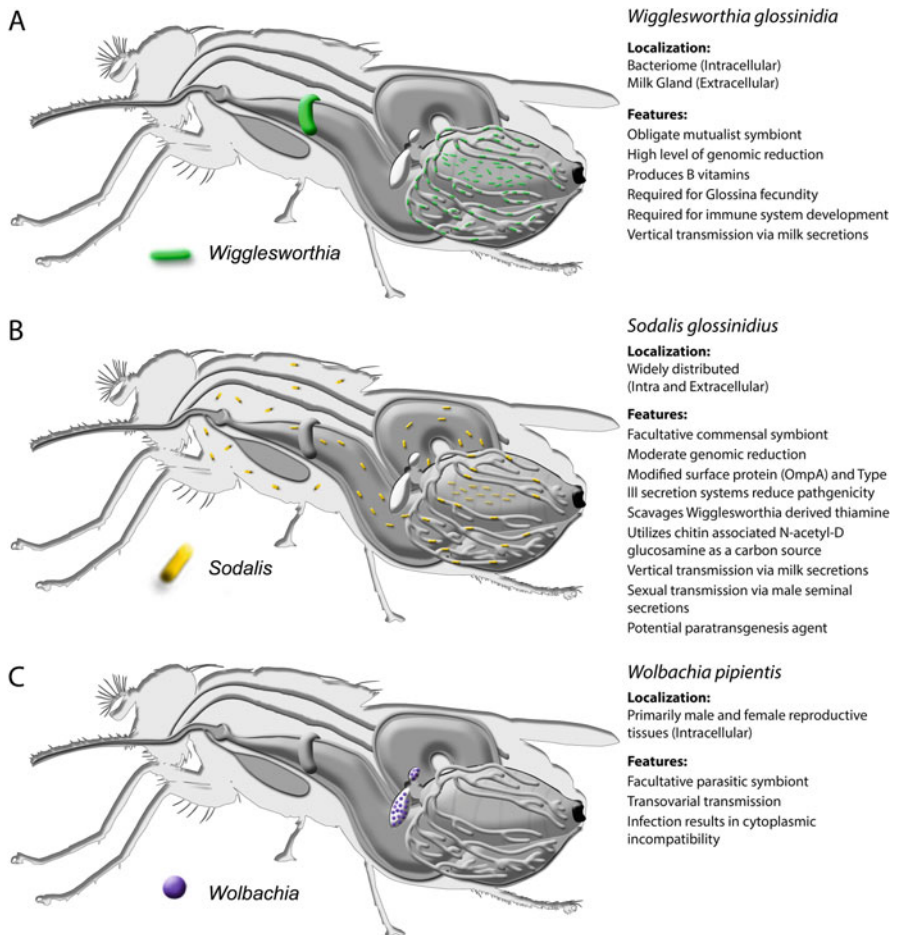


Fig. 19.1 Diagrammatic presentation of the microbiota in *Glossina*. The tissue localization and main features are reported for (a) *Wigglesworthia glossinidia*, (b) *Sodalis glossinidius*, and (c) *Wolbachia pipientis*

these bacterial species is variable. The only bacterial species consistently found in all tsetse fly species is *Wigglesworthia glossinidia*, which functions as an obligate mutualist. The interactions between vectors, their microbiota, and the parasites they vector are intricate. This chapter will explore the biology behind these relationships and the implications for tsetse physiology, vector control, and vectorial capacity.

19.2 *Wigglesworthia glossinidia*

19.2.1 *Discovery*

The first documentations of bacteria (bacteroids) living within the digestive tract of tsetse flies were made in *Glossina fuscipes* and *Glossina tachinoides* by Robert Koch in 1907 (Stuhlmann 1907). This was followed by a paper reported by Roubaud who hypothesized that the bacteria were symbiotic and aided in the digestion of blood by the fly (Roubaud 1919). These papers documented the presence of the bacteria and observed they were living intracellularly in a tissue (at the time termed the mycetome, currently referred to as the bacteriome), made up of giant hypertrophic cells. These cells contained bacteria, which Roubaud hypothesized that they were released during blood feeding to aid in digestion. The symbionts were further characterized in detail by Wigglesworth in 1929 in his analysis of the physiology of the tsetse digestive tract as well as by Buxton in his extensive 1955 memoir on *Glossina* biology for the London School of Tropical Medicine and Hygiene (Buxton 1955; Wigglesworth 1929). Wigglesworth did not observe free forms of the bacteria and suspected the observed free bacteria may have been released from the bacteriome due to damage during dissection. These papers hypothesized that *Wigglesworthia* functioned to supplement or aid in the digestion of tsetse's blood-specific diet; however, the lack of antibiotics made it difficult to target the bacteria for experimental purposes. Roubaud and Wigglesworth also hypothesized that these bacteria were vertically transmitted to the intrauterine larvae via the milk secretions generated by the female accessory (milk) glands.

The first functional evidence of *Wigglesworthia's* role as a symbiont was described when flies were fed on antibiotic-treated rabbits. The antibiotic fed flies were observed to have morphological disturbances in the bacteriome and a lack of bacteria. These flies showed decreased blood-feeding rates and appeared incapable of developing larvae, which were aborted early in their development (Hill et al. 1973). This observation was repeated by Nogge by direct antibiotic treatment of tsetse flies via an artificial blood meal (Nogge 1976). It was also shown that flies feeding on rabbits inoculated with *Wigglesworthia* become sterile. The antisera against *Wigglesworthia* from the rabbits are specifically bound and killed the bacteria in the bacteriome of flies (Nogge 1978). Nogge also noted that the loss of fecundity in tsetse in which endosymbionts had been removed (aposymbiotic) could be partially complemented by supplementation of blood meals with B-vitamins

(Nogge 1976). While the loss of symbionts has a negative impact on fecundity, it does not seem to influence lifespan (Nogge and Gerresheim 1982).

19.2.2 Localization and Transmission

The localization of *Wigglesworthia* in *Glossina* is specific to two locations. As mentioned previously, they are found living intracellularly within the giant bacteriocyte cells of the bacteriome tissue. The bacteriome is located in the anterior section of the *Glossina* midgut and from the exterior appears as a horseshoe-shaped structure that encircles the dorsal side of the midgut. The bacteriocyte giant cells protrude into the anterior midgut from the basal lamina of the gut and occupy a significant portion of the gut lumen (Wigglesworth 1929). Ultrastructural analysis of the bacteriome by transmission electron microscopy revealed many new observations about the relationship between tsetse and *Wigglesworthia*. Visualization of the bacteriome revealed that *Wigglesworthia* was gram-negative based on their membrane composition and rod-shaped up to 8 microns in length and 1–1.4 microns in width. The analysis also identified the presence of secretory vacuoles within *Wigglesworthia* connected to the plasma membrane (Reinhardt et al. 1972).

Analysis of genes enriched in the bacteriome of *Glossina morsitans* revealed tissue-specific gene expression patterns indicative of the specialization of these cells. The bacteriocytes are enriched in the expression of vesicular transport/exocytosis-related genes, sodium/potassium pumps, a lectin, and the peptidoglycan recognition protein LB (PGRP-LB) (Bing et al. 2017). The lectin is a carbohydrate-binding protein that in some systems is associated with symbiont uptake and localization (Bulgheresi et al. 2006; Chaston and Goodrich-Blair 2010; Kita et al. 2015; Wang et al. 2010). The ion pumps and secretion-associated genes suggest that the bacteriocytes are maintaining an ion gradient that may assist in the exocytosis and secretion of intracellular compounds and may function to export *Wigglesworthia*-derived cofactors. The presence of PGRP-LB is thought to have an immunosuppressive function by binding and cleaving free *Wigglesworthia*-derived peptidoglycan (Wang et al. 2009).

Initial hypotheses by Wigglesworth suggested that *Wigglesworthia* were transmitted vertically from mother to offspring via the glandular “milk” secretions produced by the female accessory gland (milk gland) that flow into the uterus and are imbibed by the developing larvae. Ultrastructural analysis of the milk gland tubules by Denlinger and Ma revealed the presence of bacteria living within the lumen of the gland and often located in close association with the openings of the secretory reservoirs from which milk is released (Ma and Denlinger 1974). The milk gland-associated bacteria showed differences from those in the bacteriome. The bacteria in the milk gland were extracellular, had a thicker cell wall, and were coated in filamentous fimbriae 5–7 nm in diameter and 2 μ m in length. These bacteria were later confirmed as *Wigglesworthia* by *in situ* staining of milk gland tissues with a specific 16S ribosomal RNA probe (Attardo et al. 2008; Balmand et al. 2013). The

morphological differences between the bacteriocyte and milk gland-derived *Wigglesworthia* appear to reflect differential morphological states required for intra and extracellular life within *Glossina*. Larval *Glossina* are exposed to *Wigglesworthia* only via their gut contents. During the larval stage, the gut is a closed system with larvae lacking an anal opening. A possible mechanism is if bacteria ingested during larvigenesis end up being partitioned, with some invading the cells of the bacteriome and others making their way to the milk gland possibly during metamorphosis. However, the route and mechanism of this colonization process remains an open question.

