AEM
Journals.ASM.org

# OmpA-Mediated Biofilm Formation Is Essential for the Commensal Bacterium *Sodalis glossinidius* To Colonize the Tsetse Fly Gut

**Michele A. Maltz, Brian L. Weiss, Michelle O'Neill, Yineng Wu, and Serap Aksoy**

Yale School of Public Health, Department of Epidemiology of Microbial Diseases, New Haven, Connecticut, USA

**Many bacteria successfully colonize animals by forming protective biofilms. Molecular processes that underlie the formation and function of biofilms in pathogenic bacteria are well characterized. In contrast, the relationship between biofilms and host colonization by symbiotic bacteria is less well understood. Tsetse flies (*Glossina* spp.) house 3 maternally transmitted symbionts, one of which is a commensal (*Sodalis glossinidius*) found in several host tissues, including the gut. We determined that *Sodalis* forms biofilms in the tsetse gut and that this process is influenced by the *Sodalis* outer membrane protein A (OmpA). Mutant *Sodalis* strains that do not produce OmpA (*Sodalis* ΔOmpA mutants) fail to form biofilms *in vitro* and are unable to colonize the tsetse gut unless endogenous symbiotic bacteria are present. Our data indicate that in the absence of biofilms, *Sodalis* ΔOmpA mutant cells are exposed to and eliminated by tsetse's innate immune system, suggesting that biofilms help *Sodalis* evade the host immune system. Tsetse is the sole vector of pathogenic African trypanosomes, which also reside in the fly gut. Acquiring a better understanding of the dynamics that promote *Sodalis* colonization of the tsetse gut may enhance the development of novel disease control strategies.**

The digestive tract of animals represents an environment that houses a dense population of microbes (18, 28, 36, 52, 53). With the advancement of molecular technologies, our awareness of the diversity and abundance of microbes in this niche has increased dramatically. It is now known that the vast majority of bacteria present in the gut are commensals and thus positively impact the physiology of their hosts (18, 28, 36, 53). However, studies to further decipher the biological mechanisms that underlie digestive tract symbioses are impeded by the taxonomic complexity of the animal microbiome, the presence of unculturable microbes, and the inability to use standard molecular biological tools to genetically manipulate many of these microorganisms. Thus, identification of efficient model systems with less diverse microbial communities can advance our understanding of host-microbe interactions and their effect on pathogenic microbes.

One model system that can be used to study commensal symbioses is the tsetse fly (Diptera: Glossinidae). This fly harbors three distinct bacterial symbionts, including the parasitic *Wolbachia*, obligate *Wigglesworthia*, and commensal *Sodalis*. Tsetse flies are unique in that they exhibit a viviparous mode of reproduction, during which juvenile larvae develop in an intrauterine environment and receive nourishment from maternal milk gland secretions. *Wigglesworthia* and *Sodalis* are maternally acquired by intrauterine larvae via milk secretions, while *Wolbachia* is transmitted through the germ line (4). In addition to residing extracellularly in the female milk gland, *Wigglesworthia* is also found intracellularly in the tsetse gut-associated bacteriome (3). As a result of the long coevolutionary history of *Wigglesworthia* and tsetse, this bacterium's genome is reduced in size and highly streamlined in composition (1, 7). Despite these genome reduction processes, *Wigglesworthia* has retained a number of vitamin biosynthesis pathways, which may provision the tsetse host with metabolites missing from its vertebrate blood-specific diet (2). Tsetse's association with commensal *Sodalis* is more recent. *Sodalis* is closely related to several free-living enteric bacteria, including *Yersinia* and *Salmonella* (44). Furthermore, phylogenetic analysis of *Sodalis* from distant tsetse species suggests that these microbes recently descended from a common ancestor and spread via horizontal transfer events (49). *Sodalis* resides intra- and extracellularly throughout its host and can be cultivated *in vitro* and genetically manipulated using conventional techniques (8). Tsetse's simple microbial community may enable us to study host-symbiont interactions, including gut colonization processes, host mechanisms that enable tolerance to beneficial microbes, and symbiont mechanisms that enable their persistence against hostile host immune responses.

Functional studies have demonstrated a direct association between bacterial outer membrane protein A (OmpA) and microbial phenotypes in the tsetse. Specifically, young adult flies (3 days posteclosion) tolerate hemocoelic superinfection with *Sodalis* but quickly perish following the same treatment with normally nonpathogenic *Escherichia coli* K-12 (50). Analysis of the *Sodalis ompA* gene sequence indicated that environmentally exposed loop domains of the OmpA protein contain amino acid insertions and/or substitutions compared to their counterparts from *E. coli* and other closely related free-living and pathogenic bacteria (44, 50). From a functional perspective, OmpA polymorphisms were determined to modulate the infection outcome in tsetse when flies were infected with either *E. coli* OmpA mutants (*E. coli* ΔOmpA) or *Sodalis* that had been genetically modified to express *E. coli* OmpA (*Sodalis* pIO$_{E.\ coli}$). These infection experiments showed that tsetse flies were able to tolerate *E. coli*ΔOmpA, while *Sodalis* pIO$_{E.\ coli}$ exhibited a pathogenic phenotype that caused all infected tsetse flies to perish. These findings suggest that modifications associated with *Sodalis* OmpA may promote tsetse's tolerance of *Sodalis* and reflect larger evolutionary adaptations that facilitate bacterial/insect symbioses.

**Received** 5 June 2012 **Accepted** 10 August 2012
**Published ahead of print** 31 August 2012
Address correspondence to Serap Aksoy, serap.aksoy@yale.edu.
Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.01858-12

OmpA is also involved in the formation of microbial biofilms (5, 14, 27, 42), which are populations of microbes that adhere to surfaces by producing a polysaccharide extracellular matrix. Biofilms play a role in bacterial colonization dynamics of the mammalian and invertebrate gastrointestinal tracts (29). In *E. coli*, OmpA influences bacterial colonization of host tissues by promoting biofilm formation on hydrophobic surfaces (27, 41). In fleas (*Xenopsylla cheopsis*), the causative agent of plague, *Yersinia pestis*, forms a biofilm that blocks the passage of blood through its host's proboscis. This process starves the host and causes it to take multiple blood meals, which in turn increases the chances of pathogen transmission to the mammalian host (11). Additionally, pathogenic *Vibrio cholerae* can form biofilms in the rectum of *Drosophila melanogaster* and eventually kill its insect host (35). It remains to be seen if OmpA also modulates the ability of *Yersinia* and *Vibrio* to form biofilms in flea and *Drosophila* host tissues, respectively.

To date, the role of biofilms with respect to symbiont colonization of the insect gut is poorly understood. In this study, we investigated whether biofilm formation is required for commensal *Sodalis* to colonize the tsetse gut and whether *Sodalis* OmpA plays a role in the process of biofilm formation. We also discuss the association between biofilms and symbiont evasion of hostile host immune responses.

## MATERIALS AND METHODS

**Tsetse and bacterial cultures.** Tsetse flies (*Glossina morsitans morsitans*) were maintained in Yale University's insectary at 24°C with 55% relative humidity. Unless specifically indicated otherwise, flies received defibrinated bovine blood through an artificial membrane feeding system every 48 h (32, 50). Aposymbiotic tsetse flies ($Gmm^{Apo}$) were generated as described previously (48). *Sodalis*-free tsetse flies ($Gmm^{Sgm-}$) were generated by feeding 200 μg of ampicillin to flies two times before bacterial inoculation. Sample sizes for all experiments are indicated in the appropriate figure legends.

All bacterial strains used in this study as described in Table S1 in the supplemental material. Wild-type (WT) *Sodalis* strains were isolated from tsetse pupae and subsequently maintained in liquid Mitsuhashi-Maramorosch (MM) medium as described previously (50). Brain heart infusion agar plus 10% defibrinated bovine blood (BHIB) was used when *Sodalis* strains were plated on solid medium. *E. coli* OmpA mutants (*E. coli* ΔOmpA) and *E. coli* OmpA mutants that express *Sodalis ompA* (*E. coli* ΔOmpA $pIO_{Sodalis}$) were generated as described previously (50). *E. coli* strains were grown at 37°C on LB agar plates or in liquid LB medium under the appropriate antibiotic selection. The growth medium was supplemented with designated antibiotics: ampicillin (Amp) (25 μg/ml for *Sodalis* and 100 μg/ml for *E. coli*), tetracycline (Tet) (5 μg/ml for *Sodalis*), and kanamycin (Kan) (50 μg/ml for *Sodalis* and 100 μg/ml for *E. coli*).

**Construction of 16S clone library.** Genomic DNA (gDNA) was extracted from six female fly guts and one male fly gut (all 8 days old) using the MasterPure DNA purification kit (Epicentre). The 16S clone library was constructed as previously described with the following modification (51): 16S rRNA genes were amplified using the primer set 27F and 1492R. The PCR amplification product was cloned into pGEM-T vector (Promega) and transformed into *E. coli* DH5α cells, and colony PCR was performed on 320 recombinant clones in 96-well plates, using T7 and SP6 primers. The PCR product was digested with *Taq*αI and HaeIII (New England BioLabs) restriction enzymes, and the generated profile was visualized on a 2.0% Metaphor agarose gel stained with ethidium bromide. Multiple clones with represented restriction profiles were then sequenced using T7 primer at the DNA analysis facility (Yale University) with BigDye Terminator. Sequences were compared to the NCBI BLAST database and the Ribosomal Database Project II (RDP) (http://rdp.cme.msu.edu/) (10). All oligonucleotide primers and PCR amplification conditions used in this study are summarized in Table S2 in the supplemental material.

**Fluorescent *in situ* hybridization (FISH) and lectin staining.** Guts were microscopically dissected from 4-day-old $Gmm^{WT}$ flies and subsequently subjected to FISH analysis and lectin staining as described previously (23, 34). In brief, a 5′-end rhodamine-labeled *Sodalis*-specific 16S rRNA probe (5′-ACGAGACTCTAGCCTGCCAG-3′) was used for FISH analysis (34). Dewaxed tissue sections, which were produced as described previously (23), were stained in a blocking solution (containing 4 nmol/ml of DAPI [4′,6-diamidino-2-phenylindole]) containing 200 ng of *Sodalis*-specific probe and 10 μg/ml of one of the following fluorescein-labeled lectins: concanavalin A, *Dolichos biflorus* agglutinin, peanut agglutinin, *Ricinus communis* agglutinin I, soybean agglutinin, *Ulex europeaus* agglutinin, and wheat germ agglutinin (WGA) (lectin kit I, Vector Laboratories, CA). Fluorescent signals were observed with a Zeiss epifluorescence microscope.

**Generation of *Sodalis ompA* mutants.** *Sodalis* mutants that do not produce OmpA (hereafter referred to as *Sodalis* ΔOmpA mutants) were generated using the TargeTron gene knockout system (Sigma-Aldrich) according to the manufacturer's protocol. In brief, the group II intron encoded on expression plasmid pACD4K-C was retargeted to insert 540 bp from the *ompA* start codon. The resulting retargeting plasmid, designated pSOM, was electroporated into *Sodalis* PT, which harbors a helper plasmid (pAR1219) that encodes the T7 RNA polymerase gene under transcriptional control of the *lac* UV5 promoter (see Table S2 in the supplemental material). Following electroporation, cells were pelleted and resuspended in MM medium supplemented with chloramphenicol and 1% glucose. Following an overnight incubation, T7 RNA polymerase gene expression was induced for 1 h with the addition of 200 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and cells were pelleted and resuspended in MM medium containing 1% glucose. After 1 h incubation, the cells were similarly pelleted and resuspended and mutant cells were selected on BHIB+Kan plates. Insertion of the intron into the correct chromosomal locus was confirmed by PCR using *ompA* gene primer pair OmpAF1 and OmpAR1. Amplification primers are listed in Table S3 in the supplemental material. Recombinant OmpA protein production was confirmed by Western blot analysis using an anti-*E. coli* OmpA antibody at a dilution of 1:20,000 (50).

***Sodalis in vitro* blood growth assay.** The mammalian complement system was inactivated by heating blood for 1 h at 56°C. *Sodalis* PT or the *Sodalis* ΔOmpA mutant at 500 CFU/ml was added to either regular or heat-inactivated bovine blood. Inoculates were maintained at 24°C. Bacterial density was monitored every day for 7 days by serially diluting and subsequently plating samples on BHIB plates. CFU were counted when they became visible (~7 days after plating).

**Biofilm formation assay.** *Sodalis* PT or *Sodalis* ΔOmpA cells were inoculated into 5 ml of MM medium and vigorously shaken at an angle in a 15-ml Falcon tube for 7 days. Seven days later, liquid medium was removed from the tubes and the "splash zone" was stained for 15 min with crystal violet. Finally, tubes were gently rinsed with water (2×) and inspected for the presence of a biofilm.

Biofilm formation was also quantified using a previously described microtiter plate biofilm assay (31). A 24-well microtiter plate (Corning Life Science) with 500 μl of MM medium was inoculated with 500 μl of log-phase culture of strains to be tested (at an optical density at 600 nm [$OD_{600}$] of ≈0.5). The microtiter plate was incubated for 11 days at 25°C, and the OD was measured at 600 nm. The relative biofilm formation was calculated with the following formula: OD of crystal violet/OD of growth yield.

***Sodalis* OmpA restores the *E. coli* biofilm formation defect.** *E. coli* strain MG1655 Δ*ompA*::Tn5Kan-2, which has an *ompA* transposon-generated mutation (21), was complemented by expressing *Sodalis* OmpA in this mutant strain. Plasmid $pIO_{Sodalis}$ was electroporated into MG1655 Δ*ompA*::Tn5Kan-2, thus generating *E. coli* strain ΔOmpA $pIO_{Sodalis}$ (21).

**TABLE 1** 16S RNA clone libraries of tsetse fly gut

| Source | No. of clones | Species | No. of isolates |
|---|---|---|---|
| Female guts w/bacteriocyte | | | |
| Gut 1 | 30 | *Wigglesworthia glossinidia* | 28 |
| | | *Sodalis glossinidius* | 2 |
| Gut 2 | 30 | *Wigglesworthia glossinidia* | 27 |
| | | *Sodalis glossinidius* | 3 |
| Gut 3 | 20 | *Wigglesworthia glossinidia* | 18 |
| | | *Sodalis glossinidius* | 2 |
| Gut 4 | 18 | *Wigglesworthia glossinidia* | 16 |
| | | *Sodalis glossinidius* | 2 |
| Female guts w/no bacteriocyte | | | |
| Gut 1 | 120 | *Wigglesworthia glossinidia* | 0 |
| | | *Sodalis glossinidius* | 120 |
| Gut 2 | 39 | *Wigglesworthia glossinidia* | 4 |
| | | *Sodalis glossinidius* | 35 |

*E. coli* strain ΔOmpA $pIO_{Sodalis}$ was subsequently monitored, as described above, to determine its biofilm formation phenotype.

***Per os* colonization assay.** To assess the ability of different bacteria to colonize the tsetse gut, 500 CFU of each strain was added to 1 ml of heat-inactivated bovine blood and provided to flies through an artificial membrane system. Following *per os* inoculation with bacteria, flies were maintained on heat-inactivated blood every 48 h. Gut tissue was microscopically dissected at 1, 3, and 5 days postinoculation with bacteria. Three guts from each group were dissected per time point, homogenized in 0.85% NaCl, serially diluted, and plated on BHIB supplemented with antibiotics. CFU per plate were manually counted. All bacterial strains, tsetse lines, and antibiotics (including concentrations) used for these experiments are indicated in the appropriate figure legends. All *per os* colonization experiments were performed in duplicate.

**Experimental induction of the tsetse immune response.** Tsetse flies were immunologically stimulated as described previously (17). In brief, $Gmm^{WT}$ flies were fed either lipopolysaccharide (LPS) and peptidoglycan (PGN) (50 μg of each/ml of blood) or live *E. coli* (500 CFU/ml blood) prior to inoculation with experimental bacteria. Flies were exposed to *E. coli* once and LPS and PGN continuously throughout the course of the colonization assay. Colonization assays were performed in duplicate as described above.