19.2.3 Phylogeny

Genetic analysis of *Wigglesworthia* by 16S ribosomal DNA analysis revealed that they fall into a discrete lineage close to members of the *Enterobacteriaceae* family within the class γ *Proteobacteria*. The *Enterobacteriaceae* are facultative anaerobes and include many species that function as gastrointestinal pathogens including *Escherichia coli* (*E. coli*) O157:H7, *Salmonella*, and *Shigella*. The γ *Proteobacteria* also contain a lineage of obligate symbionts of aphids in the genus *Buchnera* (Munson et al. 1991; Unterman et al. 1989). Analysis of *Wigglesworthia* derived from five species of *Glossina* representing three subgenera of tsetse flies revealed that these bacteria also form a specific lineage within the γ -3 subdivision of the *Proteobacteria*. Phylogenetic comparison of *Wigglesworthia* by 16S rDNA sequencing revealed them as close relatives of *Buchnera*, symbionts identified in other insects and *E. coli* (Aksoy 1995). The phylogeny of *Wigglesworthia* from the different species of *Glossina* closely matched the phylogeny of the flies themselves. This suggests the evolution of *Wigglesworthia* is parallel to that of *Glossina* reflecting an ancient relationship derived from a common ancestor (Aksoy et al. 1995; Chen et al. 1999; Symula et al. 2011). This relationship is estimated to have begun in an ancestral tsetse 50–100 million years ago.

19.2.4 Genetics, Functional Conservation, and Gene Loss

Sequencing of the *Wigglesworthia* genome revealed multiple insights into its biology and functional role as an obligate symbiont. The sequence was obtained from *Wigglesworthia* derived from the tsetse species *Glossina brevipalpis*. The genome is contained in a single chromosome and a plasmid with a total size of 697,742 base pairs containing 621 protein-coding sequences with 89.1% of the genome containing coding regions (Akman et al. 2002). The size of the genome is very small relative to most free-living bacteria and is similar in size to that of the obligate symbiont of the Pea aphid *Buchnera aphidicola*. The small size of these genomes likely reflects the symbiotic nature of these bacteria as they exist in a protected state within the

bacteriome tissues of the host (Bennett and Moran 2013; Moran and Bennett 2014). The protective environment and the presence of host-derived nutrients allow for relaxed evolutionary selection on functions required for free-living and competitive environments. In addition, obligate endosymbionts also tend to undergo rapid evolution due to the potential bottlenecks between generations during vertical transmission. To date, attempts to culture *Wigglesworthia* have failed, suggesting that these reductions have compromised their ability to survive outside the parameters of the host environment. *Wigglesworthia*'s genome also has a very low guanine/cytosine content of 22%, which is also similar to that of other intracellular bacteria. This is thought to result from the loss of genes coding for repair and recombination enzymes associated with the SOS, base excision, and nucleotide excision repair systems. However, genes with high expression levels such as ribosomal proteins and chaperonins have a bias toward the use of GC-rich amino acid codons relative to the rest of the genome (Herbeck et al. 2003). In addition, *Wigglesworthia* lacks the *DnaA* and *OriC* genes, which are essential components of the chromosomal replication complex, suggesting they are accomplishing this by an alternative method. Another interesting observation is that *Wigglesworthia* also lacks the gene coding for phosphofructokinase (*PfkA*), which is required for energy production via glycolysis and genes required for amino acid biosynthesis (Zientz et al. 2004).

The genes retained by *Wigglesworthia* are informative as to the selective pressures and unique environmental requirements within *Glossina*. These illustrate the biological demands required for maintenance of the vitality and fecundity of the host. While *Wigglesworthia* lacks a key enzyme for glycolysis, it has retained the transketolase and transaldolase enzymes utilized by the nonoxidative branch of the pentose phosphate pathway. This pathway may be facilitating the oxidation of abundant blood meal-derived amino acids for energy. In addition, they have retained the fructose biphosphatase (*fbp*) gene, which facilitates the synthesis of complex carbohydrates by gluconeogenesis as well as all the enzymatic components required for lipid, phospholipid, and nucleotide biosynthesis (Zientz et al. 2004).

The *Wigglesworthia* genome has maintained genes associated with biosynthetic pathways required for the synthesis of B-vitamins and cofactors such as pantothenate (B5), biotin (B7), riboflavin (B2), folate (B9), thiamine (B1), nicotinamide (B3), and pyridoxine (B6) (Akman et al. 2002). These compounds are deficient in blood and the retention of these biosynthetic pathways appears to be associated with nutritional supplementation of the tsetse host. This observation reinforces the original hypotheses of Roubaud, Wigglesworth, and Nogge. In addition, *Wigglesworthia* maintains all the genes required for the assembly and function of flagella. The function of the flagella in *Wigglesworthia* is unknown. The observation of fimbriae associated with extracellular milk gland *Wigglesworthia* suggests that the fimbriae are flagella and may be involved in motility and cellular invasion of the bacteriome during vertical transfer. Expression of the flagella-associated genes *motA* and *fliC* is specific to maternal milk glands, larvae and early pupal stages of development and their expression are not observed in bacteriome-associated *Wigglesworthia* (Rio et al. 2012).

High-throughput analysis of bacteriome-associated *Wigglesworthia* gene expression revealed enriched expression of functional classes of genes by the symbiont (Bing et al. 2017). The most abundant genes expressed by *Wigglesworthia* code for chaperonins, which aid in protein folding, as well as proteins associated with the degradation of misfolded proteins. These are hypothesized to compensate for the AT-rich nature of *Wigglesworthia*'s genome and the absence of rigorous DNA repair systems lost over evolutionary time. The second most highly expressed class of *Wigglesworthia* genes was associated with B-vitamin biosynthesis. Genes associated with thiamine (B1) biosynthesis were most highly expressed, followed by biotin (B7), riboflavin (B2), pantothenate and CoA (B5), nicotinamide (B3), pyridoxine (B6), and folate (B9) (Bing et al. 2017).

19.2.5 Roles in *Glossina* Digestion and Metabolism

The relationship between *Glossina* and *Wigglesworthia* is complex and is essential to multiple aspects of tsetse fly biology including digestion, metabolism, reproduction, and immunity. In the absence of *Wigglesworthia*, female *Glossina* becomes unable to develop intrauterine larval offspring, however, aposymbiotic males do not appear to suffer significant impacts to their fertility (Hill et al. 1973; Nogge 1976; Pais et al. 2008). Females in this state develop and ovulate oocytes. After fertilization, the oocytes appear to undergo embryogenesis, however, females abort their developing larvae early in intrauterine development. This suggests that either the larvae are dying due to malnutrition or that something is initiating premature parturition. Malnutrition could result from the lack of a *Wigglesworthia*-derived compound or the female's inability to effectively transfer nutrients via the milk in the aposymbiotic state. The retention of genes required for B-vitamin compound biosynthesis in the context of *Wigglesworthia*'s extreme genomic reduction highlights their significance within the context of the host and their role in dietary supplementation.

Research on the function of these compounds in *Glossina* biology has revealed important insights into this relationship. Dietary supplementation of *Glossina* blood meals with thiamine results in decreased *Wigglesworthia* population density and reduced expression of the *Wigglesworthia thiC* gene. This suggests that *Wigglesworthia* can sense environmental thiamine levels and regulate the expression of its biosynthetic pathway accordingly. Ectopic treatment with thiamine also caused changes in *Wigglesworthia* density suggesting that there is a potential population-regulatory mechanism either via the *Glossina* immune system or a regulatory mechanism within *Wigglesworthia* (Snyder et al. 2012).

In *Glossina*, the amino acid proline is the primary source of ATP production via the tricarboxylic acid (TCA) cycle. The proline is catabolized to alanine to produce ATP. The alanine is then shuttled back to lipid storage tissues where it is restored to proline by the addition of lipid-derived acetyl-CoA. This differs from many other insects that utilize carbohydrates as their primary source of ATP (Bursell 1960,

1963, 1966). A key enzyme required for the conversion of alanine back to proline is alanine-glyoxylate aminotransferase (AGAT). This enzyme requires vitamin B6 (pyridoxal 5'-phosphate) as a cofactor to function. Aposymbiotic flies have significantly lower levels of vitamin B6 as well as lower levels of free proline in their hemolymph. The lack of a key energy-associated metabolite during pregnancy is likely a significant impediment during the energetically demanding process of lactation in females (Michalkova et al. 2014).