## RESULTS

**Tsetse gut microbiome and bacterial biofilm formation in this niche.** In an effort to analyze the microbial composition of the tsetse gut, we constructed 16S rRNA libraries from this tissue and found that it houses only vertically transmitted *Wigglesworthia* and *Sodalis* (Table 1). Gut-associated *Wigglesworthia* strains are localized exclusively within a specialized organ called the bacteriome. However, *Sodalis* is found both intra- and extracellularly in several tsetse tissues, including the gut (8). As such, we set out to determine if protective biofilms form the mechanistic basis that underlies the ability of extracellular *Sodalis* to colonize the gut of wild-type tsetse flies (here designated $Gmm^{WT}$). We used DAPI (Fig. 1A) and fluorescent *in situ* hybridization (FISH) analysis with *Sodalis*-specific 16S rRNA probes (Fig. 1B) to detect bacterial clusters in this environment (Fig. 1C is a merged image of Fig. 1A and B). Additionally, these images were enlarged to identify individual bacteria (Fig. 1D to F). These data suggest that *Sodalis* resides in microcolonies in the luminal fluid of the tsetse gut. Microcolonies, which are considered to be the basic structural unit of a biofilm, were then investigated for the presence of a polysaccha-

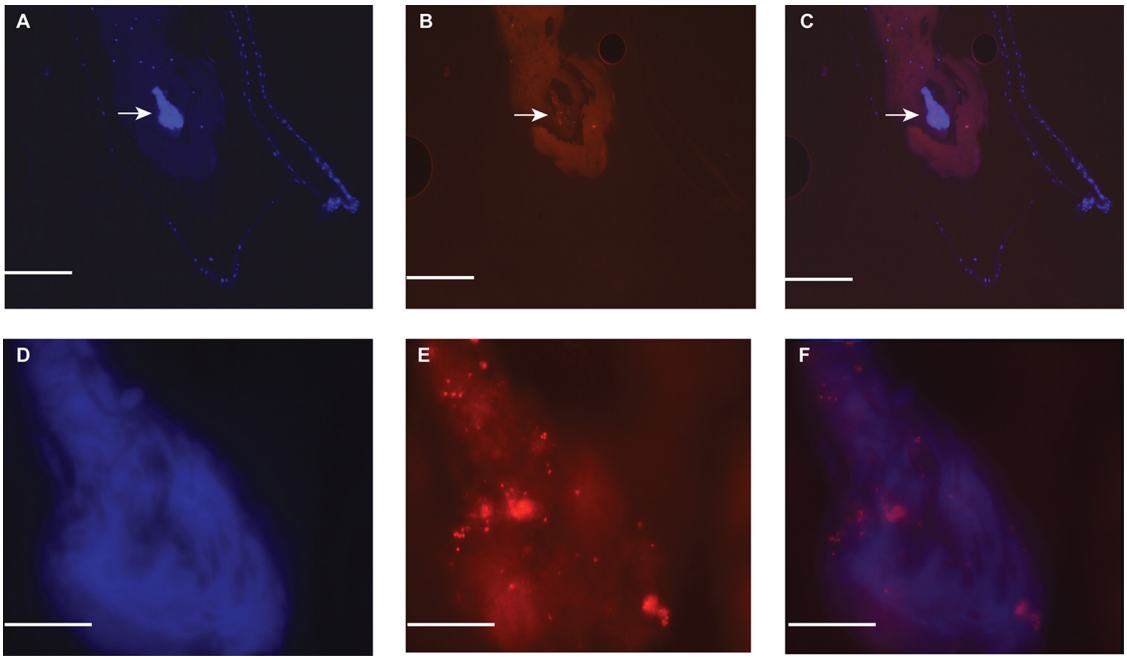


**FIG 1** *In situ* visualization of *Sodalis* clusters present in the tsetse gut. (A) Cross sections of tsetse gut tissue stained with DAPI. DNA from host epithelial tissue and *Sodalis* cells all stains blue. Bar, 50 μm. (B) Visualization of *Sodalis* clusters in the lumen of the tsetse gut using fluorescent *in situ* hybridization. Tissues were probed with a rhodamine-labeled oligonucleotide specific for *Sodalis* 16S rRNA (red). Bar, 50 μm. Autofluorescent blood meal-derived erythrocytes surround *Sodalis* cells. Bars, 50 μm. (C) Panels A and B merged to better orient the position of *Sodalis* cell clusters in relation to tsetse gut epithelial tissues. In each of panels A, B, and C, the most prominent cluster of *Sodalis* cells is indicated by a white arrow. (D to F) Enlarged views of the *Sodalis* clusters shown in panels A to C, respectively. Bars, 10 μm.

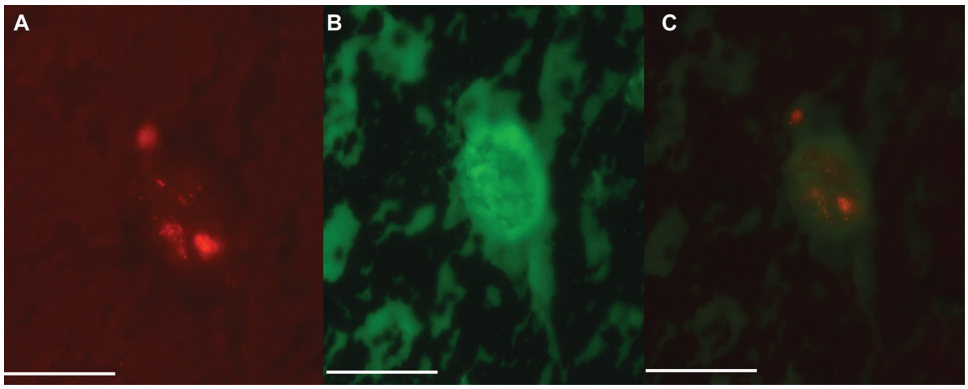


**FIG 2** Lectin staining of a biofilm-associated polysaccharide matrix surrounding a cluster of *Sodalis* cells. (A) A cross section of the tsetse gut tissue stained with a rhodamine-labeled oligonucleotide specific for *Sodalis* 16S rRNA. (B) A WGA lectin probe (green) bound to an extracellular polysaccharide matrix. (C) Panels A and B merged. The presence of a polysaccharide matrix surrounding a *Sodalis* cluster in the lumen of the tsetse gut indicates that these bacterial cells have formed a biofilm. Bars, 50 μm.

ride matrix using lectin staining. Lectins are carbohydrate-binding proteins or glycoproteins that contain at least two carbohydrate-binding sites (40). Out of the 7 different lectins we tested, only wheat germ agglutinin (WGA) reacted positively to 100% of the microcolonies found in the tsetse gut (Fig. 2). Furthermore, DAPI staining revealed that these biofilms may contain DNA in their extracellular matrix. A signal using WGA was detected along the host gut epithelial tissue, indicating that the polysaccharides could be host derived in nature (data not shown). WGA binds specifically to *N*-acetylglucosamine, a major component of peptidoglycan (39). We also tested whether WGA binds to *Sodalis* maintained in culture and detected staining in close proximity to the individual *Sodalis* cells (see Fig. S2 in the supplemental material). These findings suggest that WGA binds polysaccharides, such as the peptidoglycan and/or lipopolysaccharides that are on the bacterial cell membrane.

**Generation of *Sodalis* OmpA mutants and characterization of their growth phenotype.** The process of biofilm formation in bacteria, including *E. coli*, *Acinetobacter baumannii*, and *Salmonella enterica* serovar Typhimurium, is influenced by OmpA (14, 26, 27). In an effort to determine if OmpA exhibits a homologous function in *Sodalis*, we generated a mutant strain that does not produce this protein. To do so, an intron was inserted in frame within the *Sodalis ompA* gene, which is encoded on a single chromosomally located operon. Intron insertion was verified by PCR (Fig. 3A), and OmpA was subsequently undetectable in the mutant strain (the *Sodalis* ΔOmpA mutant; Fig. 3B). We next characterized the growth phenotype of the *Sodalis* ΔOmpA mutant in comparison to its parent strain (*Sodalis* PT). When grown in MM medium, the *Sodalis* ΔOmpA mutant exhibited no growth defect (Fig. 3C). Tsetse flies feed exclusively on vertebrate blood, and *Sodalis* resides in the tsetse gut where the blood meal accumulates and undergoes digestion. We first examined the ability of *Sodalis* PT to grow *in vitro* in complement-active and -inactive blood. Our data show that vertebrate blood contains sufficient nutrients to maintain *Sodalis in vitro* but that these bacterial cells are sensitive to the complement system when inoculated into this medium (see Fig. S3 in the supplemental material). We also found that *Sodalis* ΔOmpA exhibits a similar growth curve when cultured in heat-inactivated blood (Fig. 3D). Interestingly, *Sodalis* strains found naturally in the tsetse gut are able to survive in the presence of

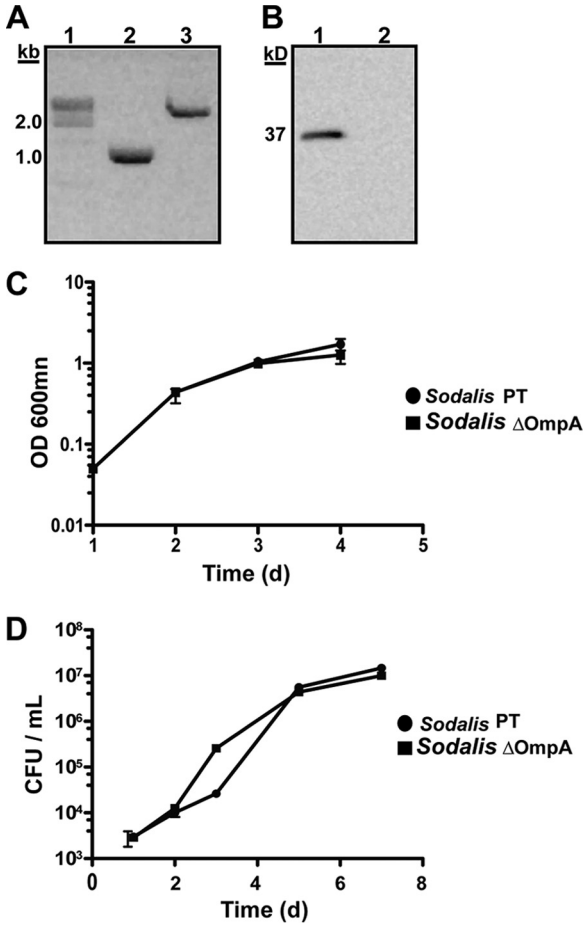


**FIG 3** Generation of a *Sodalis ompA* mutant strain. (A) PCR amplification of the *ompA* gene in the *Sodalis* parent strain (*Sodalis* PT; lane 2) and *Sodalis ompA* mutants (*Sodalis* ΔOmpA; lane 3). The ~1-kb band shift is the result of intron insertion into the *ompA* locus of the *Sodalis* chromosome. (B) A Western blot showing OmpA expression in *Sodalis* PT cells (lane 1) but not in their *Sodalis* ΔOmpA derivatives (lane 2). (C and D) *Sodalis* PT and *Sodalis* ΔOmpA growth curves in MM medium and blood, respectively. *Sodalis* ΔOmpA does not exhibit a growth defect when cultured in either medium.

vertebrate complement. This phenomenon suggests that vertebrate complement may be inactivated prior to coming in contact with endogenous *Sodalis*. Alternatively, *Sodalis* may utilize alternative mechanisms, such as the formation of protective biofilms, to circumvent this antimicrobial system.

**OmpA modulates *Sodalis* biofilm formation *in vitro*.** The *Sodalis* ΔOmpA mutant does not exhibit a growth defect in heat-inactivated vertebrate blood and will thus serve as a robust strain to characterize the relationship between OmpA and the ability of this symbiont to colonize its tsetse host. We next set out to characterize the biofilm-associated phenotype of the *Sodalis* ΔOmpA mutant. To do so, an *in vitro* assay was performed where *Sodalis* PT and *Sodalis* ΔOmpA cultures were placed in a 15-ml Falcon tube at an angle and shaken for 7 days. Subsequently, the tube's splash zone was observed for the presence of a *Sodalis* biofilm (14, 26, 31). As shown in Fig. 4A, *Sodalis* PT cells form a distinct biofilm while their *Sodalis* ΔOmpA counterparts do not. Additionally, a crystal violet microtiter plate biofilm assay was used to quantitate the biofilm formation defect presented by *Sodalis* ΔOmpA cells. This assay indicated that the *Sodalis* ΔOmpA mutant has a 91% decrease in biofilm formation compared to *Sodalis* PT. These results indicate a role for OmpA in *Sodalis* biofilm formation.

***Sodalis* OmpA reverses the biofilm defect of *E. coli* OmpA mutants.** Data from our experiments using symbiotic *Sodalis*, as well as data from previous studies using free-living *E. coli*, suggest a role for OmpA in the ability of these bacteria to form biofilms (14, 27). To further validate the functional role of OmpA in biofilm formation, we expressed the *Sodalis* OmpA protein in a mutant strain of *E. coli* (*E. coli* ΔOmpA) that does not produce this protein and thus is unable to form biofilms (27, 50). Strain *E. coli* ΔOmpA $pIO_{Sodalis}$ was previously generated by transforming *E. coli* ΔOmpA cells with a construct ($pIO_{Sodalis}$) that encodes the *Sodalis ompA* gene under transcriptional regulation of the constitutively expressed insulinase promoter (50). We used a crystal violet microtiter plate biofilm assay to monitor the biofilm formation phenotype of *E. coli* ΔOmpA $pIO_{Sodalis}$ cells. We observed that *E. coli* ΔOmpA $pIO_{Sodalis}$ and WT *E. coli* are equally competent in their ability to form biofilms *in vitro*, whereas *E. coli* ΔOmpA mutants are not (Fig. 4D). The results of our complementation assay further accentuate the role of *Sodalis* OmpA in biofilm formation processes, as biofilm-deficient *E. coli* strains are able to form these structures *in vitro* after they are genetically modified to express this protein.

**OmpA-mediated bacterial colonization of the tsetse gut.** We next developed a *per os* colonization assay and used it to examine the role of OmpA in the ability of *Sodalis* to colonize the tsetse gut. $Gmm^{WT}$ and symbiont-cured (here referred to as aposymbiotic, or $Gmm^{Apo}$) flies were given a heat-inactivated blood meal supplemented with *Sodalis* PT. Subsequently, all flies received sterile heat-inactivated blood every other day for the entirety of the 5-day experiment. At 1, 3, and 5 days after feeding with bacterial strains, gut tissues were microscopically harvested from individual flies of each group and the number of *Sodalis* isolates present was quantified. We determined that *Sodalis* PT was able to colonize the gut of both $Gmm^{WT}$ and $Gmm^{Apo}$ flies and replicate to a density of $\sim 10^6$ CFU in this niche (Fig. 5A), a density which is comparable to natural *Sodalis* levels present in $Gmm^{WT}$ guts (see Fig. S1 in the supplemental material). These data suggest that *Sodalis* PT can survive in the presence of natural *Sodalis* present in the gut of