The production of folate (Vitamin B9) by *Wigglesworthia* is important for fitness and reproductive function in *Glossina*. Folate functions as a cofactor in many pathways/processes including DNA/RNA synthesis, repair, methylation, and production of the amino acid methionine. *Wigglesworthia* upregulates genes associated with the chorismite and folate biosynthesis in young and pregnant female flies relative to males and virgin females. This suggests that *Wigglesworthia* responds to the requirements of the host. In addition, *Glossina* expresses a folate transporter protein in proportion to the folate level in the bacteriome (Snyder and Rio 2015). There are significant molecular and biochemical interactions occurring at the interface of the symbiont and host that maintain the equilibrium of the system. Female flies fed on glyphosate, an inhibitor of the chorismite and folate biosynthetic pathways, show a number of pathologies associated with folate deprivation. Offspring from folate-deficient mothers had longer larval and pupal development times, weighed less, and had a smaller adult body size (Snyder and Rio 2015).

Comparison of tetracycline-treated aposymbiotic female flies with age-matched pregnant females by untargeted metabolomic analyses revealed dysfunction in multiple metabolic pathways. As predicted by previous work, aposymbiotic females show deficiencies in B-vitamins and associated compounds resulting from the loss of *Wigglesworthia* (Bing et al. 2017). Many pathways with altered metabolite profiles are those with enzymes dependent on B vitamins as enzymatic cofactors. Metabolism of glycogen appears disrupted as aposymbiotic flies show increased levels of unprocessed glycogen metabolites relative to symbiotic flies. This is likely a result of the deficiency in vitamin B6, which is required as a cofactor by the enzyme glycogen phosphorylase. Another disruption appears in a pathway downstream of the glycogen pathway, the pentose phosphate pathway. This pathway processes glucose-6-phosphate derived from glycogen catabolism and converts it into NADPH (utilized in reduction reactions and fatty acid biosynthesis) and 5 carbon sugars (pentoses), which are required for the synthesis of aromatic amino acids and nucleotides. A key enzyme in this pathway is transketolase, which is dependent on vitamin B1 (thiamine). The disruption of this pathway results in a severe deficiency in the metabolite phosphoribosyl pyrophosphate (PRPP). This compound is an essential precursor to nucleotide biosynthesis and aposymbiotic flies appear to be impacted by this as they show significant deficiencies in purine and pyrimidine nucleotide biosynthesis (Bing et al. 2017). The deficiencies in nucleotide biosynthesis in combination with the B-vitamin deficiencies have further impacts downstream in the methionine metabolism pathway. This pathway is responsible for the production of S-adenosyl-methionine (SAM), which functions as a universal methyl donor in methylation reactions. Levels of SAM in aposymbiotic flies relative to controls were the second-

lowest among all the metabolites identified after PRPP. The biosynthesis of SAM requires adenosine, folate, and vitamin B6 all of which are deficient in aposymbiotic tsetse.

The exact cause behind why aposymbiotic female tsetse is unable to develop intrauterine larvae remains undetermined. However, the observed deficiencies indicate that lipid metabolism could be impacted. The reduced levels of NADPH could negatively impact fatty acid biosynthesis. In addition, the reduced levels of SAM could be impacting the synthesis of phospholipids, which play an important role in lipid storage, metabolism, and mobilization.

19.2.6 Roles in Immunity and Development

The interactions between *Glossina* and *Wigglesworthia* are ancient and complex affecting many aspects of the system. The role of *Glossina* species as vectors of trypanosomes makes the topic of immunity of particular interest. The obligate presence of symbionts requires fine tuning of immune responses such that potential pathogens are selectively targeted to avoid damage of symbiotic populations. Experimental treatment of female flies with the antibiotic ampicillin resulted in selective elimination of *Wigglesworthia* from the milk gland. However, the intracellular population in the bacteriome remained intact due to the inability of the antibiotic to penetrate the bacteriocytes (Pais et al. 2008). This resulted in females that maintain their fecundity; however, they do not pass on *Wigglesworthia* to their offspring. This finding allowed the study of the developmental impacts on tsetse physiology/biology associated with the loss of *Wigglesworthia*.

Glossina that develop in the absence of *Wigglesworthia* shows phenotypic effects that highlight the impacts of development in the absence of their symbionts. Aposymbiotic flies derived from ampicillin-treated mothers show a significant decrease in survival over time relative to symbiotic flies. This phenotype is exacerbated at higher temperatures. These flies also show deficiencies in digestion manifested as a reduced rate of blood meal digestion and the abundant presence of undigested hemoglobin in the gut 2 days after blood feeding. Flies lacking *Wigglesworthia* also appear more susceptible to infection by trypanosomes (Pais et al. 2008).

Analysis of immune gene expression associated with the presence or absence of *Wigglesworthia* showed that the peptidoglycan recognition protein PGRP-LB is upregulated in the bacteriome and milk gland tissues in the presence of *Wigglesworthia* and is downregulated in aposymbiotic flies (Dawadi et al. 2018; Wang and Aksoy 2012). PGRP-LB was first shown to moderate the immune system function in *Drosophila* through degradation of bacterially derived peptidoglycan and buffering activation of the Immune Deficient (IMD) immune pathway (Zaidman-Remy et al. 2006). Flies in which PGRP-LB is knocked down show higher levels of IMD-dependent antimicrobial gene expression. In *Glossina*, this protein is thought to regulate symbiont population numbers and protect the symbionts from the

immune system in the bacteriome and the milk gland. A similar role for PGRP-LB in mediating the symbiont/host relationship is also documented in the weevil (*Sitophilus zeamais*) (Anselme et al. 2006). Progeny of female PGRP-LB knock-down flies shows lower densities of *Wigglesworthia* suggesting that this is an important mechanism that ensures safe transfer of the symbiont from mother to offspring (Wang and Aksoy 2012).

Aposymbiotic *Glossina* show higher levels of IMD pathway-associated innate immune gene expression. However, if larvae develop in the absence of symbionts, they display an immunocompromised phenotype resulting from incomplete cellular immune cell development. This manifests as an inability to melanize and clot cuticular wounds and lack of mature hemocytes in their hemolymph (Weiss et al. 2011). Adult flies with this phenotype are very susceptible to hemocelic infections by *E. coli* relative to wild flies. Aposymbiotic *Glossina* appear to be deficient in plasmatocyte and crystal cell type hemocytes. These types of cells are responsible for phagocytosis of foreign bodies (plasmatocytes) and secretion of chemical components required for melanization (crystal cells). The hemocyte deficiency results from an inability of hemocyte precursor cells to differentiate into active immune cells. These findings suggest that *Wigglesworthia* provides stimuli required for proper hemocyte and immune system maturation (Weiss et al. 2011). Deeper analysis of this phenotype revealed that the presence of *Wigglesworthia* in developing larvae stimulates the expression of a gene coding for an odorant-binding protein, *obp6*. Knockdown of *obp6* expression in larval tsetse inhibited differentiation of precursor hemocytes into crystal cells specifically. The lack of crystal cells results in an inability of flies to melanize cuticular wounds or invading pathogens in a manner similar to aposymbiotic flies (Benoit et al. 2017). OBPs are thought to function as transporters for small hydrophobic molecules typically associated with olfaction (Zhou 2010). In this case, OBP6 is hypothesized to mediate the activity of a *Wigglesworthia*-derived compound required for crystal cell hemocyte differentiation. This finding expands the functional role of OBPs (previously thought to primarily function in olfaction) into the realm of development and immunity.

19.2.7 Summary

The relationship between *Wigglesworthia* and *Glossina* is intricate and essential for the survival of both organisms. The dependence of *Glossina* development, immunity, metabolic function, and reproduction on *Wigglesworthia* highlights the intricacy of this partnership. While this partnership is likely unique in some respects, the dependence of other blood-feeding insects suggests that there may be similarities in the relationships between obligate blood feeders and their associated obligate symbiotic relationships.

19.3 *Sodalis glossinidius*

19.3.1 *Discovery and Genetic Characterization*

Sodalis was first described in 1987 as a Rickettsia-like-organism isolated from the hemolymph of *Glossina* by culturing it in a cell line from the Asian Tiger mosquito *Aedes albopictus* (Welburn et al. 1987). The bacterium was classified as a member of the family *Enterobacteriaceae* within the γ -3 subdivision of the Proteobacteria. This family contains multiple species found as symbionts in other insects (Aksoy et al. 1997; Hosokawa et al. 2015; Novakova et al. 2015). The bacterium was cultured and characterized outside of the fly under microaerobic conditions using a solid-phase culture technique. A new genus, *Sodalis*, was derived from this work to contain secondary symbionts of other tsetse and insect species (Dale and Maudlin 1999). Initial analyses of the *Sodalis* genome by pulse field gel electrophoresis determined it to be ~2 Megabases (Mb) (Akman et al. 2001); however, follow up efforts with more advanced sequencing technologies revealed the genome to be 4.1 Mb (Toh et al. 2006). *Sodalis* also carries multiple extrachromosomal plasmids (Akman et al. 2001). The extrachromosomal material contains genes coding for pilus proteins required for bacterial conjugation and horizontal gene transfer as well as siderophores required for the binding and transport of iron (Darby et al. 2005).

Comparison of the genome relative to *E. coli* (another member of the *Enterobacteriaceae*) via microarray revealed that the two organisms are ~85% orthologous in terms of gene composition. The genome contains three regions encoding Type III secretion systems (SSR-1, SSR-2, and SSR-3), which appear to have independent ancestries and differences in their constitution (Toh et al. 2006). The SSR-1 region is similar in composition to the Type III secretion system *ysa* in the bacteria *Yersinia enterocolitica*, while SSR-2+3 bear similarity to SPI-1+2 in *Salmonella*. Many pseudogenes were found in the *Sodalis* genome relative to that of free-living *Salmonella typhi* and *Yersinia pestis*. Genes lacking functional orthologs in *Sodalis* are associated with anaerobic metabolism and carbohydrate transport/metabolism. In addition, there are reductions in genes coding for membrane-associated proteins as well as those involved in cell structure (Rio et al. 2003). Further analysis of the *Sodalis* genome revealed the loss of the arginine biosynthesis pathway in *Sodalis* suggesting that it scavenges this amino acid from *Glossina* (Belda et al. 2010). Another feature of the *Sodalis* genome was the loss of the pathway for synthesis of the B-vitamin thiamine, yet they have retained a thiamine transporter gene. *Wigglesworthia* is capable of thiamine production suggesting that *Sodalis* may scavenge *Wigglesworthia*-derived thiamine (Snyder et al. 2010). While *Sodalis* does not show the same level of genomic reduction as *Wigglesworthia*, the loss of these genes suggests it could be at an intermediate stage in the transition from a free-living facultative relationship to that of a symbiont.

An updated annotation of the *Sodalis* genome and development of a new culture system utilizing media with defined ingredients facilitated in-depth analysis of the nutritional and growth requirements of *Sodalis in vitro*. This work determined that

Sodalis does encode an arginine biosynthesis system and is not completely auxotrophic. Growth on media lacking arginine is reduced, but not eliminated. Addition of excess L-glutamate to this system rescued the reduced growth rate associated with arginine depletion suggesting that *Sodalis* can compensate for low environmental arginine with L-glutamate and that this compound functions as an important source of carbon and nitrogen. Another interesting finding is that the carbohydrate N-acetyl-D-glucosamine, a component of insect chitin, is an important dietary factor for *Sodalis*. This suggests that *Sodalis* may have adapted to utilize an abundant carbohydrate associated with the *Glossina* physiological environment (Hall et al. 2019).

19.3.2 Biology, Localization, and Transmission

As opposed to *Wigglesworthia*, the *Sodalis*'s range is not limited to specific tissues and are found throughout the fly. During intrauterine larval development, *Sodalis* migrates into the developing larvae via the milk secretions. Upon eclosion from the pupa, a significant increase in the numbers of *Sodalis* relative to *Glossina* host cells is observed over a two-week period. The numbers of *Sodalis* then appear to fluctuate over time in adults (Rio et al. 2006). *Sodalis* is found intra and extracellularly in tissues throughout larval and adult *Glossina* including the midgut, fat body, milk gland, uterus, and oviduct. The ovaries and developing oocytes remain uninfected (Attardo et al. 2008; Balmand et al. 2013). *Sodalis* are also capable of transfer from infected males to females via seminal secretions during mating (De Vooght et al. 2015). Work by Dale and Welburn revealed that treatment of *Glossina* with the antibiotic streptozotocin selectively eliminates *Sodalis* while leaving bacteriome-based *Wigglesworthia* intact. This treatment did not impact *Glossina* fecundity, as observed with the elimination of *Wigglesworthia*. However, flies lacking *Sodalis* showed a significant reduction in lifespan and increased susceptibility to trypanosome infection in laboratory settings (Dale and Welburn 2001).

Investigations into the role of Type III secretion systems in the relationship between *Sodalis* and *Glossina* revealed differences relative to orthologous loci in free-living and parasitic bacterial species. These systems are often associated with pathogenicity due to their role in the secretion of toxins and inflammatory agents as well as invasion of host cells. Disruption of this system by transposon-mediated mutagenesis of the *invasion protein C* (*invC*) gene (a component of the type III secretion system) prevented an invasion of insect cells cultured *in vitro* (Dale et al. 2001). SSR-2 seems to have lost gene functionality for the proteins coding for the needle structure of the secretion system. Functional analysis of these pathways suggests that SSR-1 is required for cell invasion while SSR-2 is required for intracellular division (Dale et al. 2005).

Iron acquisition is essential for bacterial survival and proliferation and *Sodalis* has retained protein-coding genes required for iron chelation, transmembrane transport, heme metabolism, and iron storage. These include an inner membrane heme ABC transporter system (*hemTUV*), an outer membrane heme transporter (*hemR*), an iron/

manganese transporter (*sitABCD*), ferritin-like proteins, and an iron-responsive negative transcriptional regulator (*Fur*). Analysis of the regulatory regions from two of these *Sodalis* genes in *E. coli* revealed that the Fur regulatory protein is required for the correct expression of these genes. Under iron-rich environmental conditions, these genes are repressed; however, in the absence of the Fur regulator, they are constitutively expressed. In *Glossina*, these genes appear to be responsive to environmental iron as in unfed teneral flies these genes are upregulated. However, at 48 hours post blood meal, the genes are significantly downregulated (Runyen-Janecky et al. 2010; Smith et al. 2013). The *hemR* outer membrane heme transporter is essential for the survival of *Sodalis* in *Glossina* as strains with mutations in this protein do not establish. The *tonB* gene codes for the protein that supplies energy to *hemR* and is also essential to this system. Strains with mutations in *tonB* show a similar phenotype to that of *hemR* mutants in that they are unable to colonize *Glossina* in its absence (Hrusa et al. 2015).

Analysis of quorum-sensing mechanisms in *Sodalis* revealed that the compound N-(3-oxohexanoyl) homoserine lactone regulates population numbers. The presence of this compound activates the transcription of genes coding for oxidative stress-response proteins that may function to reduce the oxidative burden associated with symbiosis (Pontes et al. 2008). The changes in these systems relative to free-living bacteria may reflect a reduction in mechanisms associated with pathogenesis and adaptations required for invasion by and survival of *Sodalis* in *Glossina* tissues. Transfer of *Sodalis* strains between *Glossina* species revealed that *Sodalis* originating in one species of *Glossina* are capable of colonization and survival in another species indicating that these adaptations are not species-specific. Rather, they allow *Sodalis* to be compatible across the *Glossina* genus (Weiss et al. 2006). *Sodalis* has a truncated lipopolysaccharide (LPS) structure that lacks the O-antigen, which may facilitate its ability to live within *Glossina* with the induction of an immune response (Toh et al. 2006). In depth analysis of outer membrane proteins revealed that the *OmpA* (Outer membrane protein A) gene in *Sodalis* contains polymorphisms not found in other pathogenic bacteria such as *E. coli*. The infection of *Glossina* with *E. coli* is fatal under normal conditions. However, infection with *E. coli* containing an *OmpA* mutation is nonpathogenic while infection with *Sodalis* containing the native *E. coli OmpA* gene results in a lethal infection (Weiss et al. 2008). In *Sodalis*, the *OmpA* surface protein is essential for the establishment of gut infections within *Glossina*. Mutation of the native *Sodalis OmpA* gene prevents biofilm formation that is required for the protection of bacteria from the flies' immune response (Maltz et al. 2012).

19.3.3 Distribution in Wild Populations and Relationship with Vector Competence

Analysis of wild populations of flies revealed that the presence of *Sodalis* is heterogeneous in the field. The field-collected samples of *Glossina austeni* and

Glossina pallidipes from Kenya and South Africa reveal *Sodalis* infection rates to be around 3.7% in *G. austeni* and 16% in *G. pallidipes* (Wamwiri et al. 2013). Analysis of *Glossina* populations from Luambe National Park in Zambia also revealed high levels of variability in the proportions of individuals infected with *Sodalis*. The species with the highest infection level was *Glossina brevipalpis* with 93.7% infection rate followed by *Glossina morsitans* and *G. pallidipes* with 17.5% and 1.4%, respectively (Dennis et al. 2014). Illumina sequencing-based analyses of *Glossina* microbiota of fly populations in Uganda have revealed a broader diversity of microbial taxa than previously identified across multiple species of *Glossina* including *Glossina fuscipes fuscipes* (from five distinct populations), *Glossina morsitans morsitans*, and *Glossina pallidipes*. All samples were predominantly occupied by *Wigglesworthia* with it constituting the majority of bacterial sequences. A comparison of the profiles of the remaining bacterial taxa revealed the microbiomes from the different *G. fuscipes* populations showed significant diversity between their microbial constitution. The survey also identified a high prevalence of low-intensity *Sodalis* infections across all the groups tested (Aksoy et al. 2014). Another high-throughput microbiome analysis of *Glossina palpalis palpalis* from Cameroon revealed similar results with *Wigglesworthia* being the predominant species in the flies and the investigators also found low-level infections of *Sodalis* throughout the samples (Tsagmo Ngoune et al. 2019).