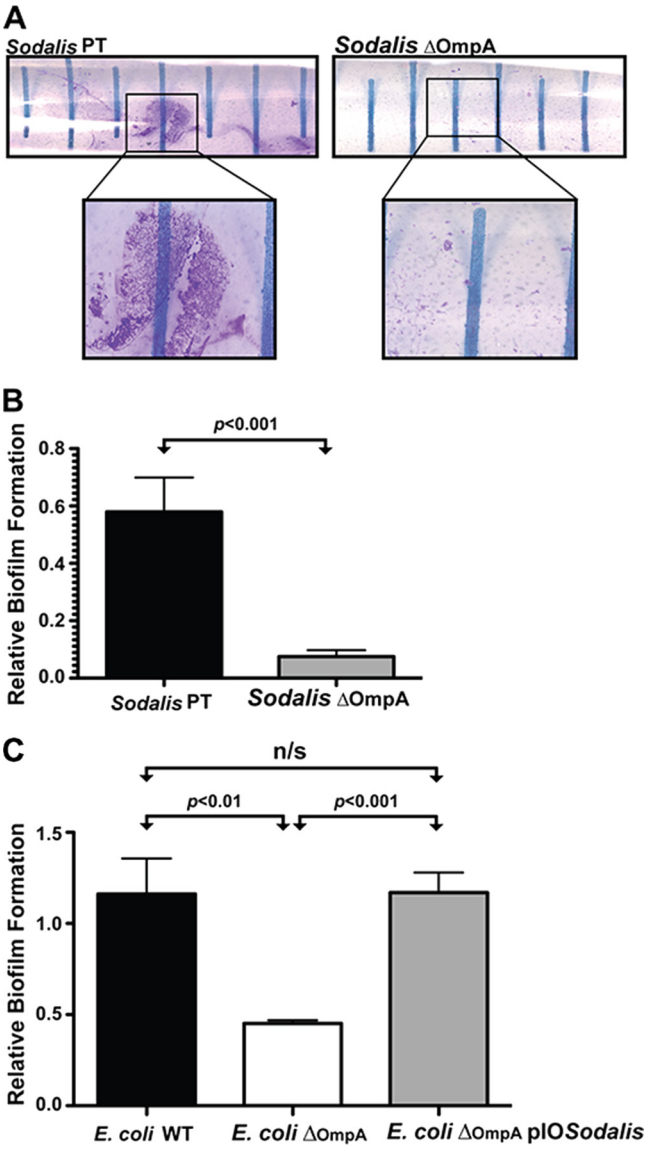


**FIG 4** OmpA regulates bacterial biofilm formation *in vitro*. (A) *Sodalis* PT and *Sodalis* ΔOmpA cells were shaken vigorously at an angle for 7 days in 15-ml Falcon tubes. The bacterial splash zone was subsequently stained with crystal violet to visualize the presence or absence of a biofilm. These structures were present in the tubes containing *Sodalis* PT cells (shown enlarged in the corresponding box). *Sodalis* ΔOmpA cells failed to form a biofilm. This experiment was performed in triplicate with 3 distinct clonal populations of each bacterial strain. (B) Spectrophotometric quantification of relative biofilm formation exhibited by *Sodalis* PT and *Sodalis* ΔOmpA cells. (C) *E. coli* ΔOmpA cells exhibit a biofilm formation defect that is restored in their counterparts that were genetically modified to express *Sodalis ompA* (*E. coli* ΔOmpA $pIO_{Sodalis}$ cells). In panels B, C and D, statistical significance was determined by the Mann-Whitney test.

$Gmm^{WT}$ flies and that these exogenous cells grow to densities similar to those of their native counterparts. *Sodalis* PT can also replace natural infections when provided to aposymbiotic flies. We next set out to determine if recombinant *Sodalis* strains can produce a biofilm in $Gmm^{WT}$ and/or $Gmm^{Apo}$ flies. We recolonized these tsetse lines *per os* with a green fluorescent protein (GFP)-expressing strain of this bacterium ($Sodalis^{pGFP}$) and then microscopically examined gut fluid for the presence of *Sodalis* clusters,

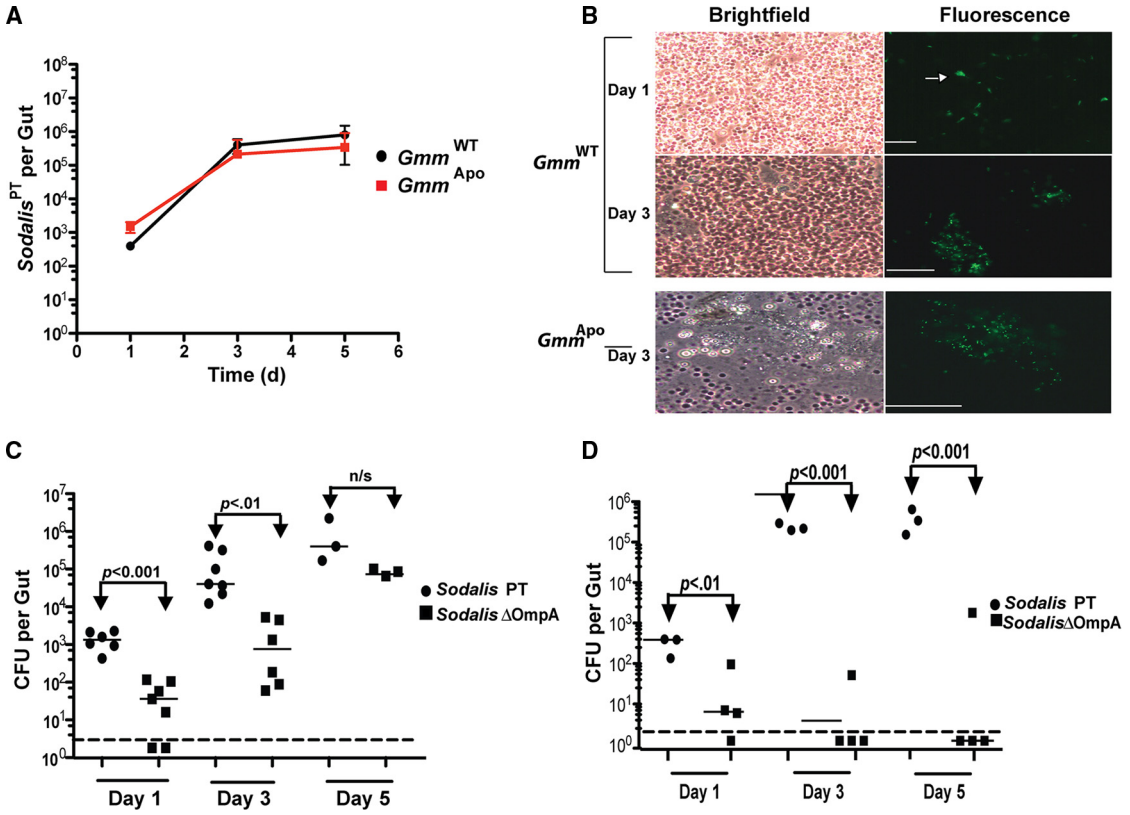


**FIG 5** OmpA modulates *Sodalis* biofilm formation in the tsetse gut. (A) A newly developed *per os* colonization assay was used to determine if *Sodalis* PT cells could establish an infection in $Gmm^{WT}$ and $Gmm^{Apo}$ fly guts. Five days postinoculation, *Sodalis* PT was found at a density of ~$10^6$ cells in both host backgrounds. (B) Biofilm formation in the tsetse gut was visualized by orally inoculating $Gmm^{WT}$ and $Gmm^{Apo}$ flies with a strain of *Sodalis* (*Sodalis* pGFP) that expresses green fluorescent protein. Fluorescing microcolonies, indicated by a white arrow, were present in $Gmm^{WT}$ flies 1 day postinoculation with *Sodalis* pGFP. Three days postinoculation, these microcolonies are more pronounced in $Gmm^{WT}$ flies. Additionally, *Sodalis* pGFP colonies are also beginning to form at this time in $Gmm^{Apo}$ flies. Bars, 50 μm. (C) Colonization of $Gmm^{WT}$ fly guts by *Sodalis* PT and *Sodalis* ΔOmpA strains. *Sodalis* ΔOmpA cells exhibit an initial colonization defect but, by 5 days postinoculation, reach a density similar to that of *Sodalis* PT cells. (D) Colonization of $Gmm^{Sgm-}$ fly guts by *Sodalis* PT and *Sodalis* ΔOmpA strains. *Sodalis* ΔOmpA cells cannot colonize tsetse when their native counterparts are absent. In panel A, colonization assays were performed in duplicate and data are presented as the means ± standard deviations. In panels C and D, each symbol represents one fly gut, and the dotted line indicates the assay's limit of detection. Statistical significance in C and D was determined by the Mann-Whitney test.

similar to the procedure for Fig. 1. We discovered that 1 day postfeeding, *Sodalis* begins to form small microcolonies in the gut of $Gmm^{WT}$ flies and that biofilms are present 3 days later. Furthermore, biofilms were also present in the gut of $Gmm^{Apo}$ flies by 3 days postinoculation (Fig. 5B). Results from a control experiment, in which gut fluid from $Gmm^{WT}$ flies (which contained no recombinant *Sodalis*) was also observed, indicated that natural populations of this symbiont do not autofluoresce (see Fig. S4 in the supplemental material). Taken together, these data demonstrate that our *per os* colonization assay can be exploited to study the molecular mechanisms that underlie bacterial colonization of the tsetse gut.