Comparison of the *Trypanosoma* infection rates in *Sodalis* infected versus uninfected field collected flies identified a significant positive correlation between the two suggesting that the presence of *Sodalis* may facilitate the establishment of blood meal-derived *Trypanosoma* infections (Farikou et al. 2010; Soumana et al. 2013; Wamwiri et al. 2013). Work on changes in *Sodalis* gene expression in permissive versus nonpermissive *Glossina pallidipes* revealed significant changes in expression profiles between the two groups. A large proportion of the changes associated with the refractory flies is associated with a viral prophage carried by *Sodalis*, which suggests that activation of this phage may be associated with an antitrypanosomal response (Hamidou Soumana et al. 2014a, b). However, recent work studying the correlation between *Sodalis* and *Trypanosoma* coinfections in wild-caught flies suggests that other factors such as geographic location, trypanosomal species, *Glossina* species, and the age and sex of the flies analyzed can be confounding factors (Channumsin et al. 2018).

Selective clearance of *Sodalis* using the antibiotic streptozotocin allowed researchers to generate a *Sodalis*-free line of *Glossina* (Sod-) in the lab for comparison with infected individuals (Sod+) by high-throughput gene expression analysis. An interesting finding from this research is that there were no significant changes in immune-responsive genes between Sod- and Sod+ flies. However, the challenge of these flies with *E. coli* or *Sodalis praecaptivus*, a free-living relative of *S. glossinidius*, resulted in a robust immune response. They also showed that activation of *Glossina*'s innate immune response did not have a significant impact on endogenous *Sodalis* numbers suggesting that the bacterium is resistant to host immune factors. Comparisons of susceptibility to *Trypanosoma brucei brucei*

infection between Sod- and Sod+ flies showed no significant difference between the two groups.

19.3.4 Potential for Use in Paratransgenesis

The ability to isolate and culture *Sodalis* outside of *Glossina* provides the opportunity for a reduction of *Glossina*'s vectorial capacity via manipulation of its symbionts. This strategy is called paratransgenesis and is a promising alternative to direct genetic manipulation of the vector species (Coutinho-Abreu et al. 2010). Current genetic transformation technologies, functional in insects such as mosquitoes and *Drosophila*, are not a viable option in *Glossina* due to their low reproductive rate and viviparous physiology. Paratransgenesis works through the genetic transformation of cultured *Sodalis* with a gene encoding an antitrypanosomal factor. The engineered *Sodalis* are then reintroduced into *Glossina* where, in principle, the presence of the modified symbiont would create a hostile environment for invading trypanosomes resulting in reduced or eliminated vectorial capacity. Paratransgenesis was demonstrated in laboratory studies to be an effective strategy in kissing bugs (Order Hemiptera, Family Triatomidae) as a way to reduce their vectorial capacity for the Chagas pathogen, *Trypanosoma cruzi* (Beard et al. 2001, 2002).

Research into the use of *Sodalis* as a paratransgenic agent shows promise. Transformation of *Sodalis* with 9 different cationic antimicrobial peptides toxic to African trypanosomes revealed that *Sodalis* is resistant to 7 of them. This finding opened the door for the utilization of *Sodalis* as a paratransgenesis agent for the delivery of antitrypanosomal compounds in vivo (Haines et al. 2003). A study demonstrated that *Sodalis* transformed with a gene for a trypanolytic nanobody can secrete this factor and that they are capable of invading tissues throughout the fly. The effective establishment of this infection was dependent on the prior treatment of the flies with the antibiotic streptozotocin to deplete native *Sodalis* numbers and reduce competition. These bacteria express the nanobody throughout the tissues of the fly; however, vertical transmission of these bacteria only occurred at a low level (De Vooght et al. 2014). Follow up work shows that the establishment of a stable infection and reliable vertical transmission with engineered bacteria is dependent upon the route of introduction. Flies given intrathoracic injections with recombinant *Sodalis* as adults were able to establish infections, but those infections are not vertically transmitted to offspring. However, the investigators found that injection of larval *Glossina* facilitated disseminated bacterial infection as well as vertical transmission to the resulting offspring (De Vooght et al. 2018).

Sterile Insect Technique (SIT) is another important strategy for population control of *Glossina*. An issue associated with SIT in *Glossina* is that radiation-sterilized males are still capable of functioning as vectors, which is an ethical issue associated with this approach. An alternative approach that minimizes this risk is the release of sterile males infected with engineered *Sodalis* to reduce or eliminate their vectorial capacity. Sterilization of paratransgenic males with gamma radiation revealed that

while the treatment caused an initial decline in the number of recombinant *Sodalis*, the bacterial numbers recovered over time making this a potentially practical approach (Demirbas-Uzel et al. 2018).

19.3.5 Summary

The relationship between *Glossina* and *Sodalis* is very different from that of *Glossina* and *Wigglesworthia*. *Sodalis* appears to be in an evolutionary transition between a free-living organism and a symbiont. Its genomic reductions are not at the level of that observed in *Wigglesworthia*; however, *Sodalis* has accumulated a significant number of pseudogenes associated with functions required for a free-living existence. Analysis of retained features suggests the development of specializations for life within the host. These include alterations to its outer membrane proteins and secretory systems to reduce its pathogenicity and immunogenicity. *Sodalis* has also made nutritional adaptations that optimize it for survival in the fly including the use of N-acetyl-D-glucosamine as a carbon source and the retention of a transporter to scavenge *Wigglesworthia*-derived thiamine. *Glossina* does not require the presence of *Sodalis* for essential functions such as reproduction. However, selective elimination of the bacteria does have a negative impact on *Glossina* lifespan suggesting a beneficial aspect to the relationship. The understanding of the relationship between *Sodalis* and the different *Trypanosoma* types remains uncertain given the conflicting results from field and lab-based studies. The ability to culture and genetically manipulate *Sodalis* makes it an ideal agent for use in paratransgenesis studies. Research on this method of control is ongoing and could be a valuable tool for integration into SIT strategies or on its own to reduce the vectorial capacity of wild *Glossina* populations.

19.4 *Wolbachia*

19.4.1 Discovery

Wolbachia is a genus of obligate intracellular gram-negative bacteria belonging to the Order Rickettsiales and were first identified from *Culex pipiens* in 1924 (Hertig and Wolbach 1924). In 1936, this finding was confirmed and described in detail (Hertig 1936). Since these early discoveries, research has shown that *Wolbachia* is widespread in arthropods (reviewed in (Serbus et al. 2008)), and is probably the most prevalent endosymbiont found in insect germlines. It is found in every insect order (Harris and Braig 2003), and is estimated to infect >65% insect species (de Oliveira et al. 2015; Hilgenboecker et al. 2008).

19.4.2 Basic Biology and Features

The widespread presence of *Wolbachia* is thought to be due to its efficient transmission and its capacity to manipulate host reproduction to favor infected females. *Wolbachia* bacteria are indeed able to colonize female germline cells, through which they are transovarially transmitted to the progeny. *Wolbachia* interspecific horizontal transmission was initially described as a rare phenomenon (O'Neill et al. 1992; Rousset et al. 1992; Turelli et al. 2018; Werren et al. 1995), but studies are increasingly showing that, in a variety of insect species, *Wolbachia* genes have been horizontally transferred to host chromosomes (Aikawa et al. 2009; Dunning Hotopp et al. 2007; Fenn et al. 2006; Klasson et al. 2009; Kondo et al. 2002; Nikoh and Nakabachi 2009; Nikoh et al. 2008; Woolfit et al. 2009).

In different arthropod hosts, *Wolbachia* infection is responsible for several mechanisms that enhance female fertility. These reproductive alterations induce different host phenotypes such as cytoplasmic incompatibility, the feminization of genetically male offspring, male-killing of infected males, and parthenogenesis by infected females (Harris and Braig 2003; Stouthamer et al. 1999; Tram et al. 2003).

Cytoplasmic Incompatibility (CI), the most prevalent and most widely investigated phenomenon, was first observed in the 1970s (Yen and Barr 1973) and results in embryonic mortality in the progeny derived from matings between insects with different *Wolbachia* infection status (Bourtzis et al. 1998; Clark et al. 2003). Unidirectional CI occurs when an infected male mates with an uninfected female; whereas the reciprocal crossing is compatible. Bidirectional CI occurs in crossings between individuals infected with different *Wolbachia* strains (Werren 1997). Embryo lethality has been related to the modifications induced by *Wolbachia* to the paternal chromosomes during spermatogenesis in a way that mitotic synchrony is lost (O'Neill and Karr 1990; Tram and Sullivan 2002). The genetic basis of CI remained unknown for a long time, and only recently LePage and colleagues identified two genes in the eukaryotic association module of prophage WO (Bordenstein and Bordenstein 2016) from *Wolbachia* strain *wMel* that acts as CI factors (Beckmann et al. 2017; LePage et al. 2017). These studies revealed that the mitotic defect is due to a deubiquitinating enzyme encoded by one of the two genes (*i.e.* CidB/CifB) and neutralized by the CidA/CifA product.

In different species, *Wolbachia* has also been proposed to play a key role in sex determination by enhancing female germline development (Cordaux and Gilbert 2017; Kageyama et al. 2017; Kageyama and Traut 2004; Sugimoto et al. 2015). For example, in the parasitoid wasp *Asobara tabida*, *Wolbachia* is essential for oogenesis as its elimination induces apoptosis in the ovaries thus impeding egg maturation (Dedeine et al. 2001; Pannebakker et al. 2007). The role of *Wolbachia* in supporting female germline development in *Drosophila melanogaster* began to be clarified when females carrying mutant alleles of the master gene in female sex determination, *sex-lethal*, rescued their fertility when infected with *Wolbachia* (Starr and Cline 2002). Following these findings, more recent work showed that *Wolbachia* interacts with RNAs encoding proteins involved in the support of germline stem cell maintenance and oocyte polarization (Ote and Yamamoto 2020). These data further

support the idea that this bacterium is a genetic manipulator of the infected arthropod hosts (Kozek and Rao 2007).

Wolbachia-mediated manipulations were shown in recent work to affect several other reproduction-related functions. For example, in *Drosophila*, *Wolbachia* affects gene transcription in larval testes (Zheng et al. 2011) as well as the expression of seminal fluid proteins (Yuan et al. 2015). Moreover, *Wolbachia* was shown to impact the expression of immunity genes in a parasitoid wasp (Kremer et al. 2012). The functions *Wolbachia* exerts in mosquitoes are achieved through manipulations of host microRNAs and the production of small RNAs regulating host gene expression (Hussain et al. 2011; Mayoral et al. 2014).

Recent studies expanded knowledge on other effects exerted by *Wolbachia* on the behavior of its hosts. These include influences on sleep, learning, memory, feeding, mating, locomotion, and aggression [for a review see (Bi and Wang 2019)]. For example, it has been suggested that *Wolbachia* is able to affect sleep in *Drosophila* by interactions with the juvenile hormone/sex-determination genes/dopamine pathway. In particular, this bacterium appears to contribute to increased sleep time in order to favor the conservation of resources and energies to support reproductive outputs, thus supporting both the host and its transmission and indicating that the coevolution between *Wolbachia* and its hosts is even more multifaced than so far discovered (Bi and Wang 2019).

19.4.3 Localization in Insect Tissues

Wolbachia primarily resides in the germline tissues of both male and female insects (Dobson et al. 1999); in males, this bacterium is found in the spermatocytes but there is no transmission through the sperm (Bressac and Rousset 1993; Clark et al. 2002; Ijichi et al. 2002). In addition, since its early detection, this bacterium was described to be present also in somatic tissues [(Dobson et al. 1999; Hertig and Wolbach 1924) also see (Pietri et al. 2016) for a review].

In the germline tissue, some *Wolbachia* strains localize at the posterior end of mature oocytes (Ote and Yamamoto 2020) thanks to the presence of RNAs and proteins transported from the nurse cells along microtubules to form a pole plasm (Serbus and Sullivan 2007). Pole cells, together with somatic gonadal cells, form the embryonic gonads, becoming primordial germ cells. During the development of the female pupa, the primordial germ cells initiate their divisions resulting in the germ cell lineage in the ovaries of adult females. *Wolbachia* is found in the germline stem cells, which are localized at the anterior end of each ovariole within the ovaries (Ote et al. 2016; Serbus et al. 2008). In particular, *Wolbachia* tends to be located close to processing bodies in the cytoplasm of these cells that supply maternal factors to the oocyte (Ferree et al. 2005; Franks and Lykke-Andersen 2008; Ote et al. 2016; Serbus et al. 2011), resulting in an association that takes place from oogenesis to embryogenesis. In particular, *Wolbachia* produces a protein able to interact with RNAs encoding proteins that are involved in the support of germline stem cells and oocyte polarization (Ote and Yamamoto 2020).

As far as it concerns *Wolbachia* distribution in somatic tissues, this bacterium is known to be particularly abundant in the nervous system in *Drosophila* and several other insect species (Albertson et al. 2013; Casper-Lindley et al. 2011; Dobson et al. 1999; Mitsuhashi et al. 2002; Moreira et al. 2009; Osborne et al. 2009; Strunov and Kiseleva 2016), as well as in the fat body, gut, salivary glands, hemocytes, and Malpighian tubules, where it has been suggested to play roles related to the host immunity and metabolic regulation (see (Pietri et al. 2016) for a review). The age of the host also impacts *Wolbachia* infection levels and tropism, further suggesting that the physiological relationships between *Wolbachia* and its hosts are extremely complex and require a case-to-case analysis (Binnington and Hoffmann 1989; Bressac and Rousset 1993; Min and Benzer 1997).

19.4.4 *Wolbachia* in *Glossina* spp.

Early hybridization experiments conducted between different species belonging to the *Glossina* genus suggested the presence of incompatibilities resulting in females with reduced fecundity and sterile males. Both bidirectional and unidirectional incompatibility events were reported ((Curtis 1972; Rawlings 1985; Vanderplank 1948) see also (Gooding 1985, 1987, 1989, 1990) for a review). These data, together with light and electron microscopy studies showing gram-negative rods in tsetse ovaries and in the periphery of the yolk of early embryos (Huebner and Davey 1974; Pell and Southern 1975; Pinnock and Hess 1974) and 16s rRNA sequence analyses (Beard et al. 1993), prompted researchers to further investigate the identity of these bacteria. The exploration of 16S rRNA phylogenetic relationships and tissue distribution in tsetse resulted in the identification of *Wolbachia* in laboratory strains of *Glossina* species in 1993 (O'Neill et al. 1993). Initially, *Wolbachia* was detected in *G. m. morsitans* and *G. m. centralis*, while it was found to be absent in *G. p. palpalis* and *G. p. gambiensis*, supporting some of the reported events of reproductive incompatibilities in the genus (O'Neill et al. 1993).

19.4.5 *Tissue Localization in Tsetse Tissues*

The tissue tropism of *Wolbachia* to the ovarian tissues suggested that transovarial transmission is the primary mode of transmission for this bacterium (O'Neill et al. 1993), which supports previous studies (Pell and Southern 1975). Subsequent work on *G. m. morsitans* laboratory flies confirmed *Wolbachia*'s absence in the milk gland secretions, as well as any other somatic tissues surrounding the uterus, further supporting its transovarial transmission (Balmand et al., 2013). *Wolbachia* was not detected extracellularly, and it was shown to infect only the trophocytes and the oocytes in the ovaries, as well as embryos and larvae (Cheng et al. 2000; Balmand et al. 2013).

However, while in certain tsetse species *Wolbachia* appeared to be restricted to the reproductive tissues, in *G. austeni*, this bacterium was detected also in somatic tissues, in particular the head, salivary glands, milk glands, and fat body (Cheng et al. 2000). These findings stimulate novel studies aimed at understanding whether these differences are due to features of different *Wolbachia* strains infecting the different tsetse species or to insect-specific factors, such as immunity-regulatory mechanisms, controlling the infections. The application of a particularly effective hybridization approach (*i.e.* high-end Stellaris® RNA-FISH) surprisingly revealed that *Wolbachia* is also present in the lumen and secretory cells of the milk glands in *G. m. morsitans* (Schneider et al. 2018). This result opens up new pathways of investigation of a potentially yet undiscovered vertical transmission mechanism for this bacterium.

19.4.6 *Wolbachia* Role in Tsetse Physiology

Earlier studies described the existence of bidirectional CI between certain *G. morsitans* subspecies and between *G. palpalis* subspecies, while unidirectional CI has been described only in crossings of certain *G. morsitans* subspecies (Cheng et al. 2000).

The first study showing the functional role of *Wolbachia* in tsetse reared in the laboratory was performed by Alam and colleagues, who showed that the infection of this bacterium is able to support the expression of cytoplasmic incompatibility (Alam et al. 2011). The females expressing CI displayed loss of fecundity due to early embryogenic failure. Currently, there is no way to selectively cure *Wolbachia* infection through antibiotic administration. Aposymbiotic flies lack all endosymbionts, including obligate bacteria responsible for obligate nutritional dialogues, in the absence of which tsetse flies are sterile. Alam and coauthors set up a method to maintain *Wolbachia*-cured *G. m. morsitans* (symbiont-free, Gmm^{APo}) fertile through dietary provisioning of blood meals supplemented with tetracycline and yeast extract, thus rescuing tsetse fecundity, which is tightly dependent on *Wigglesworthia*. Moreover, cytoplasmic incompatibility in tsetse is particularly strong, differently from many other species that are characterized by incomplete CI (Sinkins and Gould 2006).