We next investigated whether OmpA regulates the ability of *Sodalis* to colonize the tsetse gut by feeding $Gmm^{WT}$ individuals with either *Sodalis* ΔOmpA or *Sodalis* PT cells. In contrast to *Sodalis* PT, *Sodalis* ΔOmpA cells exhibited a significant colonization defect 1 and 3 days after being fed to wild-type flies, which still retain their native microbiota. However, by 5 days postinoculation, both *Sodalis* strains reached similar homeostatic densities in the gut of $Gmm^{WT}$ flies (Fig. 5C). These data suggest that OmpA is most important during initial stages of *Sodalis* gut colonization, as cells that lack this protein can reach normal densities over time. Interestingly, previous studies using other model systems have shown that mutant bacteria can "cheat" their colonization defect by cross-feeding off relevant protein products produced by endogenous bacteria also present in the system (30, 38, 45, 46). We thus investigated whether *Sodalis* ΔOmpA cells are able to colonize wild-type tsetse by cheating off the *Sodalis* naturally found in these flies. This could explain the delay in the colonization process we observed with the mutant strain. To do so, we generated *Sodalis*-free tsetse flies ($Gmm^{Sgm-}$) by feeding $Gmm^{WT}$ flies two blood meals supplemented with 200 μg of ampicillin per ml of blood. Prior to beginning the experiment, the absence of native *Sodalis* in $Gmm^{Sgm-}$ flies was verified by dissecting 4 fly guts per group and measuring the CFU by plating (data not shown). Subsequently, $Gmm^{Sgm-}$ individuals were orally inoculated with either *Sodalis* PT or *Sodalis* ΔOmpA cells. *Sodalis* PT effectively colonized $Gmm^{Sgm-}$ flies (Fig. 5D). However, in contrast to what we observed previously in $Gmm^{WT}$ flies (which have their natural microbiome), we found that *Sodalis* ΔOmpA cells exhibited a significant colonization defect in $Gmm^{Sgm-}$ individuals over the course of the entire 5-day experiment (Fig. 5D). These findings

provide further confirmation that *Sodalis* requires OmpA to colonize the tsetse gut.

**The *Sodalis* ΔOmpA mutant receives protection from host immune responses in biofilms.** We demonstrate that *Sodalis* ΔOmpA cells cannot form a biofilm *in vitro*. Furthermore, while these bacterial cells can readily colonize the gut of *Gmm*$^{WT}$ flies, they lack the ability to do so in tsetse that are missing their native microbiota (*Gmm*$^{Sgm-}$). Many bacteria evade host immunity by forming biofilms (13, 22, 43). Thus, we speculated that the biofilm formation defect presented by *Sodalis* ΔOmpA cells could leave these cells susceptible to the tsetse's innate immune system and result in their elimination from the gut environment. To test this theory, we assessed the ability of *Sodalis* ΔOmpA cells to colonize tsetse that exhibit a state of heightened immunity. As done previously, we induced a local immune response in the gut of *Gmm*$^{WT}$ flies by feeding distinct groups of flies either lipopolysaccharide (LPS) and peptidoglycan (PGN) or live *E. coli*. These treatments induce an increase in the expression of a variety of immunity-related effector molecules, including peptidoglycan recognition protein (PGRP) LB and the antimicrobial peptides (AMPs) attacin, defensin, and diptericin (17, 47). These flies were subsequently administered a heat-inactivated blood meal containing either *Sodalis* PT or *Sodalis* ΔOmpA cells. We found that host immune stimulation by either LPS and PGN or *E. coli* had no effect on *Sodalis* PT, as these cells reached a density of $10^6$ CFU within 5 days (Fig. 6A and B, respectively). These findings confirmed previous results, which showed that *Sodalis* strains are resistant to the actions of tsetse's innate immune molecules *in vitro* (16, 20) and *in vivo* (47). We next tested the ability of *Sodalis* ΔOmpA cells to colonize *Gmm*$^{WT}$ flies following host immune activation with either LPS and PGN or *E. coli*. When introduced *per os* into flies that were immune stimulated by LPS and PGN, *Sodalis* ΔOmpA strains exhibited an initial colonization defect that was significant compared to that of *Sodalis* PT. However, by 3 days postinoculation, the *Sodalis* ΔOmpA colonization defect was less severe than on day 1 (Fig. 6A). These findings indicate that *Sodalis* ΔOmpA proteins, acquired in the blood meal, are initially susceptible to tsetse's immune responses but can subsequently survive as they incorporate into native biofilms. When host immune challenge was achieved with live *E. coli* feeding, *Sodalis* ΔOmpA proteins were not able to colonize wild-type tsetse (Fig. 6B). Administration of live *E. coli* likely induces a more robust host immune response than LPS and PGN, and this response may clear *Sodalis* ΔOmpA cells before they are afforded protection by endogenous biofilms.

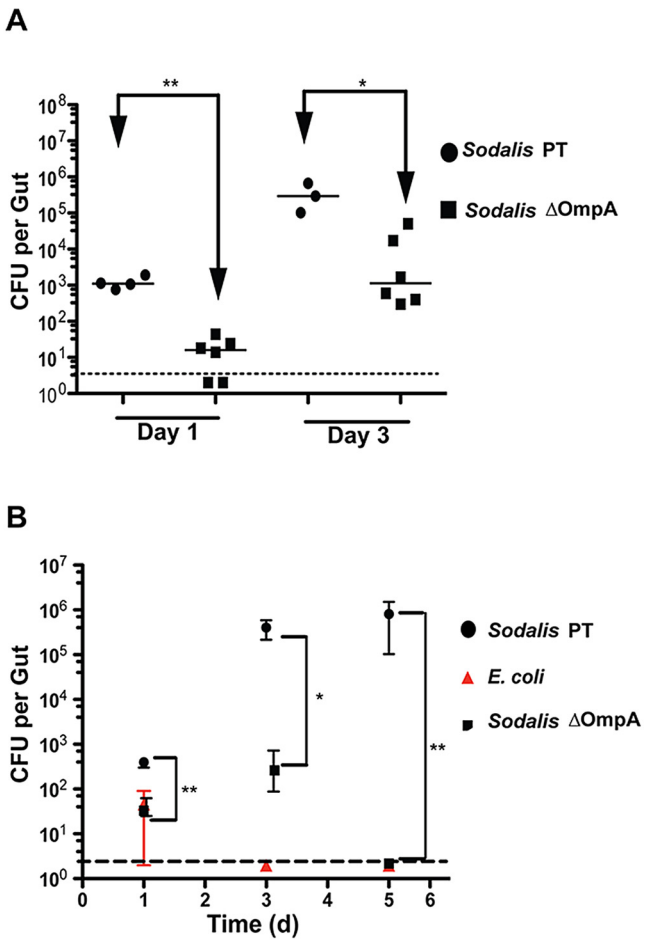


**FIG 6** Biofilms protect *Sodalis* from tsetse's innate immune response. *Sodalis* PT and *Sodalis* ΔOmpA cell colonization of immune-activated *Gmm*$^{WT}$ fly guts. LPS and PGN (A) or live *E. coli* (B) was fed to flies prior to bacterial inoculation. Guts were dissected at 1 and 3 days postinoculation to determine the number of CFU present. Each symbol represents one fly gut, and the dotted line indicates the assay's limit of detection. Statistical significance was determined by the Mann-Whitney test.

## DISCUSSION

Congregation within biofilms is essential for bacterial colonization of eukaryotic hosts. To date, most studies pertaining to this subject have focused on biofilms as they relate to host colonization by bacterial pathogens (33, 35). However, biofilms are gaining increased recognition as modulators of beneficial symbiotic associations. In this study, we investigated factors that mediate colonization of the tsetse fly gut by its commensal symbiont, *Sodalis*. We determined that *Sodalis* forms biofilms in the tsetse gut and that this process is contingent upon the presence of surface-exposed bacterial outer membrane protein A (OmpA). Furthermore, our results show that both the biofilms present in the tsetse gut and the major surface coat OmpA may provide protection to *Sodalis* against host innate immune responses. We propose that the tsetse-*Sodalis* symbiosis may serve as a useful model for better understanding the dynamics of symbiont colonization processes in the more complex mammalian systems.