Whether *Wolbachia* plays a role in trypanosome infection is still under debate. Alam and colleagues reported the presence of a negative association between *Wolbachia* and trypanosome infections in *G. f. fuscipes*, suggesting that this bacterium could prevent trypanosome infections (Alam et al. 2012). However, the tripartite association between tsetse, trypanosomes, and *Wolbachia* remains unclear. In *G. p. palpalis*, *Wolbachia* infection appeared to have no impact on the establishment of trypanosomes (Kante et al. 2018). This is similar to what was found in *G. tachinoides* and *G. m. submorsitans* (Kame-Ngasse et al. 2018). To shed light on this relevant biological aspect, more extensive data on trypanosome and *Wolbachia* infections, as well as the other symbionts, in different tsetse species and populations

are required. Moreover, the potential relationships between specific *Wolbachia* haplotypes and trypanosome infections will be essential to determine the presence and features of this dialogue.

19.4.7 *Wolbachia* Distribution in Tsetse Strains and Populations

Since its discovery, a number of studies have focused on expanding the analysis of the distribution of *Wolbachia* in tsetse species, in both laboratory strains and wild samples. The first extensive work aimed at understanding the presence and infection rates of *Wolbachia* in tsetse was performed by Cheng and colleagues, who analyzed the status of *Wolbachia* infections in laboratory colonies of *G. brevipalpis* and *G. longipinnis* (Fusca group), *G. fuscipes*, *G. tachinoides*, *G. p. palpalis*, and *G. p. gambiensis* (Palpalis group), as well as *G. m. morsitans*, *G. m. centralis*, *G. swynnertoni*, and *G. pallidipes* (Morsitans group) (Cheng et al. 2000). All individuals analyzed from the Morsitans and Fusca groups were positive for *Wolbachia* infection, while none of the flies belonging to the Palpalis group harbored *Wolbachia*, mirroring earlier findings (O'Neill et al. 1993). A 100% prevalence in laboratory strains was confirmed by a more recent study for *G. m. morsitans* and *G. m. centralis*. Similarly, the absence of *Wolbachia* was confirmed in laboratory strains of *G. f. fuscipes* and *G. tachinoides*, whereas in *G. pallidipes*, different colonies displayed different prevalences of *Wolbachia* ranging from the absence (Seibersdorf lab-colony) to low prevalence (KARI-TRC lab-colony, 3%) (Doudoumis et al. 2012). Also in this study, flies from *G. p. palpalis* and *G. p. gambiensis* laboratory colonies showed the absence of *Wolbachia* infection. Differences were reported for *G. brevipalpis*, where prevalence was not complete (41.2%) (Doudoumis et al. 2012).

Significant differences in *Wolbachia* infection frequencies are found between field populations and laboratory strains. In addition, differences are observed within wild populations, which may be dependent on ecological conditions (Mouton et al. 2007; Yun et al. 2011). For example, in the case of *G. m. morsitans*, the *Wolbachia* presence in wild populations ranges from 9.5 to 100% (Doudoumis et al. 2012). In *G. brevipalpis* sampled in South Africa *Wolbachia* appears absent, while, in Kenyan flies, infection levels are reported to be about 30% (Cheng et al. 2000). In *G. austeni*, infection rates ranged from 48 to 98%, in Kenya and South Africa, respectively (Cheng et al. 2000). More recent work also detected variations in *Wolbachia* prevalence in *G. austeni* samples in these two countries, but with the Kenyan population showing higher infection levels (Wamwiri et al. 2013). Low *Wolbachia* prevalence was detected in populations of *G. pallidipes* (below 8.5%) and in *G. gambiensis* (below 8.3%), and the absence of *Wolbachia* infection in *G. p. palpalis* and *G. f. fuscipes* populations was confirmed (Doudoumis et al. 2012). Alam and colleagues conversely showed the presence of *Wolbachia* infections in

G. f. fuscipes from Uganda, although at low density, which may have influenced its detection in previous studies (Alam et al. 2012). A similar result was obtained by Schneider and colleagues, who used sensitive PCR-based methods that allowed the identification of *Wolbachia* in *G. f. fuscipes* (Schneider et al. 2013, 2018). Recent studies identified *Wolbachia* also in *G. p. palpalis* (Kante et al. 2018), as well as in *G. tachinoides* (68.1% prevalence) and in *G. m. submorsitans* (58.5%) (Kame-Ngasse et al. 2018), further supporting the idea that the choices of molecular markers and detection methods play a key role in the sensitivity of determining the infection rates of *Wolbachia*. Indeed, when present at low titers, *Wolbachia* infections can be detected only through the integration of different tools, such as high-sensitivity blot-PCR combined with hybridizations (Schneider et al. 2018).

Finally, in *G. f. quanzensis* (Palpalis group) from Congo, the analysis of midguts revealed that 85% of the analyzed samples were infected by *Wolbachia*, with infection rates varying according to sampling sites (Simo et al. 2019). Moreover, a low number of midguts were naturally coinfecting by both *Wolbachia* and *Sodalis*, opening new questions on the potential interactions between these two tsetse symbionts.

19.4.8 *Wolbachia Integrations in Tsetse Genomes*

The genome of *G. m. morsitans* contains large segments of *Wolbachia* that were integrated via horizontal gene transfer (HGT) events. These integrated fragments contain a high degree of nucleotide polymorphisms, as well as insertions and deletions (Brelsfoard et al. 2014).

Subsequent comparative analysis of the genomes of six *Glossina* species, namely *G. morsitans morsitans*, *G. pallidipes*, *G. austeni* (Morsitans group), *G. palpalis* and *G. fuscipes* (Palpalis group), and *G. brevipalpis* (Fusca group), showed that all contain sequences with homology to *Wolbachia*, although the features of these integrations are different. Indeed, in *G. pallidipes*, *G. fuscipes*, *G. palpalis*, and *G. brevipalpis*, the homologous sequences consisted in short fragments and they were initially thought to be artifacts as PCR assays with *Wolbachia*-specific primers on these strains as well as on wild populations of these species resulted in negative results (Doudoumis et al. 2013). However, recent studies suggest that natural *G. p. palpalis* populations from Cameroon do carry *Wolbachia* symbionts (Kante et al. 2018).

However, *G. austeni* contains more extensive *Wolbachia*-derived chromosomal integrations. Both chromosomal and cytoplasmic *Wolbachia* sequences found in *G. austeni* were mapped against the reference *Wolbachia* genomes (*i.e.* wMel and wGmm), as well as the A and B chromosomal insertions found in *G. m. morsitans* (Attardo et al. 2019). The *Wolbachia* insertions in *G. austeni* range in size from 500 to 95,673 bps and display high-sequence homology not only to wMel and wGmm, but also to *G. m. morsitans* A and B insertions. The higher homology (98%) with A and B insertions from *G. m. morsitans* relative to cytoplasmic

Wolbachia sequences suggests they could be derived from an event in a common ancestor, although the absence of comparable insertions in *G. pallidipes* (a closer relative to *G. m. morsitans*) requires further investigations on wild samples from *Glossina* species/subspecies to clarify the true origin of these events.

Whether these *Wolbachia* chromosomal integrations have functional roles in *Glossina* biology is still elusive. Indeed, gene expression analyses of *Wolbachia* insertions in *G. morsitans* found very limited evidence of expression (Brelsfoard et al. 2014), suggesting they may be accidental transfer events associated with the long-term symbiosis between *Wolbachia* and these tsetse species. However, since *Wolbachia* integrations in tsetse chromosomes include genes encoding proteins carrying ankyrin repeat domains, thought to be directly related to *Wolbachia*-host interactions, further research is required to determine their potential involvement in CI (Duron et al. 2007; Iturbe-Ormaetxe et al. 2005; Tram and Sullivan 2002).

19.4.9 *Wolbachia* Diversity

Wolbachia displays a high level of diversity in arthropods and nematodes and it currently comprises 17 phylogenetic clades (or supergroups), named from A to Q (Baldo et al. 2006; Bordenstein and Rosengaus 2005; Bordenstein et al. 2009; Casiraghi et al. 2005; Glowska et al. 2015; Gorham et al. 2003; Lo et al. 2002; Paraskevopoulos et al. 2006; Ros et al. 2009; Rowley et al. 2004). Each supergroup collects strains, most frequently named after their host species (e.g. *wPip* in *Culex pipiens*, *wGff* in *G. f. fuscipes*). The most common strategy adopted for strain genotyping relies on multilocus sequence typing (MLST), which includes the sequences of the five conserved genes *fbpA*, *coxA*, *ftsZ*, *gatB*, *coxA*, and *hcpA* and the amino acid sequences of the four hypervariable regions of the WSP protein (Baldo et al. 2006). The diversity displayed in this genus is visible at different levels, including the presence of variants within the same individual host, variation among *Wolbachia* sequences sampled from different individuals belonging to the same species, as well as the molecular changes occurring in the same *Wolbachia* infection in the case of transfer to different host species (*i.e.* in the case of horizontal gene transfer) (Hoffmann et al. 2015). In the case of *Glossina*, *Wolbachia* identified in tsetse species has been regarded to belong to the supergroup A, as determined based on the *Wolbachia* surface protein (*wsp*) gene (Cheng et al. 2000; Zhou et al. 1998). A recent study investigated *Wolbachia* genetic variability in *G. f. fuscipes* from Uganda (Symula et al. 2013). Two *Wolbachia* lineages were identified, suggesting the presence of superinfection in this species, and a high diversity within and between individuals. These data suggest that different *Wolbachia* strains infected this tsetse species multiple times independently.

19.4.10 *Wolbachia as a Tool for Vector Population Control*

Wolbachia has been proposed as a tool for controlling insect disease vectors and agricultural pests through multiple approaches, which are not mutually exclusive. These methods include the release of (1) *Wolbachia*-infected males that are incompatible with females by exploiting cytoplasmic incompatibility phenotypes (O'Connor et al. 2012), (2) *Wolbachia* strains able to induce deleterious fitness effects, in particular under seasonally variable environments (Rasić et al. 2014), and (3) *Wolbachia* strains interfering with pathogen transmission and thus decreasing the ability of vectors to transmit diseases (Kambris et al. 2009; Moreira et al. 2009; Teixeira et al. 2008; Walker et al. 2011). The capacity of rapidly invading insect populations that drive maternally inherited elements into wild insects has made *Wolbachia* a promising tool for inducing genetic manipulations of detrimental species (Beard et al. 1993; Sinkins et al. 1997). The data obtained by Alam and colleagues about CI in tsetse were incorporated into a mathematical model and suggest that *Wolbachia* has the potential for use as a gene-drive mechanism. This could be used to introduce desirable phenotypes, such as resistance to trypanosome infection, into wild *Glossina* populations (Alam et al. 2011).

The exploitation of *Wolbachia*-induced CI was proposed for use in the reduction of population sizes of several insect disease vectors and agricultural pests (Apostolaki et al. 2011; Bourtzis 2008; Stouthamer et al. 1999; Xi et al. 2005; Zabalou et al. 2009). Moreover, *Wolbachia* is able to protect their hosts against viral pathogens (Cook and McGraw 2010). The initial characterization of this phenomenon was performed in *Drosophila* (Hedges et al. 2008; Osborne et al. 2009; Teixeira et al. 2008) and mosquitoes (Bian et al. 2010; Glaser and Meola 2010; Moreira et al. 2009). More recent work demonstrates that *wAlbB* infection in the C6/36 *Ae. albopictus* cell line resulted in reduced titers of several Flaviviruses and Alphaviruses, suggesting a role of *Wolbachia* in reducing transmission of pathogenic RNA viruses (Ekwudu et al. 2020). Moreover, a triple-infected *Ae. albopictus* line carrying, in addition to the two natural symbiotic strains *wAlbA* and *wAlbB*, the *wAu* from *D. simulans*, showed complete resistance to Zika and dengue infections, with moderate fitness costs (Mancini et al. 2020). Pathogen-blocking function has been associated with the upregulation of antimicrobial peptides (e.g. DEFC, defensin c) (Caragata et al. 2019; Pan et al. 2018; Pan et al. 2012).

In the case of tsetse species, as mentioned above, contrasting results have been obtained. In wild populations of *G. f. fuscipes*, the presence of *Wolbachia* has been suggested to be able to prevent trypanosome infection (Alam et al. 2012), while in *G. p. palpalis*, *G. tachinoides*, and *G. m. submorsitans*, *Wolbachia* seems not to impact establishment of trypanosome infection (Kame-Ngasse et al. 2018; Kante et al. 2018). Further studies are thus necessary to understand whether *Wolbachia* may be used in Incompatible Insect Technique (IIT)-based approaches for population control. This strategy is based on the release of *Wolbachia*-infected males that both induce CI when mated with *Wolbachia*-free wild females and refractoriness to

trypanosome infection and transmission. This concept is similar to strategies recently designed for mosquitoes (Bourtzis et al. 2016; Zhang et al. 2015a, b, 2016).

19.4.11 Summary

The *Wolbachia* symbiont is present predominantly in tsetse gonadal tissues, and it is transovarially transmitted from females to their progeny. An increasing number of studies now show that this bacterium can colonize also somatic tissues, with still unclarified functions. Horizontal transfer events have been detected in *G. m. morsitans* and *G. austeni*, but whether these integrated sequences play functional roles in tsetse biology is still unclear. *Wolbachia* induces strong cytoplasmic incompatibility in tsetse, supporting the idea that it may be exploited as a tool to control tsetse populations in the field.

19.5 Spiroplasma

The innovation of high-throughput 16S ribosomal RNA sequencing has allowed for deeper exploration of the microbiome of *Glossina* species. These studies have identified many other bacterial species in *Glossina* from laboratory colonies and from the field. From these analyses, bacteria from the Genus *Spiroplasma* were found in field-caught and lab colonies of *Glossina f. fuscipes* and *G. tachinoides*. The recent analysis of the genomes of six *Glossina* species also revealed evidence for a significant relationship between *Spiroplasma* and *G. f. fuscipes*. Analysis of the genomic scaffolds from this species revealed the presence of *Spiroplasma* genomic sequences. None of these sequences appear to be the result of a genomic integration and are likely derived from free-living *Spiroplasma* present in the flies used for sequencing (Attardo et al. 2019). Developmental stage and tissue specificity analyses revealed the *Spiroplasma* infections to be highest in the larval gut and male reproductive tissues. In addition, the analysis of flies from a collapsing colony of *G. f. fuscipes* showed that levels of *Spiroplasma* were higher in surviving flies versus recently deceased flies (Doudoumis et al. 2017). Another study found that *Trypanosoma* infection in field-collected *G. f. fuscipes* is negatively correlated with *Spiroplasma* coinfection. This finding was also demonstrated in the laboratory by comparing experimental infection rates between *Spiroplasma* infected and uninfected *G. f. fuscipes* (Schneider et al. 2019).

Spiroplasma species are found to be living in many insect and invertebrate species with relationships ranging from pathological to beneficial. Pathogenic *Spiroplasma* infections leading to death have been observed in aquatic invertebrates, such as shrimps and crabs (Nunan et al. 2005; Wang et al. 2004). In some *Drosophila melanogaster*, infection with *Spiroplasma* results in reproductive manipulation in the form of a male-killing phenotype (Montenegro et al. 2005; Paredes et al. 2015).

However, *Spiroplasma* infections in *Drosophila* can also protect against infection by the parasitic nematode *Howardula aoronymphium*. The nematode infection results in loss of fecundity in female *Drosophila*. However, the coinfection of females with *Spiroplasma* results in the restoration of fecundity and inhibition of nematode development (Haselkorn et al. 2013; Jaenike et al. 2010). The early observations of the relationship between *Spiroplasma* and *Glossina* suggest that this relationship is beneficial in terms of its ability to extend lifespan and reduce the vectorial capacity for *Trypanosoma*. However, relative to the other *Glossina* symbionts, little is known about the details of the interactions between these two species and more work will be required to develop a more comprehensive understanding of this relationship.

19.6 Conclusions

The role bacteria play in the biology of tsetse flies spans the gamut, ranging from parasitic to obligate mutualists. This system is also unique in that the microbiome of species within *Glossina* is limited relative to other insects. This is primarily due to their restricted diet and the protected nature of intrauterine larval development. These limitations simplify the system and provide opportunities to study microbe-host interactions in a way that is impossible in insects with more diverse microbiomes. The relationship between *Wigglesworthia* and *Glossina* is ancient and provides a clear example of obligate symbiosis. It also demonstrates the evolutionary mechanisms and biology behind how an organism can evolve to survive and thrive on a nutrient-rich yet limited diet. The commensal yet nonessential relationship between *Sodalis/Glossina* provides a snapshot of two organisms in the early or intermediate stages of a symbiotic relationship and can provide insights as to the adaptations made by both parties to establish a harmonious relationship. In addition, the ability to culture and manipulate *Sodalis* provides opportunities to take advantage of this relationship for purposes of controlling trypanosome transmission by *Glossina*. The nature of the relationship between *Glossina* and *Wolbachia* is more complex and shows aspects of parasitism and manipulation by *Wolbachia* similar to that observed in other insects. However, a deeper understanding of the protective nature of *Wolbachia* infections against viral infections in other insects and the inherent reproductive manipulations driving the spread of these infections may provide opportunities to utilize this relationship to reduce *Glossina* vectorial capacity. The implementation of new technologies such as high-throughput metagenomics, advanced microbial imaging, and novel *in vitro* culture techniques has opened opportunities to investigate these well-studied relationships in more depth. They also provide the ability to identify previously undescribed microbial interactions that were overshadowed by the dominant microbial fauna. Analysis of the diversity of these lesser-explored interactions could provide valuable insights into the ecology, population dynamics, and biology of this unique system.

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