Most insects, including colony-reared individuals and those captured in the field, harbor a taxonomically diverse assemblage of bacteria in their digestive tracts (12). Tsetse flies reared in our laboratory appear unique in that their guts are dominated by symbiotic *Wigglesworthia* and *Sodalis* strains. This finding implies that biofilms present in the tsetse gut lumen do not harbor mixed bacterial species like those found in other insects (24, 25). Instead, the tsetse gut lumen harbors *Sodalis* exclusively, as *Wigglesworthia* is found only intracellularly in the adjacent bacteriome. Tsetse is the sole vector of pathogenic African trypanosomes, which cause sleeping sickness in humans and nagana in domesticated animals. Laboratory-reared tsetse flies that lack their symbiotic microbes (*Gmm*$^{Apo}$) are unusually susceptible to infection with trypanosomes (47). Our recent investigations indicated that *Gmm*$^{Apo}$ flies present a severely compromised immune system that is characterized by a significantly reduced population of phagocytic hemo-

cytes (48, 50). The increased parasite transmission ability of $Gmm^{Apo}$ flies could be due to the lack of important host immune gene expression or could result from the absence of microbial biofilms in the gut. Interestingly, unlike their colony-reared counterparts, guts from field-captured tsetse flies house a more diverse bacterial population, although information on the densities of the different microbes relative to the symbiotic fauna is lacking (15). Similarly, field-based populations of other insect disease vectors are associated with taxonomically complex gut commensals that are intimately involved in their host's ability to transmit mammalian pathogens. For example, both *Anopheles* vectors of human malaria and *Aedes* vectors of dengue fever harbor gut microbiomes that influence the dynamics of parasite transmission processes (9, 37). Future studies using field-captured and laboratory-reared tsetse flies are required to determine if interactions between trypanosomes and bacterial biofilms influence this fly's vector competence.

In this study, we demonstrate that *Sodalis* colonizes the tsetse gut by forming biofilms and biofilm production is modulated by OmpA. These findings suggest that OmpA facilitates the tsetse-*Sodalis* symbiosis by promoting this bacterium's ability to reside in its host's gut. Interestingly, we demonstrated previously that OmpA also mediates the ability of *Sodalis* to reside symbiotically in tsetse hemocoel (50). This tolerance phenomenon was attributed to environmentally exposed OmpA loop domains. Specifically, the *Sodalis* OmpA strain was found to include amino acid insertions and substitutions in these regions that were absent from their homologs in *E. coli*. More so, exposed loop domains of OmpA from other insect symbionts also exhibit polymorphisms similar to those found in *Sodalis*. In tsetse, the distinct *Sodalis* and *E. coli* OmpA phenotypes corresponded with infection outcomes, as flies were found to be permissive of thoracic superinfection with *Sodalis* and *E. coli* ΔOmpA $pIO_{Sodalis}$ but highly susceptible to the same treatment with WT *E. coli*. Exposed loop domain polymorphisms appeared to differentially regulate tsetse immunity, as avirulent *Sodalis* activated a robust host immune response while virulent *E. coli* did not. We speculate that *E. coli* replication proceeded without impediment and that tsetse perished due to their inability to tolerate high densities of this bacterium in their hemocoel. In contrast, we suspect *Sodalis* was able to circumvent tsetse's hemocoelic immune response by residing within host hemocytes (50). Taken together, our studies of *Sodalis* OmpA indicate that this protein exhibits multiple functional roles that are critical to the success of this bacterium's symbiosis with its tsetse host.

Many pathogenic bacteria, including *E. coli*, *Salmonella* Typhimurium, and *Acinetobacter baumannii*, evade host immunity by cloaking themselves within protective biofilms (14, 26, 27). One way these structures function in this manner is by limiting bacterial contact with host leukocytes (13, 22, 43). This process significantly reduces host antibody production and decreases the number of bacterial cells cleared via phagocytosis. An additional way biofilms protect pathogens from their host's immune system is by shielding them from the bactericidal activity of antimicrobial peptides (13, 22, 43). In fact, this phenomenon was recently observed in *Drosophila melanogaster* following infection with *Pseudomonas aeruginosa*. In this system, biofilm-encased bacteria within the fly's crop were more resistant to host AMPs than were planktonically grown cells (33). In the tsetse gut, the expression of several potent AMPs increases significantly when flies are fed live *E. coli* (17). These effector molecules function by punching pores in the outer membrane of bacteria, which causes the cells to lyse and die (6, 17). While previous experiments demonstrated that wild-type *Sodalis* cells are resistant to host AMPs, we observed in this study that *Sodalis* ΔOmpA mutant cells are cleared from tsetse flies that exhibited heightened gut immunity (16, 20, 47). This finding suggests that a correlation exists between the susceptibility of *Sodalis* ΔOmpA cells to the tsetse immune system and this bacterial strain's inability to form biofilms. We suspect that biofilm formation-deficient *Sodalis* cells perish in the tsetse gut because they are exposed to the bacteriolytic activity of host AMPs. Our findings suggest that OmpA may provide protection to *Sodalis* cells in the tsetse's pernicious gut environment, thus allowing this bacterium to reside symbiotically in this niche.

Blood-feeding insects such as tsetse flies and mosquitoes vector highly virulent mammalian pathogens. Current methods used to reduce vector populations, such as setting traps and applying pesticides, are limited in their effectiveness (8). Thus, in addition to traditional population suppression techniques, novel control strategies that reduce the vector competence of these insects are desirable. One such approach, called paratransgenesis, involves genetically modifying midgut commensal symbionts so that they produce antiparasitic compounds. These genetically modified symbionts are subsequently passed on to future offspring (8). This potential control strategy is promising in part because these recombinant symbionts colonize their host's gut and subsequently reside in close proximity to pathogenic microbes. This intimate association suggests that effector molecules secreted by genetically altered commensals will encounter and eliminate pathogens. Our characterization of the molecular mechanisms that underlie *Sodalis* colonization of the tsetse gut will be applicable to other insect-symbiont model systems. Additionally, the newly developed *per os* colonization assay described herein accurately mimics the route that many commensals utilize to naturally colonize their host. These findings will be of fundamental importance for optimizing the efficacy of paratransgenesis in blood-feeding arthropods.

## ACKNOWLEDGMENTS

This work was generously funded by grants to S.A. from the National Institute of Allergy and Infectious Diseases (NIAID) (AI051584), the National Institute of General Medical Science (NIGMS) (069449), and the Ambrose Monell Foundation (http://www.monellvetlesen.org/). M.A.M. is the recipient of a NIH Parasitology and Vector Biology training grant (5T32AI007404).

## REFERENCES

1. **Akman L, Rio RV, Beard CB, Aksoy S.** 2001. Genome size determination and coding capacity of *Sodalis glossinidius*, an enteric symbiont of tsetse flies, as revealed by hybridization to *Escherichia coli* gene arrays. J. Bacteriol. **183**:4517–4525.
2. **Akman L, et al.** 2002. Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. Nat. Genet. **32**:402–407.
3. **Aksoy S, Pourhosseini AA, Chow A.** 1995. Mycetome endosymbionts of tsetse flies constitute a distinct lineage related to *Enterobacteriaceae*. Insect Mol. Biol. **4**:15–22.
4. **Attardo GM, et al.** 2008. Analysis of milk gland structure and function in *Glossina morsitans*: milk protein production, symbiont populations and fecundity. J. Insect Physiol. **54**:1236–1242.
5. **Barrios AF, Zuo R, Ren D, Wood TK.** 2006. Hha, YbaJ, and OmpA regulate *Escherichia coli* K12 biofilm formation and conjugation plasmids abolish motility. Biotechnol. Bioeng. **93**:188–200.
6. **Carlsson A, Nystrom T, de Cock H, Bennich H.** 1998. Attacin—an insect immune protein—binds LPS and triggers the specific inhibition of bacterial outer-membrane protein synthesis. Microbiology **144**:2179–2188.

7. **Chen X, Li S, Aksoy S.** 1999. Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia glossinidia*. J. Mol. Evol. **48**:49–58.
8. **Cheng Q, Aksoy S.** 1999. Tissue tropism, transmission and expression of foreign genes *in vivo* in midgut symbionts of tsetse flies. Insect Mol. Biol. **8**:125–132.
9. **Cirimotich CM, Ramirez JL, Dimopoulos G.** 2011. Native microbiota shape insect vector competence for human pathogens. Cell Host Microbe **10**:307–310.
10. **Cole JR, et al.** 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. **33**: D294–D296.
11. **Darby C.** 2008. Uniquely insidious: *Yersinia pestis* biofilms. Trends Microbiol. **16**:158–164.
12. **Dillon RJ, Dillon VM.** 2004. The gut bacteria of insects: nonpathogenic interactions. Annu. Rev. Entomol. **49**:71–92.
13. **Foster TJ.** 2005. Immune evasion by staphylococci. Nat. Rev. Microbiol. **3**:948–958.
14. **Gaddy JA, Tomaras AP, Actis LA.** 2009. The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. Infect. Immun. **77**:3150–3160.
15. **Geiger A, et al.** 2011. Bacterial diversity associated with populations of *Glossina* spp. from Cameroon and distribution within the Campo sleeping sickness focus. Microb. Ecol. **62**:632–643.
16. **Haines LR, Hancock RE, Pearson TW.** 2003. Cationic antimicrobial peptide killing of African trypanosomes and *Sodalis glossinidius*, a bacterial symbiont of the insect vector of sleeping sickness. Vector Borne Zoonotic Dis. **3**:175–186.
17. **Hao Z, et al.** 2001. Tsetse immune responses and trypanosome transmission: implications for the development of tsetse-based strategies to reduce trypanosomiasis. Proc. Natl. Acad. Sci. U. S. A. **98**:12648–12653.
18. **Hooper LV, Gordon JI.** 2001. Commensal host-bacterial relationships in the gut. Science **292**:1115–1118.
19. **Hu C, Aksoy S.** 2006. Innate immune responses regulate trypanosome parasite infection of the tsetse fly *Glossina morsitans morsitans*. Mol. Microbiol. **60**:1194–1204.
20. **Hu Y, Aksoy S.** 2005. An antimicrobial peptide with trypanocidal activity characterized from *Glossina morsitans morsitans*. Insect Biochem. Mol. Biol. **35**:105–115.
21. **Kang Y, et al.** 2004. Systematic mutagenesis of the *Escherichia coli* genome. J. Bacteriol. **186**:4921–4930.
22. **Kharazmi A.** 1991. Mechanisms involved in the evasion of the host defence by *Pseudomonas aeruginosa*. Immunol. Lett. **30**:201–205.
23. **Kikuchi Y, Graf J.** 2007. Spatial and temporal population dynamics of a naturally occurring two-species microbial community inside the digestive tract of the medicinal leech. Appl. Environ. Microbiol. **73**:1984–1991.
24. **Kikuchi Y, Hosokawa T, Fukatsu T.** 2011. Specific developmental window for establishment of an insect-microbe gut symbiosis. Appl. Environ. Microbiol. **77**:4075–4081.
25. **Kikuchi Y, Meng XY, Fukatsu T.** 2005. Gut symbiotic bacteria of the genus *Burkholderia* in the broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis* (Heteroptera: Alydidae). Appl. Environ. Microbiol. **71**: 4035–4043.
26. **Kint G, De Coster D, Marchal K, Vanderleyden J, De Keersmaecker SC.** 2010. The small regulatory RNA molecule MicA is involved in *Salmonella enterica* serovar Typhimurium biofilm formation. BMC Microbiol. **10**: 276.
27. **Ma Q, Wood TK.** 2009. OmpA influences *Escherichia coli* biofilm formation by repressing cellulose production through the CpxRA two-component system. Environ. Microbiol. **11**:2735–2746.
28. **Maccaferri S, Biagi E, Brigidi P.** 2011. Metagenomics: key to human gut microbiota. Dig. Dis. **29**:525–530.
29. **Macfarlane S, Dillon JF.** 2007. Microbial biofilms in the human gastrointestinal tract. J. Appl. Microbiol. **102**:1187–1196.
30. **Maltz M, Graf J.** 2011. The type II secretion system is essential for erythrocyte lysis and gut colonization by the leech digestive tract symbiont *Aeromonas veronii*. Appl. Environ. Microbiol. **77**:597–603.
31. **Merritt JH, Kadouri DE, O'Toole GA.** 2005. Growing and analyzing static biofilms. Curr. Protoc. Microbiol. **Chapter 1**:Unit 1B 1.
32. **Moloo SK.** 1971. An artificial feeding technique for *Glossina*. Parasitology **63**:507–512.
33. **Mulcahy H, Sibley CD, Surette MG, Lewenza S.** 2011. *Drosophila melanogaster* as an animal model for the study of *Pseudomonas aeruginosa* biofilm infections in vivo. PLoS Pathog. **7**:e1002299. doi:10.1371/journal.ppat.1002299.
34. **Pais R, Lohs C, Wu Y, Wang J, Aksoy S.** 2008. The obligate mutualist *Wigglesworthia glossinidia* influences reproduction, digestion, and immunity processes of its host, the tsetse fly. Appl. Environ. Microbiol. **74**:5965–5974.
35. **Purdy AE, Watnick PI.** 2011. Spatially selective colonization of the arthropod intestine through activation of *Vibrio cholerae* biofilm formation. Proc. Natl. Acad. Sci. U. S. A. **108**:19737–19742.
36. **Qin J, et al.** 2010. A human gut microbial gene catalogue established by metagenomic sequencing. Nature **464**:59–65.
37. **Ramirez JL, et al.** 2012. Reciprocal tripartite interactions between the *Aedes aegypti* midgut microbiota, innate immune system and dengue virus influences vector competence. PLoS Negl. Trop. Dis. **6**:e1561. doi: 10.1371/journal.pntd.0001561.
38. **Sandoz KM, Mitzimberg SM, Schuster M.** 2007. Social cheating in *Pseudomonas aeruginosa* quorum sensing. Proc. Natl. Acad. Sci. U. S. A. **104**:15876–15881.
39. **Sizemore RK, Caldwell JJ, Kendrick AS.** 1990. Alternate gram staining technique using a fluorescent lectin. Appl. Environ. Microbiol. **56**:2245–2247.
40. **Slifkin M, Doyle RJ.** 1990. Lectins and their application to clinical microbiology. Clin. Microbiol. Rev. **3**:197–218.
41. **Smith SG, Mahon V, Lambert MA, Fagan RP.** 2007. A molecular Swiss army knife: OmpA structure, function and expression. FEMS Microbiol. Lett. **273**:1–11.
42. **Snoussi S, et al.** 2012. Adaptation of *Salmonella enterica* Hadar under static magnetic field: effects on outer membrane protein pattern. Proteome Sci. **10**:6.
43. **Thurlow LR, et al.** 2011. *Staphylococcus aureus* biofilms prevent macrophage phagocytosis and attenuate inflammation in vivo. J. Immunol. **186**: 6585–6596.
44. **Toh H, et al.** 2006. Massive genome erosion and functional adaptations provide insights into the symbiotic lifestyle of *Sodalis glossinidius* in the tsetse host. Genome Res. **16**:149–156.
45. **Venturi V, Bertani I, Kerenyi A, Netotea S, Pongor S.** 2010. Co-swarming and local collapse: quorum sensing conveys resilience to bacterial communities by localizing cheater mutants in *Pseudomonas aeruginosa*. PLoS One **5**:e9998. doi:10.1371/journal.pone.0009998.
46. **Vulic M, Kolter R.** 2001. Evolutionary cheating in *Escherichia coli* stationary phase cultures. Genetics **158**:519.
47. **Wang J, Wu Y, Yang G, Aksoy S.** 2009. Interactions between mutualist *Wigglesworthia* and tsetse peptidoglycan recognition protein (PGRP-LB) influence trypanosome transmission. Proc. Natl. Acad. Sci. U. S. A. **106**: 12133–12138.
48. **Weiss BL, Maltz M, Aksoy S.** 2012. Obligate symbionts activate immune system development in the tsetse fly. J. Immunol. **188**:3395–3403.
49. **Weiss BL, et al.** 2006. Interspecific transfer of bacterial endosymbionts between tsetse fly species: infection establishment and effect on host fitness. Appl. Environ. Microbiol. **72**:7013–7021.
50. **Weiss BL, Wu Y, Schwank JJ, Tolwinski NS, Aksoy S.** 2008. An insect symbiosis is influenced by bacterium-specific polymorphisms in outer-membrane protein A. Proc. Natl. Acad. Sci. U. S. A. **105**:15088–15093.
51. **Worthen PL, Gode CJ, Graf J.** 2006. Culture-independent characterization of the digestive-tract microbiota of the medicinal leech reveals a tripartite symbiosis. Appl. Environ. Microbiol. **72**:4775–4781.
52. **Xu J, et al.** 2003. A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. Science **299**:2074–2076.
53. **Xu J, Chiang HC, Bjursell MK, Gordon JI.** 2004. Message from a human gut symbiont: sensitivity is a prerequisite for sharing. Trends Microbiol. **12**:21–28.